Advances in Biochemical Engineering/Biotechnology 156
Series Editor: T. Scheper

Rajni Hatti-Kaul
Gashaw Mamo
Bo Mattiasson *Editors*

Anaerobes in Biotechnology

Springer

156
Advances in Biochemical Engineering/Biotechnology

Aims and Scope

This book series reviews current trends in modern biotechnology and biochemical engineering. Its aim is to cover all aspects of these interdisciplinary disciplines, where knowledge, methods and expertise are required from chemistry, biochemistry, microbiology, molecular biology, chemical engineering and computer science.

Volumes are organized topically and provide a comprehensive discussion of developments in the field over the past 3–5 years. The series also discusses new discoveries and applications. Special volumes are dedicated to selected topics which focus on new biotechnological products and new processes for their synthesis and purification.

In general, volumes are edited by well-known guest editors. The series editor and publisher will, however, always be pleased to receive suggestions and supplementary information. Manuscripts are accepted in English.

In references, Advances in Biochemical Engineering/Biotechnology is abbreviated as *Adv. Biochem. Engin./Biotechnol.* and cited as a journal.

More information about this series at http://www.springer.com/series/10

Rajni Hatti-Kaul • Gashaw Mamo • Bo Mattiasson
Editors

Anaerobes in Biotechnology

With contributions by

F. Burton · R.A. Börner · A. Dabir · J. Daniell · P.K. Dhakephalkar · H. El Enshasy · E.A. Elsayed · M.E. Ersahin · N.-U. Frigaard · C.T.M.J. Frijters · R. Hatti-Kaul · V. Honkalas · M.E. Kokko · M. Köpke · S. Lal · D.B. Levin · M. Lin · A.E. Mäkinen · K. Malik · R.A. Malek · G. Mamo · B. Mattiasson · R. Munir · S. Nagaraju · N.Z. Othman · J.A. Puhakka · A. Schnürer · S.D. Simpson · R. Sparling · M. Taillefer · F.P. van der Zee · J.B. van Lier · E.W.J. van Niel · M. Wadaan · J. Wang · M. Xu · S.-T. Yang

Springer

Editors
Rajni Hatti-Kaul
Biotechnology, Department of Chemistry
Center for Chemistry & Chemical Engineering
Lund University
Lund, Sweden

Gashaw Mamo
Biotechnology, Department of Chemistry
Center for Chemistry & Chemical Engineering
Lund University
Lund, Sweden

Bo Mattiasson
Biotechnology, Department of Chemistry
Center for Chemistry & Chemical Engineering
Lund University
Lund, Sweden

ISSN 0724-6145 ISSN 1616-8542 (electronic)
Advances in Biochemical Engineering/Biotechnology
ISBN 978-3-319-45649-2 ISBN 978-3-319-45651-5 (eBook)
DOI 10.1007/978-3-319-45651-5

Library of Congress Control Number: 2016952250

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

Preface

Anaerobes, the microorganisms that live and grow in environments deficient in oxygen, play a significant role in many processes which occur in nature, such as the production of biological dinitrogen, methane and hydrogen sulphide, the degradation of wastes, fermentation of organic matter and carbon dioxide fixation. They also play a vital role in human health in terms of causing infections but also constitute important microflora inside humans and animals that determines the state of their health.

Long before the nature of anaerobes was understood, man has been using these microorganisms for the production of alcoholic beverages and for fermenting foods by lactic acid bacteria for preservation. Because of their complex metabolism, special cultivation requirements and slow growth, anaerobes have been less explored than their aerobic counterparts. Over the years, however, the applications of these microorganisms have been growing dramatically. Several of the initial well, known large-scale applications include biogas production, wastewater treatment, and vinegar and wine production. In spite of these important processes, anaerobes have often been neglected in favour of aerobes, which are easier to handle. However, the anaerobic microflora promise an interesting metabolic potential which seems to show promise for future development in industrial biotechnology. Lately, driven by the emergence of new techniques and research and development activities to enable a transition from fossil-based to biobased economy to lower greenhouse gas emissions and toxic wastes, there has been a surge in the amount of information concerning anaerobes and in exploiting their potential in depolymerisation of biomass and production of different chemicals and energy carriers.

This volume on "Anaerobes in Biotechnology" was thus planned to bring attention to this important group of diverse microorganisms and to highlight their potential beyond what is commonly known.

As many anaerobes are living in very complex microbial consortia, isolation of individual organisms has been rather cumbersome. While next-generation sequencing technologies have started to provide new insights into the total community of

microorganisms, new technologies to isolate and cultivate anaerobes have also been developed which are covered in this book. It is clearly seen from this volume that dramatic developments have taken place during a few decades in understanding the diversity of anaerobes in different anoxic environments, including deep sea sediments associated with enormous methane hydrate deposits, and identification of new enzymes and metabolic pathways used by the anaerobes for the breakdown of complex organic matter and metabolism of sugars and gases that are now utilized to develop processes. There is also an improved understanding of the role of anaerobes as engines that run the biogeochemical cycles, which greatly benefit environmental technology such as bioremediation.

This volume not only updates the roles of anaerobes in the production of energy carriers but also elucidates the advantages of anaerobe-based microbial fuel cells that extract energy from organic matter and generate electricity. The relatively recent progress made in the applications of anaerobes and their enzyme systems for production of solvents and organic acids is highlighted. Furthermore, the important role of anaerobes as probiotics and as a source of novel antimicrobial drugs for providing health benefits and overcoming the problem of bacterial drug resistance is described. The human microbiome is playing a far more important role in the wellbeing of individuals than anticipated earlier.

All the applications of anaerobes require good cultivation systems and hence progress made in anaerobic bioreactors has been included. Novel process concepts have been applied to develop high cell density fermentations and for harvesting the inhibitory products in situ while maintaining the cells in a viable, active state.

One can expect much more focus on anaerobes in the years to come with respect to the discovery of novel organisms, a better understanding of their genome, metabolic pathways, enzymes, etc. and the development of tools for their genetic manipulation concomitantly with the emergence of new applications using pure or mixed cultures, and their molecules.

We, as editors of this volume, have learnt a lot from the various contributions from scientists who are experts in the field. We would like to thank all the authors for their efforts and for making this book an interesting, valuable source of information on anaerobic biotechnology. We would also like to thank the Series editor of *Advances in Biochemical Engineering/Biotechnology* for believing in the importance of anaerobes for biotechnology and encouraging us to edit this volume.

Lund, Sweden — Rajni Hatti-Kaul
Lund, Sweden — Gashaw Mamo
Lund, Sweden — Bo Mattiasson

Contents

Adv Biochem Eng Biotechnol (2016) 156: 1–34
DOI: 10.1007/10_2016_10

Published online: 9 June 2016

Anaerobes in Industrial- and Environmental Biotechnology

Rajni Hatti-Kaul and Bo Mattiasson

Abstract Anaerobic microorganisms present in diverse ecological niches employ alternative strategies for energy conservation in the absence of oxygen which enables them to play a key role in maintaining the global cycles of carbon, nitrogen, and sulfur, and the breakdown of persistent compounds. Thereby they become useful tools in industrial and environmental biotechnology. Although anaerobes have been relatively neglected in comparison to their aerobic counterparts, with increasing knowledge about their diversity and metabolic potential and the development of genetic tools and process technologies to utilize them, we now see a rapid expansion of their applications in the society. This chapter summarizes some of the developments in the use of anaerobes as tools for biomass valorization, in production of energy carriers and chemicals, wastewater treatment, and the strong potential in soil remediation. The ability of several autotrophic anaerobes to reduce carbon dioxide is attracting growing attention as a means for developing a platform for conversion of waste gases to chemicals, materials, and biofuels.

Keywords Anaerobic fermentation, Anaerobic respiration, Anammox process, Biofuels, Glycerol fermentation, Heavy metal removal, Nitrogen removal, Sulfate reducing bacteria

Contents

The original version of this chapter was revised. In figure 2, Cellulos has been corrected to Cellulose.

R. Hatti-Kaul (✉) and B. Mattiasson
Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, Box 124, 221 00 Lund, Sweden
e-mail: rajni.hatti-kaul@biotek.lu.se; bo.mattiasson@biotek.lu.se

Abbreviations

ATP	Adenosine triphosphate
E^0	Reduction potential
EGSB	Expanded granular sludge bed
GAP	Glyceraldehyde-3-phosphate
3-HPA	3-Hydroxypropionaldehyde
3-HP	3-Hydroxypropionic acid
NADH	Nicotinamide adenine dinucleotide reduced
1,3-PDO	1,3-Propanediol
RTCA	Reductive tricarboxylic acid
RuBP	Ribulose-1,5-biphosphate
SRB	Sulfate reducing bacteria
UASB	Upflow anaerobic sludge bed
ΔGo´	Free energy change under standard conditions

1 Introduction

Anaerobic microorganisms, present in different ecological niches deficient in free oxygen ranging from compost heaps and mammalian gut (see [1]) to deep sea sediments (see [2]) and volcanoes, play important roles in global carbon, nitrogen and sulfur cycles, and in extracting energy from organic matter [3–5]. The earliest industrial applications of anaerobes have been in the production of foods such as bread, yoghurt, cheese and sauerkraut, and wines and beer. They have also been used for the production of chemicals including solvents, organic acids [6], vitamins, and pharmaceutical products [7]. An area of great importance where anaerobes have made enormous impact is that of environmental remediation wherein their metabolic diversity in transforming a range of organic and inorganic compounds is utilized for treatment of solid and liquid municipal and industrial waste streams [8]. In the current trend in shift from fossil-based to biobased economy, anaerobes are

attracting increasing interest as tools for transformation of renewable resources such as biomass and gases (e.g., synthesis gas [9] into diverse chemicals and both liquid (this chapter and [9]) and gaseous biofuels [3, 4]. They serve as sources for enzymes catalyzing the degradation of complex biomass or the uptake of gases such as CO, CO_2, and H_2, and of metabolic pathways for different products in both wild type and engineered microbial hosts.

Anaerobic microorganisms also produce more complex compounds which might be of importance for their survival in the ecosystems in which they operate. Anaerobes as sources of antimicrobial and bioactive substances, for example, have long been overlooked. The increasing understanding of the potential of anaerobes for human health is covered in [1] of this volume.

The advantages of anaerobic processes are the lower capital and operational costs and energy consumption as compared to aerobic processes. The main limitation, however, is their low growth rate and hence long process times caused by the limited amount of energy generated in the absence of O_2. Hence process intensification by, for example, implementing approaches for maintaining high cell density, good mass transfer, and removal of product inhibition is needed to develop efficient processes [6, 8].

This chapter provides a brief overview of the existing and potential applications of the anaerobes in industrial and environmental biotechnology based on different means of energy conservation by the microorganisms.

2 Anaerobic Fermentation and Respiration

In the absence of O_2, microorganisms utilize two mechanisms for energy conservation: *anaerobic fermentation* (an internally balanced oxidation-reduction reaction) in which the redox process occurs in the absence of exogenous electron acceptor and the oxidation is coupled to the reduction of the compound derived from an electron donor, and *anaerobic respiration* which uses an electron acceptor other than O_2, such as NO_3^-, SO_4^{2-}, fumarate, etc. The amount of energy produced, especially during fermentation, is limited, and hence the anaerobes are slow growers.

In anaerobic fermentation, ATP needed for growth is often formed as a result of *substrate-level phosphorylation* during catabolism of an organic compound. For example, two molecules of ATP are formed per molecule of glucose fermented to pyruvate by glycolysis (Embden–Meyerhof–Parnas (EMP) pathway in, for example, *Saccharomyces cerevisiae*), whereas the Entner–Duodoroff (ED) pathway (in, for example, *Zymomonas mobilis*) results in net generation of only 1 mol ATP per mol glucose [7]. The pyruvate is subsequently reduced to the fermentation products as a way to balance the reduction of NAD^+ at an earlier enzymatic step with the oxidation of NADH, and as a result fills up the pool of NAD^+, allowing the glycolysis to continue (Fig. 1). In yeast, pyruvate is reduced to ethanol with release of CO_2, whereas in lactic acid bacteria it is reduced to lactate, in clostridia to

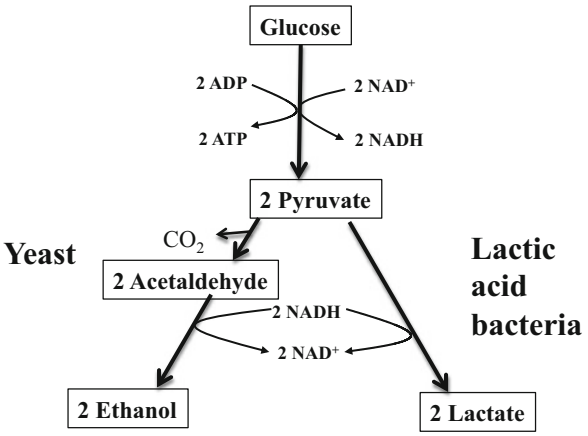


Fig. 1 Examples of anaerobic fermentation in yeast and lactic acid bacteria. ATP is formed as a result of substrate-level phosphorylation

butanol or 2,3-butanediol, etc., and in other fermentative prokaryotes to other products. Fermentation is thus more than just an energy-yielding process; it provides a means of producing natural products useful to society.

Formation of fermentation products is directly linked to the specific growth rate of the organism, and the product yield is related to the yield of the cell mass, which in turn is determined by the metabolic pathway of the organism and the cultivation conditions. The highest product yields are expected when the cells are using carbon and energy primarily for maintenance and nearly all of the available electrons are being converted to product, and the product yields are lowered with increase in specific growth rate [7].

Anaerobic respiration involving electron acceptors other than O_2 is enabled by electron transport systems containing cytochromes, quinones, iron–sulfur proteins, and other electron transport proteins in the cell membrane analogous to the situation in conventional aerobes [10]. Most of these organisms are obligate anaerobes, but in some cases, such as in denitrifying bacteria, anaerobic respiration competes in the same organism with the aerobic variety, and is favored in the absence of O_2. The reduced chemical compounds such as NADH, generated during the microbial metabolism, pass on the electrons to proteins in the electron transport system in the membrane with sequentially increasing reduction potentials (E^0) until reaching the final electron acceptor. The energy in the electrons is utilized to pump the protons across the membrane to establish a transmembrane electrochemical gradient (a proton gradient). The flux of protons across the membrane with the help of ATP synthase provides energy for ADP to react with inorganic phosphate to generate ATP. The energy released from the electron acceptors other than O_2 is lower because of their lower reduction potentials; hence the anaerobic respiration is less energy efficient. The electron acceptors with reduction potentials near that of O_2 are Fe^{3+}, NO_3^-, and NO_2^-, whereas the more electronegative acceptors are SO_4^{2-}, S^0, and CO_2 (Table 1). The use of inorganic compounds as electron acceptors in energy metabolism is called dissimilative metabolism and the reduced products are excreted into the environment (in contrast to assimilative metabolism

Table 1 Examples of anaerobic respiration using different electron acceptors (adapted from [10])

Respiration type	Electron acceptor	Product	E^0[V]	Organisms	Examples
Aerobic respiration	O_2	H_2O	+0.82	Obligate aerobes and facultative aerobes	Aerobic prokaryotes and eukaryotes
Iron reduction	Fe(III)	Fe(II)	+0.75	Facultative and obligate anaerobes	*Geobacter* sp., *Shewanella* sp., *Desulfovibrio* sp.
Nitrate reduction (denitrification)	NO_3^-	NO_2^-, N_2O, N_2	+0.40	Facultative anaerobes	*Paracoccus denitrificans*, *Pseudomonas stutzeri*, *Escherichia coli*
Dehalogenation	Halogenated organic compounds, R-X	Halide ions, X^- and dehalogenated compound, R-H	+0.25–+0.60	Facultative- and obligate anaerobes	*Trichlorobacter*, *Dehalobacter*
Fumarate respiration	Fumarate	Succinate	+0.03	Facultative anaerobes	*Escherichia coli*
Sulfate reduction	SO_4^{2-}	HS^-	−0.22	Obligate anaerobes	*Desulfobacter*
Methanogenesis (carbonate respiration)	CO_2	CH_4	−0.25	Methanogenic archaea, obligate anaerobes	*Methanothrix thermophila*
Sulfur reduction	S^0	HS^-	−0.27	Facultative- and obligate anaerobes	*Desulfuromonadales*
Acetogenesis (carbonate respiration)	CO_2	CH_3COO^-	−0.30	Homoacetogenic bacteria, obligate anaerobes	*Acetobacterium woodii*

in which the compounds are reduced for use in biosynthesis). Anaerobic respiration plays an important role in biogeochemical cycling and biodegradation of anthropogenic organic pollutants, thus having strong environmental significance.

3 Anaerobes and Industrial Biotechnology

Except for a few examples, anaerobes as tools for industrial production have been largely neglected until recently. The growing interest in the production of fuels and chemicals from renewable resources and knowledge of the microbial diversity of anoxic environments and their metabolic potential have led to an increase in research and development and even industrial cases involving their use directly or their enzymes in industrial processes, some examples of which are provided here and in other chapters of this volume [3–6, 9, 11, 12]. There are also increasing research activities on developing systems for genetic manipulation of several anaerobes, e.g., to prevent the formation of by-products or to increase their substrate spectrum.

3.1 Enzymes for Biomass Hydrolysis

Anaerobic bacteria and archaea from extreme environments and their thermostable enzymes have been the focus of studies for several years because of their potential as industrial biocatalysts for hydrolysis of polysaccharides and other biopolymers and for synthesis of chemicals and pharmaceutical intermediates [13, 14]. These enzymes are expected to withstand the relatively harsh reaction conditions required for improving the solubility of reactants and products, mass transfer, and productivity. Both conventional screening and bioinformatics approaches for screening from the vast genomic and metagenomic sequences available from both extreme and mesophilic sources are now providing access to several novel interesting candidates.

Currently interest is dominated by a search for enzymes for lignocellulose depolymerization. Anaerobes degrade lignocelluloses using free enzyme systems or multidomain enzyme complexes anchored to the bacterial cell wall called cellulosomes, the latter being in the majority (see [11]) [15]. Several free enzymes possess cellulose binding modules (CBMs) for facilitating binding to the cellulose fiber. Members of the genus *Caldicellulosiruptor* and *Thermotoga* are well-known examples of anaerobic extreme thermophiles producing extracellular thermostable enzymes which degrade biomass polysaccharides [16, 17]. (Hemi)cellulolytic systems from several thermophilic and mesophilic clostridial species, e.g., *Clostridium cellulolyticum*, *C. thermocellum*, *C. cellulovorans*, *C. stercorarium* isolated from anoxic environments in, e.g., sewage, compost, soil, manure, have also been studied.

The gut ecosystem of both herbivorous and omnivorous mammals is among the novel sources being explored for lignocellulolytic enzymes. It comprises a diverse population of obligately anaerobic bacteria, fungi, and protozoa which have evolved the capacity for efficient utilization of complex and recalcitrant plant polymers [18]. The enzyme activities found in the rumen of herbivores are diverse, including plant cell wall polymer-degrading enzymes (cellulases, xylanases, beta-glucosidases, pectinases), amylases, phytases, proteases, and specific plant toxin-degrading enzymes, e.g., tannases. The variety of the enzymes obtained is attributed not only to the diversity of microbial community but also the multiplicity of fibrolytic enzymes produced by individual microbes, which act synergistically to facilitate the hydrolysis of biomass polymers.

The major cellulolytic species isolated from rumen for several decades have been the Gram-negative *Fibrobacter succinogenes* and Gram-positive *Ruminococcus flavefaciens* and *R. albus* [18]. *R. flavefaciens* and related species are also well represented in sequences from human fecal fiber samples. Relatively recently, a novel anaerobic bacteria *Cellulosilyticum ruminicola* isolated from yak rumen was described to possess diverse catalytic potential with multiple fibrolytic enzymes including cellulases, xylanase, pectinase, mannanase, feruloyl- and acetyl-esterases, the majority of them with CBMs [19].

There are also highly abundant species of bacteria in rumen which, even if lacking the ability to degrade intact plant cell walls, produce multiple polysaccharide hydrolases including xylanase, pectinase, and cellulase. The predominant hemicellulose-digesting bacteria such as *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* degrade xylan and pectin but not cellulose.

Anaerobic fungi and protozoa in the rumen have an important role in the initial degradation of biomass particles. The cellulolytic enzymes produced by the fungi such as *Neocallismastix* sp. and *Piromyces* sp., also include both multicomponent complexes and free enzymes which are highly active and are able to degrade wider range of substrates than the bacteria [20, 21]. The cellulases and xylanases produced by the protozoans lack the multimodular organization displayed by the bacterial and fungal enzymes, and are secreted into food vacuoles [18].

The increasing use of metagenomic analyses continues to provide information on the diversity of biomass-degrading enzymes including novel ones, without being limited by the constraints of cultivability of the organisms, and we can see their use in the processing of biomass through individual enzymes, enzyme complexes, or consolidated bioprocessing.

3.2 Anaerobic Fermentation of Sugars to Alcohols

3.2.1 Ethanol

Ethanol from biomass feedstocks is among the largest fermentation product available globally, estimated volume in 2013 being 88.69×10^9 L [22]. The primary

motive underlying the enormous interest in the production of ethanol has been for its use as a biofuel in spite of the fact that it does not have a high energy content. Nevertheless, it is a good platform for the chemicals and plastics industries; for example, the partly biobased plastic, polyethylene terephthalate (PET), PlantBottle, introduced recently by Coca-Cola is made from ethylene produced from bio-ethanol.

Saccharomyces cerevisiae remains the preferred organism for production of ethanol even though the bacterium *Zymomonas mobilis* is suggested to possess superior characteristics in giving three- to fourfold higher productivity than *S. cerevisiae* [23]. *Z. mobilis* is not suitable for industrial production because of its narrow substrate specificity and the cell mass not being suitable as animal feed. The glycolytic pathway of glucose metabolism gives 2 mol of pyruvate which are converted to 2 mol of ethanol and CO_2, giving a theoretical yield of 0.511 for ethanol and 0.489 for CO_2 per unit mass of glucose. Besides ethanol, glycerol, acetate, and lactate are also formed as by-products which reduce somewhat the ethanol yield. The ethanol yield in the industrial processes is kept at 90–93% of the theoretical value, with low concentrations of the residual sugar.

Feedstock and energy consumption are the main cost-contributing factors in the production of biobased ethanol. The current industrial production of ethanol is based on sugarcane (in Brazil), cornstarch (in USA), and industrial wheat (in Europe) but processes based on lignocellulosic feedstocks, e.g., from agricultural or forest residues or grasses, are being developed which implies greater challenges in terms of biomass pretreatment and resourceful and efficient use of its components. Furthermore, various strategies for process improvements to increase product yields, and energy and cost efficiency are being investigated both at the level of the microbe and the process [24]. Efforts to develop organisms capable of assimilating both hexoses and pentoses arising from cellulose and hemicellulose, and with higher tolerance to the inhibitory products in the lignocellulose hydrolysate, are being made [25]. However, the rate and yield of ethanol production from xylose still remain much lower than those from glucose.

Simultaneous saccharification and fermentation (SSF), i.e., combining the enzymatic degradation of cellulose or starch with fermentation of glucose obtained from these polysaccharides, has shown higher productivities [26]. On the other hand, consolidated bioprocessing (CBP) involving direct microbial conversion of the biomass to bioethanol achieved by engineering *S. cerevisiae* with genes-encoding cellulases and hemicellulases is gaining recognition as a highly integrated and cost-effective system [27, 28] (Fig. 2). Some studies on CBP involving the bacterium *Clostridium thermocellum* used for cellulase production, cellulose hydrolysis, and glucose fermentation, and simultaneous conversion of pentoses obtained from hemicellulose hydrolysis by co-fermentation with *C. thermosaccarolyticum* have shown higher substrate conversion [29], but are limited by the low tolerance of clostridia to ethanol and formation of other organic acids as by-products, resulting in lower ethanol yields. Obtaining strains of *C. thermocellum* with high ethanol tolerance (exceeding 60 g/L ethanol) has been shown to be possible [27].

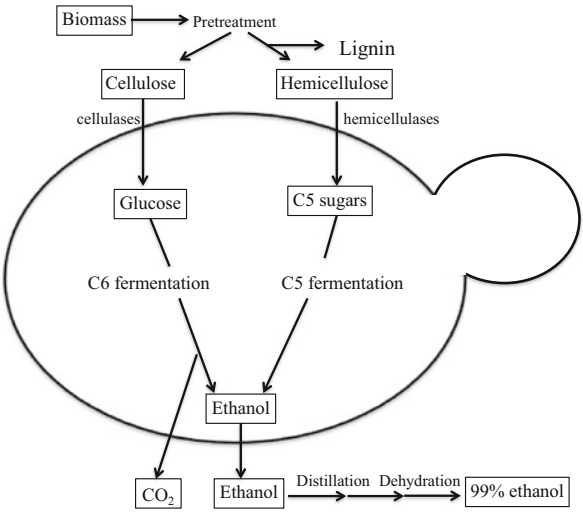


Fig. 2 Consolidated bioprocessing concept for ethanol production from lignocellulosic raw material using engineered *Saccharomyces cerevisiae*

Several studies on ethanol fermentation using immobilized cells have been reported but with limited success. The method most often used for cell immobilization, i.e., entrapment in gel matrices, affects cell growth while ethanol production is linked to the production of cell mass. Moreover, cell growth results in the disruption of the gel matrix. On the other hand, adsorption onto the surface of an inert carrier does not affect the cell growth significantly and presents a more reasonable choice for cell immobilization. On the whole, immobilization protects the cells from the inhibitory conditions of ethanol fermentation [30]. Spontaneous self-flocculation of yeast cells has provided a simple means for their separation and a much superior alternative to the immobilization on carriers. The flocculated cells are more tolerant than the free cells [31], can be purged from the fermentor under controlled conditions, and can finally be recovered by sedimentation rather than by centrifugation. A process for ethanol fermentation with an annual production capacity of 200 kt using self-flocculating yeast has been operated commercially in China since 2005 [30].

In situ product recovery is yet another approach investigated for process improvement by alleviating product inhibition, e.g., by integration of pervaporation or performing vacuum fermentation. Use of thermophilic organisms for performing ethanol fermentations at high temperatures and hence facilitating continuous distillation or "stripping" of ethanol from the fermentation broth has also been reported [32]. To make this approach commercially viable requires development of strains tolerant to ethanol and other inhibitors, and ideally possessing broader substrate specificity. Although efforts on improving the cost efficiency of the in situ product recovery approaches are ongoing, currently bioreactor engineering strategies to alleviate ethanol inhibition by decreasing backmixing are being used, e.g., by replacing a single continuous stirred tank reactor with tanks in series [30, 33].

The approach for process improvement by increasing fermentation rate and ethanol concentration that seems to have gained attention is that of very high

gravity (VHG) fermentation performed by using a fermentation medium with sugar concentration in excess of 250 g/L to achieve higher than 15 vol% ethanol compared with 10–12 vol% generally obtained in industrial fermentations [34, 35]. Many strains of *S. cerevisiae* tolerate much higher concentrations of ethanol than previously believed; this is strongly dependent on the nutritional conditions, e.g., availability of assimilable nitrogen, etc. and protective functions of some nutrients such as glycine as an osmoprotectant against high substrate concentration [35]. Furthermore, biomass accumulation and cell viability is improved by controlling redox potential during VHG fermentations [36]. VHG is expected to result in water savings and reduced distillation costs, reduced effluent and treatment costs, which comprise the major fraction of energy costs, accounting for 30% of the total production costs of ethanol production.

3.2.2 Butanol

Butanol has superior fuel properties compared to ethanol, which has led to renewed interest in its production by fermentation which was discontinued around the mid-twentieth century because of competition from the petrochemical industry. The solventogenic clostridia, *Clostridium acetobutylicum* and *C. beijerinckii* have been most commonly used for acetone-butanol-ethanol (ABE) fermentations, although the use of other strains including *Clostridium pasteurianum*, *C. sporogenes*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* has also been reported [37]. The ABE fermentation is characterized by low productivity because of low cell concentration and product inhibition giving a maximum total solvent (ABE) concentration in the range of 20 g/L. The metabolism of clostridia strains comprises an acidogenic phase characterized by conversion of the substrate to acids (acetic and butyric acids) and exponential cell growth and ATP formation, followed by a solventogenic phase in which the substrate and acids are converted to the solvents (Fig. 3).

The potential of improving the microbial strains and also the fermentation and downstream recovery operations for obtaining processes with higher yield and productivity has been shown [38]. For example, a hyper-amylolytic, hyper-butanol-producing strain of *C. beijerinckii* BA101, developed using chemical mutagenesis, was shown to produce and tolerate 33 g/L total solvents in batch culture, an increase of 69% over the parental strain [39]. In another study, disruption of the pathway of acetone production in *C. acetobutylicum* increased the ratio of butanol in the solvent from 71% to 80% [40].

Improved cell growth and reactor productivities have even been reported by integrating product recovery with the fermentation step. Product recovery by gas stripping has been performed by bubbling CO_2 or H_2 through the reactor for capturing the solvent followed by passing the gas through a condenser to recover the solvent and recycling the gas back to the fermentor [41]. Using fed-batch fermentation with *C. beijerinckii* BA101 integrated with gas stripping, consumption of 500 g glucose with production of 233 g total solvent (ABE) and productivity of

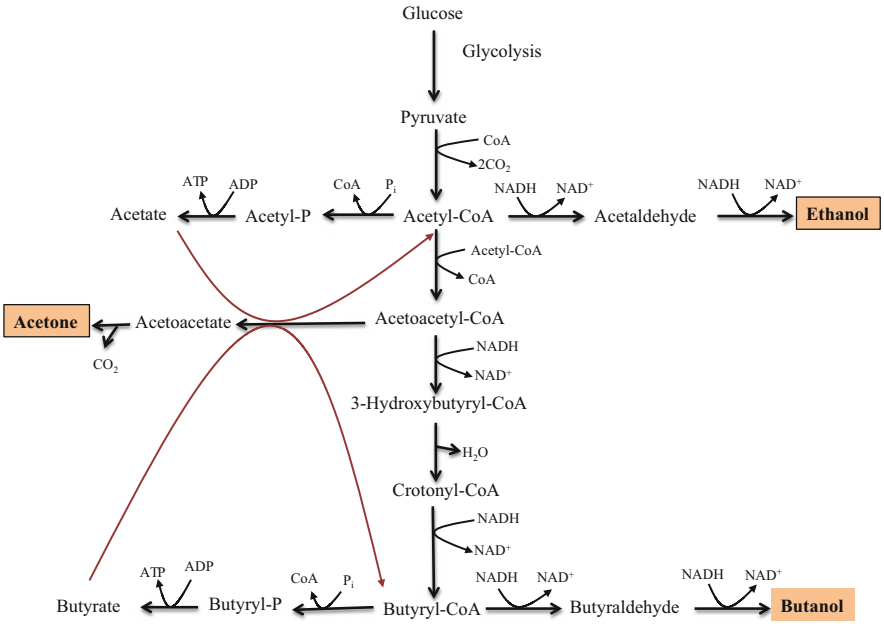


Fig. 3 Metabolic pathway for the formation of acetone–butanol–ethanol by clostridial species. Acetic- and butyric acids are the predominant products during the early stages of fermentation. With drop in pH, the synthesis of the acids is stopped and solvents start to accumulate

1.16 g/L.h as compared to 162 g glucose consumption and about 76 g solvent obtained in a batch system with gas stripping was reported [41]. In an alternative fed-batch system integrated with pervaporation, 165 g total solvent was obtained by consumption of 384 g glucose at a productivity of 0.98 g/L.h [38, 42]. Use of membrane assisted extractive fermentation in which the extractant is separated from the fermentation broth by a porous membrane increased the glucose consumption from 59.4 to 86 g/L and butanol production from 16 to 20.1 g/L [43]. Flash fermentation is yet another way used for in situ butanol recovery and to decrease its concentration in the fermentation broth [44]. The liquid fraction from the separator is returned to the fermentor and the butanol-enriched vapor is sent for product recovery by distillation.

In continuous fermentations, although the productivities are improved the product concentrations are lower and do not remain stable for long periods. On the other hand, continuous fermentations using immobilized cell reactors provide higher cell densities and productivities. The reactor productivity could be improved 40–50 times in fermentations using *C. beijerincki* cells adsorbed onto clay brick particles [45]. The problem was, however, the blockage of the reactor with time because of excessive cell growth, and a significant fraction of the cell biomass was inactive as spores. More recently, a biofilm reactor containing *C. acetobutylicum* growing on fibrous matrices integrated with product recovery by adsorption onto a resin was reported to give improved production efficiency of butanol [46]. Co-adsorption of

acetone to the resin improved the reactor performance and redox modulation by methyl viologen maintaining a high butanol:acetone ratio.

As in the case of ethanol and other products, substrate cost is the prime determinant factor for commercial production, and hence lignocellulosic substrates such as wheat straw and corn stover are being investigated as raw materials, even for butanol production [47, 48].

3.3 Anaerobic Biotransformation of Glycerol

The interest in valorization of glycerol gained momentum with its ready availability as a by-product (10% of the total product) of biodiesel production. Glycerol is also obtained in large amounts during production of bioethanol and processing of oils and fats. Many microorganisms are able to utilize glycerol in the presence of external electron acceptors, although few are able to do so fermentatively. Because of the reduced nature of carbon atoms in glycerol, its fermentation generates twice the amount of reducing equivalents than that produced from sugars, giving glycerol an advantage for the production of reduced chemicals and fuels [49, 50].

Much of the focus on microbial transformation of glycerol has been on products naturally produced by anaerobic microorganisms. 1,3-Propanediol (1,3-PDO) is the most extensively investigated product of glycerol fermentation although the industrial production of the diol is achieved from glucose using engineered *Escherichia coli*. 1,3-PDO is used as a renewable monomer for the polyester polymethylene terephthalate (PTT) used in fibers, textiles, and carpets. Production of 1,3-PDO from glycerol was reported as early as 1881 in a glycerol fermenting mixed culture containing *C. pasteurianum* [51]. Subsequently, quite a few microorganisms have been shown to possess the ability to convert glycerol to 1,3-PDO.

Several species of the Enterobacteriaceae family of the genera *Klebsiella* (*K. pneumonia*), *Enterobacter* (*E. agglomerans*), *Citrobacter* (*C. freundii*), and clostridia (*C. butyricum* and *C. pasteurianum*) convert glycerol to 1,3-PDO through two pathways [52]. In the oxidative pathway, glycerol is dehydrogenated by NAD-dependent glycerol dehydrogenase to dihydroxyacetone, which is then phosphorylated and enters the glycolytic pathway to form pyruvate which is further converted to different products depending on the bacterial species. In a parallel reductive pathway, also known as the propanediol utilization (Pdu) pathway, glycerol is dehydrated by glycerol dehydratase (a coenzyme B_{12}-dependent enzyme except in *C. butyricum*) to 3-hydroxypropionaldehyde (3-HPA), which is reduced to 1,3-PDO by NADH-dependent 1,3-PDO dehydrogenase. This pathway provides a means to achieve a redox balance in the absence of electron acceptors by regeneration of NAD^+ required for sugar metabolism. In *K. pneumonia* and *C. freundii*, glycerol fermentation yields 1,3-PDO and ethanol or acetic acid as the main products [53], whereas the clostridial strains produce different co-products such as butyric acid, butanol, lactic acid, acetic acid, ethanol, etc., depending on the species [49, 52]. Even the co-production of 1,3-PDO with hydrogen at high yields

by fermentation of crude biodiesel derived glycerol using heat-treated mixed cultures has been demonstrated [54].

High levels of 1,3-PDO have been reported during glycerol fermentation by natural and mutant strains. For example, mutant strains of *K. pneumonia* were able to reach 1,3-PDO titers of 103 g/L as compared to 59 g/L for the wild type strain [55]. However, the pathogenicity of the organism poses limitation for large-scale production. A non-pathogenic strain of *K. pneumonia* BLh-1 immobilized in calcium alginate beads was used for repeated transformation of glycerol residue from biodiesel manufacturing plant, showing good operational stability and 1,3-PDO productivities as high as 4.48 g/L.h [56].

Among the non-pathogenic organisms, a number of *Clostridium* strains are known to produce 1,3-PDO; *C. butyricum* produces 94 g/L 1,3-PDO when grown on glycerol as sole carbon source [57]. Engineering of the 1,3-PDO generation pathway from *C. butyricum* into *C. acetobutylicum*, a strain that does not naturally ferment glycerol has also been successfully achieved [58].

Members of the strain *Lactobacillus*, which are commonly used in the food industry, have also been shown to produce 1,3-PDO. Lactobacilli (e.g., *Lactobacillus brevis*, *L. buchneri*, *L. reuteri*) have only the Pdu pathway for conversion of glycerol to 1,3-PDO as they lack the glycerol dehydrogenase needed for glycerol oxidation, and hence need an additional substrate for growth and generation of reducing equivalents. High productivity (0.85 g/L.h) and product concentrations (85.4 g/L) have been reached with *Lactobacillus diolivorans* cultivated under anaerobic conditions in a medium containing a mixture of glucose and glycerol at 0.1:1 M ratio [59]. However, lactic acid, acetic acid, and ethanol are formed as by-products. On the other hand, studies with *L. reuteri* showed that resting cells could be used for glycerol transformation without the presence of glucose, hence avoiding the metabolic products of glucose metabolism. However, 3-hydroxypropionic acid (3-HP) is formed as a co-product at an equimolar ratio with 1,3-PDO, which is ascribed to the oxidative branch of the Pdu pathway that converts 3-HPA via three enzymatic steps to 3HP and also enables cofactor regeneration needed for continuous glycerol transformation [60] (Fig. 4).

L. reuteri has also been used for the transformation of glycerol to 3-HPA, an antimicrobial agent and also a potential platform for other C3 chemicals. The Pdu pathway is encapsulated inside protein microcompartments in the microbial cells which prevents the exposure of cytosol to the toxic effects of the aldehyde [61]. 3-HPA mediated product inhibition and its further conversion to the downstream products during its production is also reduced by in situ complexation of the aldehyde with free or immobilized semicarbazide or bisulfite [62, 63]. Subsequent recovery of 3HPA from the complex is still a challenge.

Studies on fermentation of glycerol to other alcohols such as *n*-butanol by *C. pasteurianum* [64], ethanol and formate by *Klebsiella planticola* [65], and ethanol and hydrogen by *Enterobacter aerogenes* mutant [66] have also been reported.

Organic acids are other products formed by anaerobic fermentation of glycerol [6]. The bacteria belonging to the genera Propionibacteria produce propionic acid

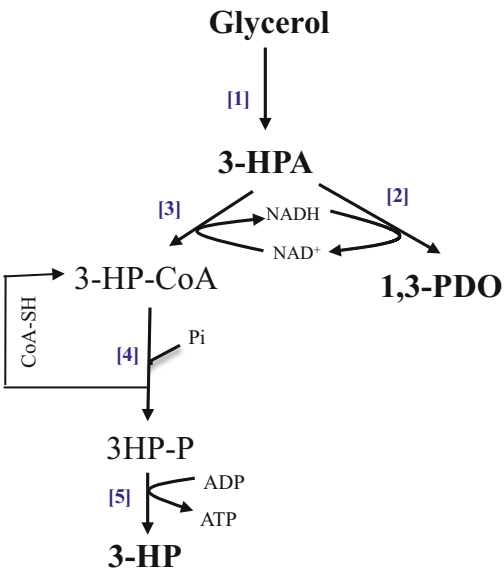


Fig. 4 The propanediol utilization pathway in *Lactobacillus reuteri* and some other anaerobic microorganisms. The numbers indicate reactions catalyzed by: (*1*) glycerol dehydratase, (*2*) 1,3-propanediol oxidoreductase, (*3*) propanediol dehydrogenase (PduP), (*4*) phosphotransacylase (PduL), and (*5*) propionate kinase (PduW)

using the Wood Werkman cycle, and, as indicated above, the use of glycerol is advantageous because of its reduced nature and formation of less by-products even though the growth rate is lower compared to the sugar substrate. High cell density fermentations by recycling the free or immobilized cells for repeated fermentations result in higher propionic acid productivity [67, 68]. Production of succinic acid using *Anaerobiospirillum succiniproducens* and *Actinobacillus succinogenes* from glycerol in the presence of CO_2 has also been investigated [69, 70], with reduced formation of the acetic acid by-product. The product yield in the latter case was increased by directed evolution of the organism.

Because the use of several wild type organisms for industrial production involves bottlenecks attributed to their pathogenicity, the need for complex media and lack of knowledge of physiology of the organisms and the genetic tools necessary for their manipulation, metabolic engineering of *E. coli* with the desired pathways has been suggested to provide a platform for the production of chemicals from glycerol. *E. coli* has been shown to ferment glycerol when grown anaerobically to ethanol and 1,2-propanediol (1,2-PDO) [71, 72]. It has been proposed that 1,2-PDO production from glycerol provides a means to consume the reducing equivalents generated during the production of cell mass, whereas ethanol production through a redox balanced pathway provides energy by generating ATP by substrate-level phosphorylation.

3.4 Production of Chemicals and Fuels from Carbon Dioxide

Gas feedstocks are attracting increasing attention as raw materials for synthesis of fuels and chemicals. CO_2 is present in abundance in anoxic environments and also formed as a by-product of industrial activities. Microbial reduction of CO_2 is a process of ecological significance and is forecasted to have great industrial potential. Both prokaryotes and archaea possess different metabolic pathways for CO_2 fixation utilizing H_2, H_2S, S, CO, NH_3, metal sulfides such as pyrite (FeS_2), and reduced metal ions as electron donors. ATP is generated by a chemiosmotic mechanism, i.e., the proton motive force generated by the electron flow from the reduced inorganic substrate to the oxidized electron acceptor coupled to the transduction of H^+ or Na^+ across the cell membrane, and the reducing power is provided by the oxidation of the reduced inorganic substrates.

Fermentation of syngas (containing CO, CO_2, and H_2), produced from biomass or fossil feedstocks, described in [9], is an important example of the microbial route for conversion of gas mixtures to a variety of products. Several strictly anaerobic acetogenic bacteria belonging to Proteobacteria, Planctomycetes, Spirochaetes, and Euryarchaeota (e.g., certain *Acetobacterium* spp., *Clostridium* spp., *Desulfobacterium* spp., *Eubacterium* spp., and *Moorella* spp.) are capable of syngas/CO_2 fermentation using the *reductive acetyl-CoA pathway* or *Wood-Ljungdahl* (WL) metabolic pathway to acetate [9, 73, 74]. Electrons required for the metabolic process are obtained from H_2 via the hydrogenase enzyme and/or from CO via the CO dehydrogenase enzyme:

$$2CO_2 + 4H_2 \longrightarrow C_2H_4O_2 + 2H_2O,$$
$$4CO + 2H_2O \longrightarrow C_2H_4O_2 + 2CO_2.$$

Although Lanza Tech has developed syngas fermentation based on the WL pathway for commercial scale production of ethanol [9], Evonik uses a CO_2-based acetone fermentation process in which the acetogens are genetically modified to divert the acetyl-CoA from the WL pathway to acetone via acetoacetyl-CoA and acetoacetate [75]. Recently, *Moorella thermoacetica*, a thermophilic acetogenic bacterium which produces acetic acid as the only end product of the WL pathway, was used in a bubble column reactor for conversion of syngas to acetic acid with improved productivity [76]. Fermentation of CO by *Clostridium carboxidivorans* has also been performed, which showed the formation of acetic acid, butyric acid, and ethanol when pH was not regulated, whereas with pH regulation ethanol and butanol were formed both from CO fermentation and from the bioconversion of acetic- and butyric acids [77]. Furthermore, CO has even been used as a raw material for the production of polyhydroxyalkanoate bioplastic by enabling its biological conversion to CO_2 using carbon monoxide dehydrogenase and a CO-binding protein bound to the cell surface of the CO_2 utilizing *Ralstonia eutropha* [78].

Besides the WL pathway, other pathways are used by different organisms for CO_2 fixation [73, 74, 79]. The most widespread and well studied CO_2 fixation pathway is the *Calvin–Benson–Bassham* (CBB) cycle in autotrophic bacteria and eukaryotes which utilizes the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) for catalyzing the reaction between CO_2, ribulose-1,5-biphosphate (RuBP) and water to give two molecules of 3-phosphoglycerate which are phosphorylated and reduced to glyceraldehyde-3-phosphate (GAP), a key intermediate in sugar metabolism. After three molecules of CO_2 are fixed by involving three molecules of RuBP, six molecules of GAP are formed, five of which are used for regeneration of RuBP and one is used for biosynthesis of cell material. Among the anaerobes, the CBB cycle is utilized by purple nonsulfur (e.g., *Rhodobacter*, *Rhodospirillum*, and *Rhodopseudomonas*) and purple sulfur bacteria (e.g., *Chromatium*) and hydrogen bacteria (e.g., *Ralstonia* and *Hydrogenovibrio*), but is absent in archaea.

The *reductive tricarboxylic acid* (RTCA) or *Arnon–Buchanan* cycle involves the reverse flux of the aerobic TCA cycle for CO_2 fixation. Present in chemoautotrophic bacteria, green sulfur bacteria (*Chlorobium limicola* and *Chlorobaculum tepidum*) and sulfate reducing *Desulfobacter hydrogenophilus*, the RTCA cycle produces one molecule of acetyl-CoA from two molecules of CO_2 and eight reducing equivalents (Fig. 5). The acetyl-CoA is subsequently converted to pyruvate and phosphoenol-pyruvate used to regenerate the intermediates of the TCA cycle using four CO_2 fixing enzymes. The net result is the formation of one oxaloacetate molecule from four molecules of CO_2.

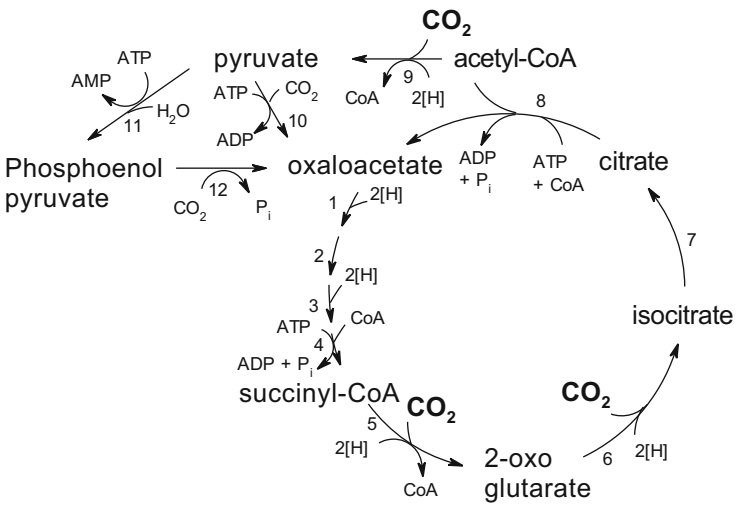


Fig. 5 The reductive tricarboxylic acid cycle. The enzymes catalyzing the different reactions are: (*1*) malate dehydrogenase, (*2*) fumarate hydratase, (*3*) fumarate reductase, (*4*) succinyl-CoA synthetase, (*5*) oxoglutarate synthase, (*6*) isocitrate dehydrogenase, (*7*) aconitate hydratase, (*8*) ATP-citrate lyase, (*9*) pyruvate synthase, (*10*) pyruvate carboxylase, (*11*) phosphoenolpyruvate synthase, and (*12*) phosphoenolpyruvate carboxylase [74]

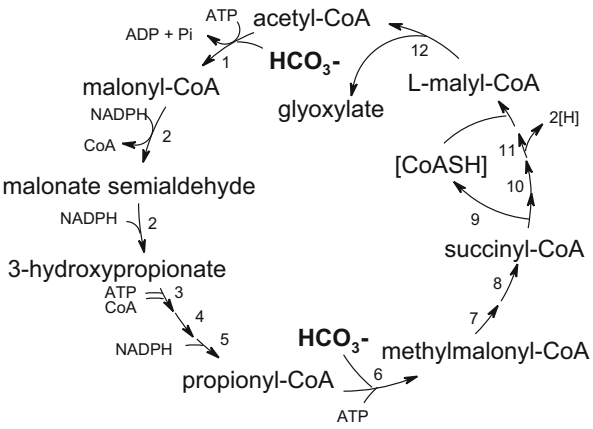


Fig. 6 The 3-hydroxypropionate/malyl-CoA cycle. The enzymes catalyzing the different reactions are: (*1*) acetyl-CoA carboxylase, (*2*) malonyl-CoA reductase, (*3*) 3-hydroxypropionyl-CoA synthetase, (*4*) 3-hydroxypropionyl-CoA dehydratase, (*5*) acryloyl-CoA reductase, (*6*) propionyl-CoA carboxylase, (*7*) methylmalonyl-CoA epimerase, (*8*) methylmalonyl-CoA mutase, (*9*) succinyl-CoA-L-malate-CoA transferase, (*10*) succinate dehydrogenase, (*11*) fumarate hydratase, and (*12*) L-malyl-CoA lyase [80]

The 3-hydroxypropionate/malyl-CoA cycle occurs in some green non-sulfur bacteria of the family Chloroflexaceae. The process consists of two overlapping metabolic cycles as observed in *Chloroflexus aurantiacus*. Two molecules of bicarbonate and acetyl CoA are used as starting materials to generate succinyl-CoA through several enzymatic steps. The CoA group of succinyl-CoA is transferred to malate to give maloyl-CoA which undergoes cleavage for regeneration of acetyl-CoA, yielding glyoxylate as a side product (Fig. 6).

The hydroxypropionate-hydroxybutyrate cycle is functional in facultative and strictly anaerobic Sulfolobales species comprising extreme thermoacidophiles (e.g., *Metallosphaera sedula*) growing on sulfur, pyrite, or H_2 under microaerobic conditions. In this cycle, one acetyl group is formed from two molecules of bicarbonate; the key carboxylating enzyme is the bifunctional biotin-dependent acetyl-CoA-propionyl-CoA carboxylase. The first half of the cycle involves conversion of acetyl-CoA to succinyl-CoA in a manner similar to 3-hydroxypropionate/maloyl-CoA cycle, and in the second half of the cycle, two molecules of acetyl-CoA are regenerated from succinyl-CoA through 4-hydroxybutyrate (Fig. 7).

The most recently discovered CO_2 fixing pathway, the dicarboxylate/4-hydroxybutyrate cycle was first reported in hyperthermophilic archaeon *Ignococcus hospitalis* in 2008 [82] and is found in anaerobic or microaerobic autotrophic members of Thermoproteales and Desulfurococcales. The cycle also comprises two stages: conversion of acetyl-CoA to succinyl-CoA (as in the RTCA cycle) and vice versa (as in the 3-HP/4-hydroxybutyrate cycle), with a net result of

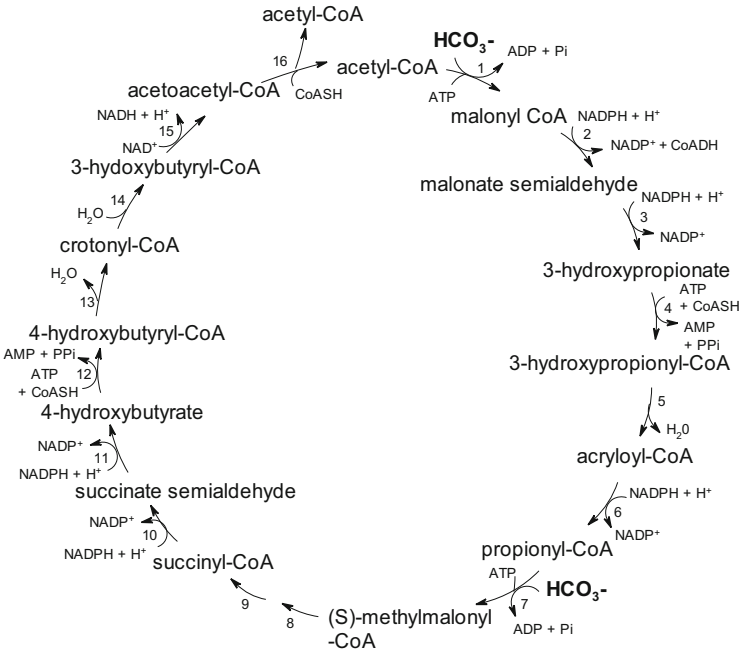


Fig. 7 The hydroxypropionate-hydroxybutyrate cycle. The reactions are catalyzed by the following enzymes: (*1*) acetyl-CoA carboxylase, (*2*) malonyl-CoA reductase, (*3*) malonate semialdehyde reductase, (*4*) 3-hydroxypropionyl-CoA synthetase, (*5*) 3-hydroxypropionyl-CoA dehydratase, (*6*) acryloyl-CoA reductase, (*7*) propionyl-CoA carboxylase, (*8*) methylmalonyl-CoA epimerase, (*9*) methylmalonyl-CoA mutase, (*10*) succinyl-CoA reductase, (11) succinate semialdehyde reductase, (*12*) 4-hydroxybutyryl-CoA synthetase, (*13*) 4-hydroxybutyryl-CoA dehydratase, (*14*) crotonyl-CoA hydratase, (*15*) (S)-3-hydroxybutyryl-CoA dehydrogenase, and (*16*) acetoacetyl-CoA β-ketothiolase [81]

formation of acetyl-CoA from one molecule of CO_2 and one molecule of bicarbonate.

It is evident that these CO_2 fixation pathways involve several intermediates besides acetyl-CoA which can serve as important building blocks for the biobased industry.

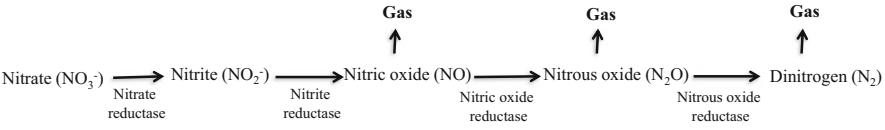
4 Anaerobes and Environmental Remediation

Anaerobic degradation is widely used in wastewater treatment to degrade the solids from primary and secondary treatment, in the treatment of industrial effluents and in bioremediation of contaminated soil and groundwater. The degradation processes are dependent on anaerobic respiration in the presence of the electron acceptors (Table 1). When several electron acceptors are present, the organism selects the one

that yields the largest amount of energy by repressing the formation of reductase enzymes for the other electron acceptors. In the absence of O_2, nitrate is the electron acceptor of choice, and when both are absent SO_4^{2-} is the favored electron acceptor. Methanogenesis, a form of carbonate respiration, used in anaerobic digestion to produce methane, is inhibited in the presence of SO_4^{2-} [7]. These applications of anaerobes and their potential for dehalogenation are described in this section. Further details of anaerobic digestion are given in [4]. Frigaard [12] discusses the use of anoxygenic phototrophic bacteria for the removal of hydrogen sulfide from wastewater and gas streams and for the bioremediation of recalcitrant dyes, pesticides, and heavy metals.

4.1 Nitrogen Removal from Wastewaters

The most common inorganic nitrogen compounds in nature besides nitrogen are ammonia and nitrate formed by chemical processes. Their removal is an important process in the treatment of municipal- and industrial wastewaters and is generally achieved by a combination of nitrification and denitrification processes. Nitrification is an aerobic process involving oxidation of ammonium to nitrate via nitrite. In denitrification, NO_3^- or NO_2^- is converted to dinitrogen gas via NO_2, NO, and N_2O under anaerobic conditions in the presence of organic carbon [10], the main biological route for atmospheric N_2 formation. Production of all the enzymes involved in this pathway is repressed by oxygen.



NO_3^- is one of the most common alternative electron acceptors in anaerobic respiration. The first step in the dissimilative nitrate reduction to nitrite is catalyzed by membrane-bound enzyme nitrate reductase. The lower reduction potential of the NO_3^-/NO_2^- couple (+0.43 V) as compared to the O_2/H_2O couple (+0.82 V) results in only two proton translocating steps. In *E. coli*, NO_3^- is reduced only to NO_2^- whereas in other denitrifying organisms, such as *Paracoccus denitrificans* and *Pseudomonas stutzeri*, NO_2^- is further reduced via three reductive steps to N_2. The proton motive force established as a result of electron transport leads to ATP formation, as described above. Complete denitrification is accompanied by generation of an additional ATP molecule resulting from proton extrusion in the reaction catalyzed by NO reductase.

Some organisms can reduce the NO_2^- to NH_3 in a dissimilative process. Most denitrifying bacteria are facultative aerobes and under anaerobic conditions can use even other electron acceptors such as Fe^{3+} and certain organic compounds for energy generation, and can also grow by fermentation.

During the past two decades, an alternative, less energy intensive process compared to the conventional nitrification-denitrification has been developed [83]. The anammox (anaerobic ammonium oxidation) process involves oxidation of ammonium to N_2 gas under anoxic conditions [84, 85]:

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O \quad \left(\Delta Go' = -357 \text{ kJ per reaction}\right)$$

NO_2^- is the preferred electron acceptor, which is reduced to N_2 via hydroxylamine and hydrazine as intermediates [86]. The source of NO_2^- is from the oxidation of ammonia by aerobic nitrifying bacteria, which coexist with the anaerobic anammox bacteria in ammonia-rich wastewaters with suspended particles providing oxic and anoxic zones. The anammox process is mediated by a specialized group of bacteria belonging to the phylum Planctomycete, of which five genera have been identified up to now: *Candidatus* Brocadia, *Ca*. Kuenenia, *Ca*. Anammoxoglobus, *Ca*. Jettenia isolated from activated sludge plants, and *Ca*. Scalindua obtained from the natural habitat [87]. The bacteria can use CO_2 as the sole carbon source although the mechanism of CO_2 fixation is not fully understood. They have an extremely low growth rate with an estimated doubling time of 11 days [88]. The anammox reactions have been found to occur inside a cytoplasmic membrane-bound compartment called anammoxosome in *Brocadia anammoxidans* [86].

The anammox process design involves several considerations, including partial oxidation of NH_4^+ to NO_2^-, inhibitory effects of NO_2^- (above 50–150 mg N/L) and dissolved O_2 [87, 89]. This requires a balance between the different microbial groups involved; the most practical approach to limit nitrite oxidation is the reactor operation under oxygen limited conditions that favors growth of ammonium oxidizing bacteria vs NO_2^- oxidizing bacteria whose oxygen affinity is higher and face additional competition for nitrite by the anammox bacteria.

Several technologies have been developed and successfully implemented in about 100 full scale plants during the past decade. Early processes using two-stage reactor configurations have been replaced by single-stage systems, e.g., moving bed biofilm reactor (MBBR), granular sludge bioprocesses, and sequential batch reactor (SBR). The latter is the most commonly applied reactor type (more than 50% of all partial nitritation and anammox processes) followed by granular systems and MBBRs. The different treatment facilities differ in the control strategies for feed control, intermittent or continuous feeding, suspended or attached biomass, etc. [87]. The ANAMMOX® process tested since 2002 has become a cost-effective and sustainable way of removing ammonium from effluents low in organic matter and ammonia from waste gas. Compared to conventional nitrification/denitrification, it leads to savings on operational costs of up to 60%, and

decrease in the need for organic carbon by 100%, aeration requirements by 60%, and sludge production by about 90% [87].

4.2 Sulfate Reduction and Heavy Metal Removal

Sulfate-reducing bacteria (SRB) are obligate anaerobes that decompose simple organic compounds (e.g., lactate, acetate, propionate, butyrate, hydrogen, etc.) using sulfate as the terminal electron acceptor, resulting in the formation of H_2S and bicarbonate. The eight-electron reduction of sulfate is initiated by activation of sulfate by ATP to form adenosine phosphosulfate (APS) catalyzed by ATP sulfyrase, followed by reduction to sulfite by APS reductase, and sulfite in turn is reduced to sulfide by sulfite reductase. As described above, the electron transport results in a proton motive force and ATP synthesis.

$$2CH_2O + SO_4^{2-} \longrightarrow H_2S + 2HCO_3^-,$$
$$Me^{2+} + H_2S \longrightarrow MeS + 2H^+.$$

The formation of bicarbonate during sulfate reduction helps to control the pH of the microenvironment. Depending on the pH, H_2S can be present in the soluble HS^- or S^{2-} form, or can react with metals such as Cu^{2+}, Zn^{2+}, or Ni^{2+} to precipitate them as metal sulfides. The metal ions can even be precipitated as hydroxides and oxides if the pH becomes high.

Wastewaters containing high concentrations of dissolved heavy metals and with low pH from mining and industrial processing (metallurgical, electronic, electroplating and metal finishing industries, flue gas scrubbing) pose significant environmental hazard as the heavy metals can be distributed over wide areas and then be assimilated by plants and other living organisms. Such waters (acid mine drainage) contain high concentrations of sulfate formed by chemical or biological oxidation of the exposed sulfide minerals, and high acidity in the form of sulfuric acid which can dissolve other minerals, releasing cations. Many methods have been used for treatment of heavy metal contaminated wastewaters such as complexation, precipitation, and absorption which are expensive and leave large amounts of residual sludge. Microbial metal sulfide precipitation has shown promise as an attractive alternative over the physico-chemical methods. Its in concentration of advantages are the low amounts of residual sludge and lower solubility of the sulfides even at low pH (as compared to the hydroxides), facilitating separation and cost effectiveness. SRB are even able to facilitate reduction of metals such as ferric iron, manganese, arsenic, selenium, chromium, uranium, technetium, gold, etc. which is useful not only for waste treatment but also for concentrating metals from low-grade ores. The reduction of metals occurs either as a result of anaerobic respiration or is mediated enzymatically [90–94].

There is quite a rich flora of SRB, which have most probably been important when metal sulfide minerals were formed long ago [95]. In waters with high SO_4^{2-} concentration and high biological oxygen demand (BOD) the process of sulfate reduction might be spontaneous, but at the expense of methane yield because of inhibition of methanogenesis in the presence of SO_4^{2-}. It might be advantageous to reduce the SO_4^{2-} concentration and facilitate methane production simultaneously [96], although it is important first to remove heavy metals because formation of metal sulfides during the digestion process would result in a sludge contaminated with heavy metals. Such a product cannot be used as a fertilizer and is regarded as a risk waste that needs to be deposited under safe conditions.

In the treatment of acid mine drainage, the rate of SO_4^{2-} reduction to sulfide has been found to be the most crucial step, determined by the activity of the SRB [97]. Besides the strict anaerobic environment, a narrow pH range (pH 5–8) is required for optimal SO_4^{2-} reduction by the SRB. Several acidophilic SRB have been isolated that are suitable for remediation of acidic wastewaters [98] Several different sources of SRB have been studied in laboratory set-ups, and a few in larger-scale processes [99]. Other parameters influencing heavy metal precipitation using anaerobic sulfate reduction include the electron donor, sulfate concentration, heavy metal species, and temperature. Toxicity of the heavy metals to SRB may pose a limitation [100], for which more tolerant strains such as *Klebsiella planticola* (which is tolerant to high cadmium concentrations) provide attractive alternatives [101]. H_2S is also toxic for the microorganisms producing it, and hence one cannot reach high concentrations of sulfide in the microbial process (up to 14 mM is reported). Because the solubility of metal sulfides is very low, one can inspite of the low sulphate concentration reach very efficient removal of heavy metals from wastewater.

The carbon is an extra cost, which necessitates the choice of inexpensive material, e.g., organic waste. Use of composted cow manure with hay has been found to be a good substrate with acid neutralization capacity and organic nutrients for growth of the bacteria. The hay also acts as a bulking agent and helps to maintain hydraulic conductivity [97]. Besides hay, porous ceramics and decomposed wood chips also serve as good bulking agents.

SRB have been successfully used in treatment of waters and leachates in large-scale bioreactors and pilot scale studies, mixed SRBs being more effective than pure bacterial cultures. Continuous reactor systems for heavy metal removal with freely suspended cells require high residence time to prevent washout of the cells, although the use of immobilized cells on carrier matrix with high surface area allows short residence times and maintains high biomass retention and reaction rates [102]. Various types of bioreactors have been used for studying the process including (semi-)continuous stirred tank reactors (CSTR) [100, 103], upflow anaerobic sludge blanket reactors (UASB) [104–106], fluidized bed reactors (FBR) [107], permeable reactive barriers (PRB) [108], etc.

The precipitation of metal sulfides can be controlled such that each metal sulfide precipitates as an almost pure metal sulfide before conditions are changed and the next metal is precipitated. Such stepwise precipitation is controlled by changing the

redox potential during the precipitation step [109]. Sequential precipitation would make it possible to recycle metal sulfides, thereby reducing but not eliminating the need for exploiting fresh mines. So far, however, it has been regarded as too expensive and therefore metal sulfide sludge is deposited. When oxygen gets access to such a deposit, then the metal ions start leaking, and the process repeats itself.

SRB have even been used in the removal of heavy metals from soils in an integrated process in which the metals were first mobilized by sulfur-oxidizing bacteria followed by precipitation of the mobilized metals in an anaerobic reactor with SRB [110]. SRB and iron-reducing bacteria such as *Shewanella* and *Geobacter* species are considered to have potential for in situ bioremediation in natural sediments [111].

4.3 Anaerobic Digestion to Produce Biogas: Acetogenesis and Methanogenesis

Anaerobic digestion is a sustainable approach that combines waste treatment with the production of biogas for use as renewable energy and biofuel along with recovery of useful by-products including fertilizer and chemicals [112]. Furthermore, it reduces spontaneous formation and release to the atmosphere of methane from decomposing biomass left in the fields, and also reduces the volumes of waste, making it simpler and cheaper to handle.

The microbiology and technology of biogas production is covered in [4] in this volume. In two of the important steps of this complex process, acetogenesis and methanogenesis, the strictly anaerobic prokaryotes, homoacetogens and methanogens, can use CO_2 as an electron acceptor in energy metabolism and autotrophic growth via the acetyl-CoA pathway, with H_2 as the major electron donor. Acetogenesis uses even other electron donors such as different C_1 compounds, sugars, organic acids, amino acids, etc., yielding acetate as the main product. In methanogenesis, CO_2 is converted to CH_4 with the help of several coenzymes used for binding the CO_2 and redox reactions. In both acetogenesis and methanogenesis, ATP is produced by the generation of proton or sodium motive force, while acetogenesis also involves energy conservation by substrate-level phosphorylation [10].

The most attractive feature of anaerobic digestion is that it can operate on most types of biomass in contrast to the other biofuels, ethanol, butanol, and biodiesel produced from carbohydrates and lipid, respectively. To meet the standards of vehicle fuel, biogas is upgraded by removal of carbon dioxide and hydrogen sulfide, to around 96% methane; in Sweden a minor amount of propane is also added to achieve the same energy content as that of natural gas before it is injected into the natural gas grid.

Production of biogas is one of the largest biotechnology processes operated worldwide besides treatment of water (both wastewater and water for

Fig. 8 Automatic methane potential test system (AMPTS) for monitoring BMP of up to 15 samples simultaneously. Material to be evaluated is mixed with an inoculum, and the gas formed is passed via a scrubber where CO_2 and H_2S are removed. The remaining gas is fed to gas flow meters. Courtesy: Bioprocess Control AB, Lund, Sweden

consumption), and has moved from being an activity to reduce the sludge volume at wastewater treatment plants to being used in industrial plants treating a variety of different substrates: municipal solid waste, food wastes, waste material from agriculture, energy crops, etc. With this transition came several interesting challenges for improvement in productivity by, e.g., optimization of the substrate feed and possibilities to use better process monitoring and control to achieve high volumetric organic loading rates. High-rate anaerobic reactors such as UASB, Expanded Granular Sludge Bed (EGSB), anaerobic filter, anaerobic sequencing batch reactor (ASBR) and FBR can provide loading rates of 10–40 kg COD/m^3 day [8, 112, 113]. Such systems are characterized by high solids retention time caused by biomass immobilization or granulation, and can operate under high hydraulic retention times without any fear of biomass washout [112].

By proper process control one can improve the organic loading rate and maintain control over the process. However, because of the heterogeneity of the raw material and the complexity of the process, monitoring and control of the process becomes a challenging task. As the different components in the crude raw material are digested differently, it is useful to measure the total biogas potential, or more importantly biomethane potential (BMP) of the material before formulating the recipe for the substrate feed to the reactor. An instrument developed recently for measuring the BMP of several samples is shown in Fig. 8. By running several such incubations in parallel one can optimize the feed composition or even monitor the effects of inhibitory compounds present in the feed. Figure 9 shows the monitoring of methane production with respect to time for some well defined substrates and their mixtures.

The types of substrates metabolized during anaerobic digestion have an impact on the methane yield; carbohydrate and protein give high yield because both acetogens and methanogens are involved in metabolizing these substrates to

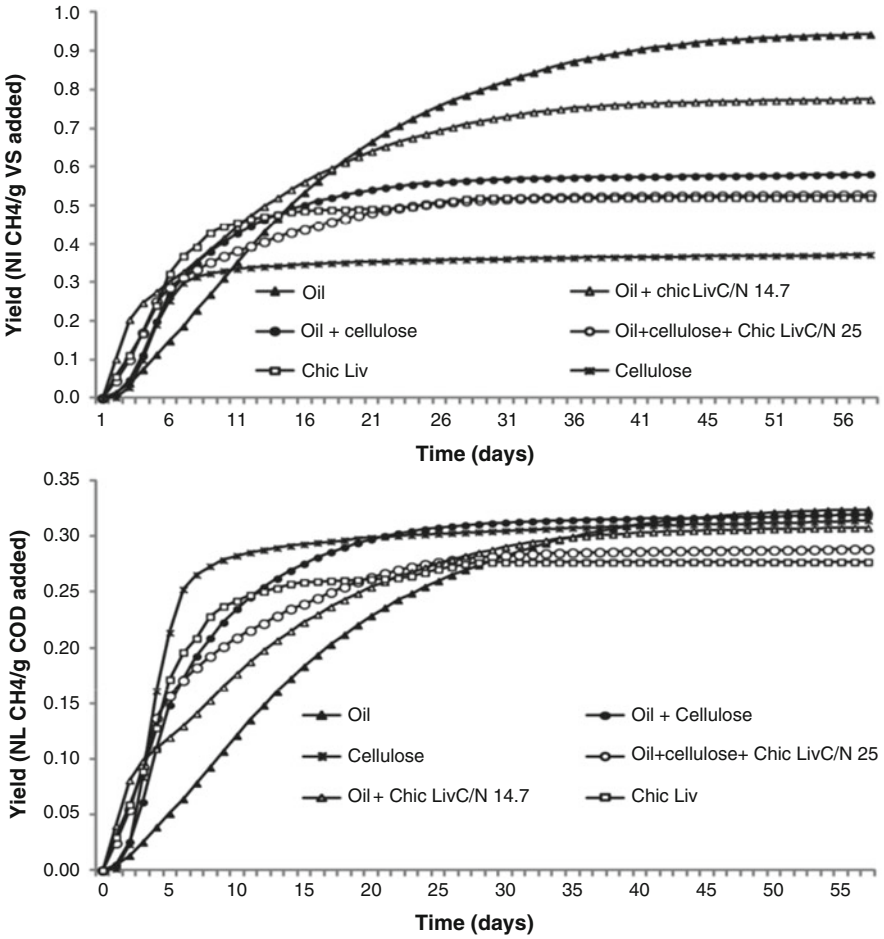


Fig. 9 Methane yield as a function of volatile solids and chemical oxygen demand (COD), respectively, in various synthetic media containing carbohydrates, fats, and protein when used separately and in different combinations. The different substrate mixtures were: oil *filled diamonds*; chicken liver *open squares*; cellulose *filled squares*; oil + cellulose *filled circles*; oil + chicken liver (C/N ratio 14,7) *open triangles*; oil + cellulose + chicken liver (C/N ratio 25) *open circles*

methane, whereas acetate and hydrogen give relatively low yields because only methanogens are involved. Intensification of methanogenesis has been demonstrated by increasing the hydrogen-producing capacity of the microbial consortium by adding the natural hydrogen-producing bacterium *Caldicellulosiruptor saccharolyticus* to the reactor [114]. Increase in biogas formation by 160–170% was achieved. The bacterium has the added advantage of having cellulolytic activity and hence would be suitable for digestion of lignocellulosic biomass.

It is possible to monitor many parameters characterizing the performance of an anaerobic digestion process such as temperature, stirring speed, contents of organic

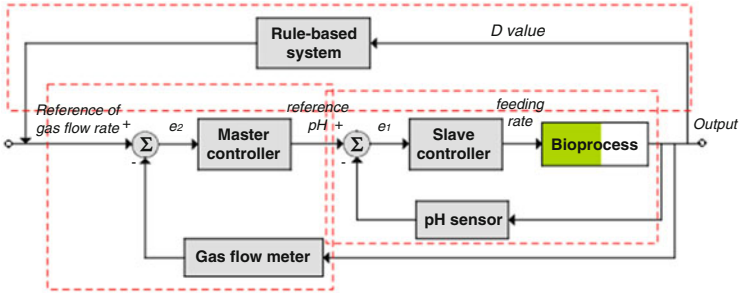


Fig. 10 Component block diagram of a rule based-system with extremum-seeking feature. (From [115], with permission)

acids, pH, alkalinity, hydrogen concentration, volume of gas formed, and gas composition (see [4]). One needs to have a very good model to be able to integrate the different signals in an efficient way. There is no single solution to the problem of defining optimal control strategies. A strategy to control based on gas volume and pH is shown in Fig. 10. By using two controllers with different weight (one master controller and one slave) it is possible to integrate two or more signals via a control algorithm. This gives a more accurate process control than applying control based on just one signal. Proper process control can improve productivity by at least 100%.

4.4 Anaerobic Dehalogenation

Several chlorinated compounds function as electron acceptors for anaerobic respiration by certain microorganisms capable of dehalogenating or completely mineralizing halogenated hydrocarbons by fermentative-, oxidative-, or reductive pathways [10, 116]. The chlorinated compounds are of natural or anthropogenic origin, and can be harmful to human health and environment because of their high toxicity, persistence, and bioaccumulation. During the past decades, research has been focused on halorespiring microbes which couple the reductive dehalogenation using specific enzyme systems to energy conservation via electron transport coupled phosphorylation, yielding between −130 and −180 kJ/mol of chlorine removed and a corresponding redox potential of +0.25 − +0.60 V [116] (Table 1). Halorespiring microbes have been isolated from both pristine and contaminated environments. Isolates belonging to the genera *Desulfitobacterium*, *Desulfuromonas*, *Desulfovibrio*, *Desulfomonile*, and *Trichlorobacter* are versatile with respect to their electron acceptors and donors. *Desulfomonile* sp. is a sulfate-reducing bacterium which uses H_2 or organic compounds as electron donors and chlorobenzoate as an electron acceptor. Several *Dehalococcoides* and *Dehalobacter* spp. isolates are dependent on halorespiration for growth, often

coupled to hydrogen as the sole electron donor; the former converts tri- and tetrachloroethylene to ethane, and the latter converts dichloromethane to acetate and formate [10].

5 Concluding Remarks

It is obvious from the examples above and from other chapters in this volume that anaerobes offer many possibilities within both industrial- and environmental biotechnology. The coming years should see rapid developments concerning deeper understanding of diversity, physiology, and biocatalytic potential, development of genetic tools, metabolic engineering of anaerobes, and their growing use as mono- and mixed cultures for various applications.

Acknowledgements The authors are grateful to the Swedish Research Council for financial support, and to Dr. Sang-Hyun Pyo for help with some of the figures.

References

1. Mamo G (2016) Anaerobes as sources of bioactive compounds and health promoting tools. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_15
2. Honkalas V, Dabir A, Dhakephalkar P (2016) Life in anoxic sub-seafloor environment: linking microbial metabolism and mega reserves of methane hydrate. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_9
3. van Niel EWJ (2016) Biological processes for hydrogen production. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_7
4. Schnurer A (2016) Biogas production - microbiology and technology. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_8
5. Kokko ME, Mäkinen AE, Puhakka JA (2016) Anaerobes in bioelectrochemical systems. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_10
6. Wang J, Lin M, Xu M, Yang S-T (2016) Anaerobic fermentation for production of carboxylic acids as bulk chemicals from renewable biomass. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_12
7. Erickson LE, Fung DYC (2010) Anaerobes. In: Flickinger MC (ed) Encyclopedia of industrial biotechnology: bioprocess, bioseparation and cell technology. Wiley, Hoboken
8. van Lier JB, van de Zee FP, Frijters CTMJ, Ersahin ME (2016) Development of anaerobic high rate reactors, focusing on sludge bed technology. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_13
9. Daniell J, Nagaraju S, Köpke M, Simpson SD (2016) Low carbon-fuel and chemical production by anaerobic gas fermentation. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_11
10. Madigan MT, Martinko JM (2006) Brock biology of microorganisms, 11th edn. Pearson Prentice Hall, New Jersey
11. Munir R, Levin DB (2016) Enzyme systems of anaerobes for biomass conversion. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_5

12. Frigaard N-U (2016) Biotechnology of anoxygenic phototrophic bacteria. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_6
13. Bragger JM, Daniel RM, Coolbear T, Morgan HW (1989) Very stable enzymes from extremely thermophilic archaebacteria and eubacteria. Appl Microbiol Biotechnol 31:556–561
14. Littlechild JA (2015) Archaeal enzymes and applications in industrial biocatalysts. Archaea 2015, 10.1155/2015/147671
15. Himmel ME, Xu Q, Luo Y, Ding S-Y, Lamed R, Bayer EA (2010) Microbial enzyme systems for biomass conversion: emerging paradigms. Biofuels 1:323–341
16. Gibbs MD, Reeves RA, Farrington GK, Anderson P, Williams DP, Bergquist PL (2000) Multidomain and multifunctional glycosyl hydrolases from the extreme thermophile *Caldicellulosiruptor* isolate Tok7B.1. Curr Microbiol 40:333–340
17. Ying Y, Meng D, Chen X, Li F (2013) An extremely thermophilic anaerobic bacterium *Caldicellulosiruptor* sp. F32 exhibits distinctive properties in growth and xylanases during xylan hydrolysis. Enzyme Microb Technol 53:194–199
18. Flint HJ, Bayer EA (2008) Plant cell wall breakdown by anaerobic microorganisms from the mammalian digestive tract. Ann NY Acad Sci 1125:280–288
19. Cai S, Li J, Hu FZ, Zhang K, Luo Y, Janto B, Boissy R, Ehrlich G, Dong X (2010) *Cellulosilyticum ruminicola*, a newly described rumen bacterium that possesses redundant fibrolytic-protein-encoding genes and degrades lignocellulose with multiple carbohydrate-borne fibrolytic enzymes. Appl Environ Microbiol 76:3818–3824
20. Selinger LB, Forsberg CW, Cheng K-J (1996) The rumen: a unique source of enzymes for enhancing livestock production. Anaerobe 2:263–284
21. Teunissen MJ, Op den Camp HJM (1993) Anaerobic fungi and their cellulytic and xylanolytic enzymes. Antonie van Leewen 63:63–76
22. Gupta A, Verma JP (2015) Sustainable bio-ethanol production from agro-residues: a review. Renew Sustain Energy Rev 41:550–567
23. Sprenger GA (1996) Carbohydrate metabolism in *Zymomonas mobilis*: a catabolic pathway with some scenic routes. FEMS Microbiol Lett 145:301–307
24. Cardona CA, Sánchez ÓJ (2007) Fuel ethanol production: process design trends and integration opportunities. Bioresour Technol 98:2415–2457
25. Matsushika A, Inoue H, Kodaki T, Sawayama S (2009) Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl Microbiol Biotechnol 84:37–53
26. Wyman CE, Spindler DD, Grohmann K (1992) Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol. Biomass Bioenergy 3:301–307
27. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583
28. Van Zyl WH, Lynd LR, den Haan R, McBride JE (2007) Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. Adv Biochem Engin/Biotechnol 108:205–235
29. Wyman CE (1994) Ethanol from lignocellulosic biomass: technology, economics, and opportunities. Bioresour Technol 50:3–16
30. Bai FW, Anderson WA, Moo-Young M (2008) Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv 26:89–105
31. Hu CK, Bai FW, An LJ (2005) Effect of the flocculating of a self-flocculating yeast on its ethanol tolerance and corresponding mechanisms. Chin J Biotechnol 21:123–128
32. Taylor MP, Eley KL, Martin S, Tuffin MI, Burton SG, Cowan DA (2009) Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. Trends Biotechnol 27:398–405
33. Madson PW, Monceaux DA (1999) Fuel ethanol production, in the alcohol textbook, 3rd edn. Nottingham University Press, UK

34. Joannis-Cassan C, Riess J, Jolibert F, Taillandier P (2014) Optimization of very high gravity fermentation process for ethanol production from industrial sugar beet syrup. Biomass Bioenergy 70:165–173
35. Puligundla P, Smogrovicova D, Obulam VSR, Ko S (2011) Very high gravity (VHG) ethanolic brewing and fermentation: a research update. J Ind Microbiol Biotechnol 38:1133–1144
36. Liu C-G, Wang N, Lin Y-H, Bai F-W (2012) Very high gravity ethanol fermentation by flocculating yeast under redox potential-controlled conditions. Biotechnol Biofuels 5:61
37. Visioli LJ, Enzweiler H, Kuhn RC, Schwaab M, Mazutti MA (2014) Recent advances on biobutanol production. Sustain Chem Proc 2:15
38. Ezeji TC, Qureshi N, Blaschek HP (2004) Butanol fermentation research: upstream and downstream manipulations. Chem Record 4:305–314
39. Chen CK, Blaschek HP (1999) Acettae enhances solvent production and prevents degeneration in *Clostridium beijernckii* BA101. Appl Microbiol Biotechnol 52:170–173
40. Jiang Y, Xu C, Dong F, Yang Y, Jiang W, Yang S (2009) Disruption of the acetoacetate decarboxylase gene in solvent producing *Clostridium acetobutylicum* increases the butanol ratio. Metab Eng 11:284–291
41. Ezeji TC, Qureshi N, Blaschek HP (2004) Acetone butanol ethanol (ABE) production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. Appl Microbiol Biotechnol 63:653–658
42. Qureshi N, Blaschek HP (1999) Production of acetone butanol ethanol (ABE) by a hyper-producing mutant strain of *Clostridium beijerinckii* BA101 and recovery by pervaporation. Biotechnol Prog 15:594–602
43. Tanaka S, Tashiro Y, Kobayashi G, Ikegami T, Negishi H, Sakaki K (2012) Membrane-assisted extractive butanol fermentation by *Clostridium saccharoperbutylacetonicum* N1-4 with 1-dodecanol as the extractant. Bioresour Technol 116:448–452
44. Mariano AP, Angelis DF, Maugeri F, Atala DIP, Maciel MRW, Maciel Filho R (2008) An alternative process for butanol production: continuous flash fermentation. Chem Prod Process Mode 3:34
45. Qureshi N, Lai LL, Blaschek HP (2004) Scale-up of a high productivity continuous biofilm reactor to produce butanol by adsorbed cells of *Clostridium beijerinckii*. Food Bioprod Proc 82:164–173
46. Liu D, Chen Y, Ding F-Y, Zhao T, Wu J-L, Guo T, Ren H-F et al (2014) Biobutanol production in a *Clostridium acetobutylicum* biofilm reactor integrated with simultaneous product recovery by adsorption. Biotechnol Biofuels 7:5
47. Li J, Baral NR, Jha AK (2014) Acetone-butanol-ethanol fermentation of corn stover by *Clostridium* species: present status and future perspectives. World J Microbiol Biotechnol 30:1145–1157
48. Qureshi N, Saha BC, Cotta MA (2008) Butanol production from wheat straw by simultaneous saccharification and fermentation using *Clostridium beijerinckii*: Part II – Fed batch fermentation. Biomass Bioenergy 32:176–183
49. Clomburg JM, Gonzalez R (2013) Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. Trends Biotechnol 31:20–28
50. Yazdani SS, Gonzalez R (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 18:213–219
51. Freund A (1881) Über die bildung und darstellung von trimethylenealkohol aus glycerin. Monatsh Chem 2:636–641
52. Biebl H, Menzel K, Zeng A-P, Deckwer W-D (1999) Microbial production of 1,3-propanediol. Appl Microbiol Biotechnol 52:289–297
53. Homann T, Tag C, Biebl H, Deckwer W-D, Schink B (1990) Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. Appl Microbiol Biotechnol 33:121–126

54. Selembo PA, Perez JM, Lloyd WA, Logan BE (2009) Enhanced hydrogen and 1,3-propanediol production from glycerol by fermentation using mixed cultures. Biotechnol Bioeng 104:1098–1106
55. Oh B-R, Seo J-W, Heo S-Y, Hong W-K, Luo LH, Kim S, Park D-H, Kim CH (2012) Optimization of culture conditions for 1,3-propanediol production from glycerol using a mutant strain of *Klebsiella pneumoniae*. Appl Biochem Biotechnol 166:127–137
56. De Souza EA, Rossi DM, Ayub MAZ (2014) Bioconversion of residual glycerol from biodiesel synthesis into 1,3-propanediol using immobilized cells of *Klebsiella pneumoniae* BLh-1. Renew Energy 72:253–257
57. Wilkens E, Ringel AK, Hortig D, Willke T, Vorlop KD (2012) High-level production of 1,3-propanediol from crude glycerol by *Clostridium butyricum* AKR102a. Appl Microbiol Biotechnol 93:1057–1063
58. Gonzalez-Pajuelo M, Meynial-Salles I, Mendes F, Andrade JC, Vasconcelos I, Soucaille P (2005) Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. Metab Eng 7:329–336
59. Pflügl S, Marx H, Mattanovich D, Sauer M (2012) 1,3-Propanediol production from glycerol with *Lactobacillus diolivorans*. Bioresour Technol 119:133–140
60. Dishisha T, Pereyra LP, Pyo S-H, Britton RA, Hatti-Kaul R (2014) Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol. Microb Cell Fact 13:76
61. Sriramulu DD, Liang M, Hernandez-Romero D, Raux-Deery E, Lünsdorf H, Parsons JB, Warren MJ, Prentice MB (2008) *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation. J Bacteriol 190:4559–4567
62. Sardari RRR, Dishisha T, Pyo S-H, Hatti-Kaul R (2013) Improved production of 3-hydroxypropionaldehyde by complex formation with bisulfite during biotransformation of glycerol. Biotechnol Bioeng 110:1243–1248
63. Sardari RRR, Dishisha T, Pyo S-H, Hatti-Kaul R (2013) Biotransformation of glycerol to 3-hydroxypropionaldehyde: improved production by in situ complexation with bisulfite in a fed-batch mode and separation on anion exchanger. J Biotechnol 168:534–542
64. Malaviya A, Jang Y-S, Lee SY (2012) Continuous butanol production with reduced byproducts formation from glycerol by a hyper producing mutant of *Clostridium pasteurianum*. Appl Microbiol Biotechnol 93:1485–1494
65. Jarvis GN et al (1997) Formate and ethanol are the major products of glycerol fermentation produced by *Klebsiella planticola* strain isolated from red deer. J Appl Microbiol 83:166–174
66. Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N (2005) Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. J Biosci Bioeng 100:260–265
67. Dishisha T, Alvarez MT, Hatti-Kaul R (2012) Batch- and continuous propionic acid production from glycerol using free and immobilized cells of *Propionibacterium acidipropionici*. Bioresour Technol 118:553–562
68. Dishisha T, Ståhl Å, Lundmark S, Hatti-Kaul R (2013) An economical biorefinery process for propionic acid production from glycerol and potato juice using high cell density fermentation. Bioresour Technol 135:504–512
69. Lee PC, Lee WG, Lee SY, Chang HN (2001) Succinic acid production with reduced byproduct formation in the fermentation of *Anaerobiospirillum succiniproducens* using glycerol as a carbon source. Biotechnol Bioeng 72:41–48
70. Vlysidis A, Binns M, Webb C, Theodoropoulos C (2011) Glycerol utilization for the production of chemicals: conversion to succinic acid, a combined experimental and computational study. Biochem Eng J 58–59:1–11
71. Dharmadi Y, Muraka A, Gonzalez R (2006) Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 94:821–829

72. Gonzalez R, Murarka A, Dharmadi Y, Yazdani SS (2008) A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in *Escherichia coli*. Metab Eng 10:234–245
73. Berg IA, Kockelkorn D, Ramos-Vera WH, Say RF, Zarzycki J, Hügler M, Alber BE, Fuchs G (2010) Autotrophic carbon fixation in archaea. Nat Rev 8:447–460
74. Sato T, Atomi H (2010) Microbial inorganic carbon fixation. Encycl Life Sci. doi:10.1002/9780470015902.a0021900
75. Ampelli C, Perathoner S, Centi G (2015) CO_2 utilization: an enabling element to move to a resource- and energy-efficient chemical and fuel production. Philos Trans R Soc Lond A Math Phys Eng Sci 373:20140177
76. Hu P, Rismani-Yazdi H, Stephanopoulos G (2013) Anaerobic CO_2 fixation by the acetogenic bacterium *Moorella thermoacetica*. AIChE J 59:3176–3183
77. Fernández-Naveira Á, Abubackar HN, Veiga MC, Kennes C (2016) Efficient butanol-ethanol (B-E) production from carbon monoxide fermentation by *Clostridium carboxidivorans*. Appl Microbiol Biotechnol 100:3361–3370
78. Hyeon JE et al (2015) Efficient biological conversion of carbon monoxide (CO) to carbon dioxide (CO_2) and for utilization in bioplastic production by *Ralstonia eutropha* through the display of an enzyme complex on the cell surface. Chem Commun 51:10202–10205
79. Fuchs G, Länge S, Rude E, Schäfer S, Schauder R, Scholtz R, Stupperich E (1987) Autotrophic CO_2 fixation in chemotrophic anaerobic bacteria. In: van Verseveld HW et al. (eds) Microbial growth on C1 compounds. Martinus Nijhoff Publishers, Dordrecht, pp 39–43
80. Hügler M, Menendez C, Schägger H, Fuchs G (2002) Malonyl-coenzyme A reductase from *Chloroflexux auranticus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO_2 fixation. J Bacteriol 184:2404–2410
81. Teufel R, Kung JW, Kockelkorn D, Alber BE, Fuchs G (2009) 3-Hydroxypropionyl-coenzyme A dehydratase and acryloyl-coenzyme A reductase, enzymes of the autotrophic 3-hydroxypropionate/4-hydroxybutyrate cycle in the *Sulfolobales*. J Bacteriol 191:4572–4581
82. Huber H, Gallenberger M, Jahn U, Eylert E, Berg IA, Kockelkorn D, Eisenreich W, Fuchs G (2008) A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic archaeum *Ignicoccus hospitalis*. Proc Natl Acad Sci U S A 105:7851–7856
83. Van de Graaf AA, Mulder A, de Bruijn P, Jetten MSM, Robertson LA, Kuenen JG (1995) Anaerobic oxidation of ammonium is a biologically mediated process. Appl Environ Microbiol 61:1246–1251
84. Hu BL, Shen LD, Xu XY, Zheng P (2011) Anaerobic ammonium oxidation (anammox) in different natural ecosystems. Biochem Soc Trans 39:1611–1616
85. Van de Graaf AA, de Bruijn P, Robertson LA, Jetten MSM, Kuenen JG (1996) Autotrophic growth of anaerobic ammonium-oxidizing microorganisms in a fluidized bed reactor. Microbiol 142:2187–2196
86. Jetten MSM, Wagner M, Fuerst J, van Loosdrecht M, Kuenen G, Strous M (2001) Microbiology and application of the anaerobic ammonium oxidation ("anammox") process. Curr Opin Biotechnol 12:283–288
87. Lackner S, Gilbert EM, Vlaeminck SE, Joss A, Horn H, van Loosdrecht MCM (2014) Full-scale partial nitritation/anammox experiences – an application survey. Water Res 55:292–303
88. Strous M, Heijnen JJ, Kuenen JG, Jetten MSM (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl Microbiol Biotechnol 50:589–596
89. Strous M, Kuenen JG, Jetten MSM (1999) Key physiology of anaerobic ammonium oxidation. Appl Environ Microbiol 65:3248–3250
90. De Luca G, de Philip P, Dermoun Z, Rousset M, Vermeglio A (2001) Reduction of technetium (VII) by *Desulfovibrio fructosovorans* is mediated by the nickel-iron hydrogenase. Appl Environ Microbiol 67:4583–4587

91. Goulhen F, Gloter A, Guyot F, Bruschi M (2006) Cr(VI) detoxification by *Desulfovibrio vulgaris* strain Hildenborough: microbe-metal interaction studies. Appl Microbiol Biotechnol 71:892–897
92. Lovley DR, Phillips EJ (1994) Reduction of chromate by *Desulfovibrio vulgaris* and its c_3 cytochrome. Appl Environ Microbiol 60:726–738
93. Lovley DR, Roden EE, Phillips EJP, Woodward JC (1993) Enzymatic iron and uranium reduction by sulfate-reducing bacteria. Marine Geol 113:41–53
94. Valls M, de Lorenzo V (2002) Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiol Rev 26:327–338
95. Shen Y, Buick R (2004) The antiquity of microbial sulphate reduction. Earth Sci Rev 64:243–272
96. Mohan SV, Rao NC, Prasad KK, Sarma PN (2005) Bioaugmentation of an anaerobic sequencing batch biofilm reactor (AnSBBR) with immobilized sulphate reducing bacteria (SRB) for the treatment of sulphate bearing chemical wastewater. Process Biochem 40:2849–2857
97. Cohen RRH (2006) Use of microbes for cost reduction for metal removal from metals and mining industry waste streams. J Clean Prod 14:1146–1157
98. Kolmert A, Johnson DB (2001) Remediation of acidic wastewaters using immobilized, acidophilic sulphate-reducing bacteria. J Chem Technol Biotechnol 76:836–843
99. Gadd GM, White C (1993) Microbial treatment of metal pollution – a working biotechnology? Trends Biotechnol 11:353–359
100. Kieu HTQ, Müller E, Horn H (2011) Heavy metal removal in anaerobic semi-continuous stirred tank reactors by a consortium of sulfate-reducing bacteria. Water Res 45:3863–3870
101. Sharma PK, Balkwill DL, Frenkel A, Vairavamurthy MA (2000) A new *Klebsiella planticola* strain (Cd-1) grows anaerobically at high cadmium concentrations and precipitates cadmium sulfide. Appl Environ Microbiol 66:3083–3087
102. Baskaran V, Nemati M (2006) Anaerobic reduction of sulfate in immobilized cell bioreactors, using a microbial culture originated from an oil reservoir. Biochem Eng J 31:148–159
103. Sahinkya E (2009) Biotreatment of zinc-containing wastewater in sulfidogenic CSTR: performance and artificial neural network (ANN) modeling studies. J Hazard Mater 164:105–113
104. Goncalves MMM, da Costa ACA, Leite SGF, Sant´Anna GL (2007) Heavy metal removal from synthetic wastewaters in an anaerobic bioreactor using stillage from ethanol distilleries as a carbon source. Chemosphere 69:1815–1820
105. Jong T, Parry DL (2003) Removal of sulfate and heavy metals by sulfate reducing bacteria in short-term bench scale upflow anaerobic packed bed reactor runs. Water Res 37:3379–3389
106. Lenz M, Hullebusch EDV, Hommes G, Corvini PFX, Lens PNL (2008) Selenate removal in methanogenic and sulfate-reducing upflow anaerobic sludge bed reactors. Water Res 42:2184–2194
107. Kaksonen AH, Vanhanen MLR, Puhakka JA (2003) Optimization of metal sulphide precipitation in fluidized bed treatment of acidic wastewater. Water Res 37:255–266
108. Bartzas G, Komnitsas K, Paspaliaris L (2006) Laboratory evaluation of Fe^0 barriers to treat acidic leachates. Mineral Eng 19:505–514
109. Pott B-M, Mattiasson B (2004) Separation of heavy metals from water solutions in lab scale. Biotechnol Lett 26:451–456
110. White C, Sharman AK, Gadd GM (1998) An integrated microbial process for the bioremediation of soil contaminated with toxic metals. Nat Biotechnol 16:572–575
111. Fredrickson JK, Gorby YA (1996) Environmental processes mediated by iron-reducing bacteria. Curr Opin Biotechnol 7:287–294
112. Khanal SK (2008) Overview of anaerobic technology. In: Khanal SK (ed) Anaerobic biotechnology for bioenergy production. Principles and applications. Wiley-Blackwell, Arnes, pp 1–28

113. Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domiguez-Espinosa R (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 22:477–485
114. Bagi Z, Ács N, Bálint B, Horváth L, Dobó K, Perei KR, Rákhely G, Kovács KL (2007) Biotechnological intensification of biogas production. Appl Microbiol Biotechnol 76:473–482
115. Liu J, Olsson G, Mattiasson B (2006) Extremum-seeking with variable gain control for intensifying biogas production in anaerobic fermentation. Water Sci Technol 53:35–44
116. Smidt H, de Vos WM (2004) Anaerobic microbial dehalogenation. Ann Rev Microbiol 58:43–73

Adv Biochem Eng Biotechnol (2016) 156: 35–54
DOI: 10.1007/10_2016_1

Published online: 31 March 2016

Isolation and Cultivation of Anaerobes

Rosa Aragão Börner

Abstract Anaerobic microorganisms play important roles in different biotechnological processes. Their complex metabolism and special cultivation requirements have led to less isolated representatives in comparison to their aerobic counterparts. In view of that, the isolation and cultivation of anaerobic microorganisms is still a promising venture, and conventional methodologies as well as considerations and modifications are presented here. An insight into new methodologies and devices as well as a discussion on future perspectives for the cultivation of anaerobes may open the prospects of the exploitation of these microorganisms as a source for biotechnology.

Keywords Anaerobic microorganisms, Cultivation, Cultivation devices, Isolation, Microcosms, New methodologies, Single cell

Contents

R.A. Börner (✉)
Division of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden

The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Hørsholm, Denmark
e-mail: rosabo@biosustain.dtu.dk

Abbreviations

FCM Flow cytometry
NGS Next generation sequencing
PVC Poly(vinyl chloride)

1 Introduction

Anaerobic microorganisms, and especially bacteria, have played an outstanding role in the rise and expansion of industrial biotechnology. Anaerobic bacteria and archaea have had a long history of industrial application for the production of fuels and chemicals [1, 2]. The pioneer process in the fermentation industry was the use of *Clostridium acetobutylicum* to produce alcohols and solvents since 1920 [3]. Nowadays, anaerobic microorganisms have regained attention for their unique biosynthetic capabilities and advantages regarding substrate flexibility and toxicity tolerance for their industrial application [4]. However, because of their complex metabolism and often special cultivation requirements, they have been less explored than their aerobic counterparts.

In natural environments, anaerobic microorganisms thrive where oxygen has been depleted and play a crucial role in the carbon cycle. They usually form consortia where organic matter is degraded in sequential steps in a synergetic action [5]. Examples of such environments are sediments from lakes and rivers and the gastrointestinal tract of ruminants, where a highly specialized microbial system has evolved to convert biomass. Microorganisms of industrial relevance for the biorefinery sector, for example, are likely to be found in such environments. To harness these potentials, advancement in microbial diversity studies and bioprospection are needed.

Lately, rapid advances in next-generation sequencing (NGS) technologies have dramatically increased the knowledge of natural processes based on genetic information, providing new insights into the total community of microorganisms. Added information on environmental gene tags (EGTs) achieved with NGS can be used to elucidate the population's functionality [6] and/or be used for bioprospection. Nevertheless, the isolation and cultivation of microorganisms is still relevant because it provides reference strains for in-depth physiological studies, broadens our view in the area of basic microbial research, and provides access to new organisms for production of metabolites of commercial interest.

An analysis of the large number of novel cultivation methods described over the last 15 years indicates that at least some so-called "unculturable" microorganisms are not fastidious, but they might be too rare to be captured [7]. One of the strategies to overcome this is simply to increase the conventional cultivation effort and geography of sampling [8], as many environments on Earth have not been explored. Considering that the access to new isolates can potentially bring new metabolites and applications, the isolation effort is justified. Moreover, novel and smart isolation and cultivation techniques can help to improve the access to so far "unculturable" microorganisms and improve our overall knowledge on anaerobes. Conventional and new approaches for isolation and cultivation techniques for anaerobic microorganisms are discussed herein.

2 Natural Habitats

Anaerobic bacteria are widespread in almost all environments on Earth. Natural systems such as the sediment of rivers, lakes, and oceans or the gastrointestinal tract (GI) of animals are habitats for these microorganisms. They can also be found in micro-environments where oxygen has been depleted by other aerobic organisms, such as in soil and decaying plant material [9]. Some microorganisms are enriched by human activities, such as in sewage plants, compost piles, and anaerobic digesters for the production of biogas [10]. These microorganisms play an important role in the carbon cycle by contributing to organic biomass degradation and by converting insoluble organic material to soluble compounds and gases that can circulate back to aerobic environments. With prospects for biotechnological applications in, for example, the biorefinery sector, it is a promising venture to search for interesting microorganisms in systems that have evolved under high organic load, such as lignocellulosic material.

3 Cultivation of Anaerobes Still Important in the Omics Era

With the latest developments in sequencing technologies, culturing of anaerobic microorganisms has been gradually abandoned in favor of molecular methods. This is mainly because of the time demanded, the technical difficulty of culturing anaerobes, and the fact that molecular methods have allowed a broader phylogenetic identification than what was obtainable with conventional cultivation techniques.

The advent of omics approaches has provided a vast amount of information on microbial diversity, its function in a given environment [11, 12], and identification of new genes for potential biotechnological uses [13, 14]. Although the analysis of

the vast generated data is also under current development and promising tools have been suggested [15–17], the identification of proteins or genes for a specific function is mainly done by comparing the results to existing databases. Thus, the discovery of proteins or metabolites for new functions using sequence-based data is limited. By having access to cultures of the microorganisms, such impediments can be circumvented by function assays and by physiological and genomic studies.

A pure culture of the microorganism thus remains essential for in-depth physiological studies, including its genome sequence [18]. Single cell isolation and genomic sequencing from environmental samples has surged as an optional approach to culturing [19]. Sequencing of isolated single cells has provided a highly valuable approach for a better understanding of its phylogenetic and metabolic markers [20, 21]. However, access to more cells for repeated and extended experimental tests to validate sequencing data and perform function assays, for example, is still important.

It is also relevant to point out that diverse pyrosequencing studies of anaerobic environmental samples have revealed that most sequences to date were assigned to "unculturable" species [18]. One example is the human gut bacteria, where most abundant phyla are Firmicutes and Proteobacteria with many not-yet-cultured species within the Clostridiales order, which are mainly strict anaerobes [22, 23]. This discrepancy reveals a future challenge to cultivation of microorganisms. Efforts for cultivation of anaerobes should also be extended to Archaea and Eukarya, as such organism groups play important role in ecosystems and have in general been overlooked compared to prokaryotes [24–26].

Data from sequencing technologies can provide information for modifying cultivation approaches to obtain cultures of not-yet-cultured microorganisms [27, 28]. Development of simple techniques for cultivation of anaerobes with potential to be implemented in different laboratories is under development by different research groups around the world and is a promising venture.

4 Manipulation of Anaerobic Microorganisms

Strict anaerobes require an oxygen-free environment and a low redox potential for growth. Thus, a critical aspect for manipulation and handling of anaerobes is to create an environment where oxygen has been depleted and/or replaced. Care should be taken at all times when handling viable anaerobic microorganisms, including sample collection, isolation, cultivation, screening assays, and characterization of the microorganisms.

For sample collection of a liquid material, best collection procedure would be to use a syringe and hypodermic needle with precaution to exclude air. The sample can then be placed in a sterile and rubber sealed glass transport tube containing oxygen-free carbon dioxide or nitrogen gas [29]. Solid or mixed samples such as sediment or rumen content can be collected fresh in sterile glass containers and filled up to the top. Optionally, a solution of reducing chemicals such as cysteine

hydrochloride can be injected into the transport container after placing the sample to improve reducing capacity. The processing of material from sample collection can be handled on-site using an inflatable polyethylene glove box alternative and a portable gas tube (nitrogen or carbon dioxide) to fill up the bag atmosphere.

There are different techniques available for maintaining an oxygen-free environment during the processing of samples for anaerobic cultivation and assays. This can be done by preparing cultivation media in glass vials, tubes, or reactors where oxygen is exhausted, replaced by other gases such as N_2 or CO_2, and trapped or depleted by addition of reducing chemicals. Another alternative is to handle the microorganisms in chambers where the O_2 has been replaced by other gases (as in media preparation) and/or depleted by the aid of catalysts [30, 31]. The use of anaerobic jars is common practice in clinical studies and can also be used for the transport of sample material or cultures. In all cases, colorimetric indicators can be used to evaluate the presence of oxygen in the system, such as resazurin, in solution or in stripes.

Anaerobic chambers for handling of anaerobes offer the great advantage that conventional microbiological and laboratory procedures can be carried out under oxygen free environment. Anaerobic chambers are commercially available in the option as rigid or flexible chambers. The most common chambers in anaerobe labs are made of plexiglass (PlasLab) or vinyl (Coy). Other temporary solutions for anaerobic work are the polyethylene Aldrich® AtmosBag (Sigma-Aldrich) or the PVC pyramid glove bag (Erlab Captair). These chambers are of small size, transparent, easily stored, and could also be useful for field work. They have connections for inlet/outlet of gas and, in some cases, also for power outlet. It allows for work under a saturated gas environment of a chosen gas, and with enough gas flush a reduced or oxygen free gas phase can be achieved. If the commercial options are not available, it is feasible to construct an anaerobic chamber. Important facts to be considered are the material, the sealing of the connections, lasting and integrity of the construction, pre-chamber characteristics, maximum pressure, and volume. It is thus possible to initiate or extend the work with anaerobic microorganisms in different laboratories by setting up the basic equipment for gas exchange and oxygen replacement plus an anaerobic chamber.

The anaerobic chamber can be highly beneficial for performing different experiments such as screening, enzyme assays, single cell manipulation [32], or encapsulation [33] for example. For assays using spectrophotometric measurements, microtiter plates and spectrophotometer cuvettes can be transferred from the anaerobic chamber by sealing the microtiter plates with petroleum jelly [34] or using rubber stoppered cuvettes [35]. Another alternative is to use the AnaeroPack® or AnaeroPouch® Systems (Mitsubishi Gas Chemical Company) for transport between the anaerobic chamber and other analytic devices. Care should be taken regarding the sterility of the environment inside the chamber and to avoid cross contaminations. All material transferred into the chamber should be cleaned with bacteriostatic ethanol solution (70 vol.%). Anaerobic chambers can be cleaned using disinfectants such as Virkon (DuPont) or Biocidal ZF (Accurate Chemical & Scientific) to avoid cross contamination.

Anaerobic cultures in serum vials are manipulated with hypodermic syringes and needles. Air in the syringe should be avoided when collecting or inoculating samples. It is also important to bear in mind that some anaerobes, especially bacteria, produce large amount of gases during growth, and thus overpressure is a risk to be considered in the cultures which might also affect sampling from the vials. Sufficient overhead space in the vials should be maintained to minimize overpressure risk; approximately 40% is used in cultivation of anaerobe bacteria, but it should be increased for microorganisms known to produce large amount of gas, such as the archaea *Methanosarcina* [36]. Overpressure can be alleviated from glass vials by, for example, introduction of a thin needle through the rubber septum to vent the produced gas. This should be better performed in a laminar flow cabinet and with consideration of volatile compounds released from the culture vials.

For maintenance of cultures, glycerol stocks can be prepared inside an anaerobic chamber. An alternative for less sensitive microorganisms is to prepare the glycerol stocks in a sterile flow chamber while constantly flushing the tube with anoxic and sterile gas, followed immediately by placing the tube in dry ice or liquid nitrogen. Anaerobic spore-forming bacteria are best stored as spore suspensions. Spore suspensions are prepared by centrifugation of old liquid cultures followed by a rinsing step with cold and anoxic PBS buffer. The obtained pellet containing spores is suspended in PBS buffer and aliquoted in small serum vials for storage in cold or in cryogenic tubes for frozen glycerol stocks. Alternatively, spore suspensions can be prepared from old cultures in solid media (2–20 days incubation time). The plate is scraped with a sterile loop and suspended in cold sterile water or buffer (5 mL) for aliquot and storage. An extra heat treatment (70°C for 20 min) can be employed on the spore suspension to kill remaining vegetative cells [37]. The washing, suspension and aliquot of the spores should be carried out in an anaerobic chamber. A heat shock step (70°C for 1–3 min) should be employed on the defrosted or cold spore stocks to reactivate the spores prior to inoculation onto the culture medium.

5 Cultivation Techniques

5.1 Conventional Cultivation Techniques

Strict anaerobes require an oxygen-free environment and a low redox potential for growth. The anaerobic technique developed by Robert Hungate [38] was later modified [39, 40] and is now commonly used in all major laboratories dealing with strict anaerobic cultures. It is based on the formation of anoxic gases by the passage of an inert gas such as N_2 and CO_2 through a heated copper column (pre-reduced with H_2) [30]. Commercially available equipment for aiding the gas exchange in media preparation is the Automated gassing machine (GR Instruments BV). The anoxic gas produced is then used to replace air in glass vials containing culture medium, which are sealed with rubber stoppers and closed with aluminum

caps. To generate a low redox potential in the medium, reducing agents such as cysteine hydrochloride are commonly used, although other compounds can also be employed.

To isolate anaerobic bacteria, solid media are usually prepared according to the roller-tube technique, although classical microbiological techniques on Petri dishes can also be used if handled inside an anaerobic chamber. In the roller-tube method, the inoculum is added to melted cooled agar prepared according to the Hungate technique. The tube or vial is carefully rolled to obtain a thin agar layer on its surface. To roll the tubes under a water tap can help to obtain an even thinner layer. Selective media can be used for the selection of cultures with desired activities, e.g., cellulose is included as the only carbon source for isolation of cellulose-degrading microorganisms. Colonies can be picked with needles and inoculated in liquid medium inside the anaerobic chamber.

In general, the steps involved in obtaining a microbial culture can be challenging. Many factors can increase the isolation rate and allow cultivability of bacteria from an environmental sample. It has been suggested that the best approach, given a limited time and budget to obtain cultures of interest, is to maximize the cultivation success by simulating conditions of the natural environment, as this is considered the most critical step [41]. Overall, it is important to consider the choice of media composition and environmental conditions as the main factors to determine the growth of the microorganisms [42].

5.2 Constituents of Anaerobic Media

The constituents of media for the cultivation of anaerobic microorganisms are based on a carbon source, energy source, and an electron donor and acceptor [31]. Media can be complex or defined, selective or non-selective, according to the aim of the study. There are innumerous descriptions of media for anaerobic microorganisms in the scientific literature and many protocols for media composition and preparation can be found in the main culture collections such as the German Culture Collection (www.dsmz.de) or the American Type Culture Collection (www.atcc.com).

For cultivation and isolation of anaerobic microorganisms for biotechnological purposes it has been common to work with defined media and to employ selective media constituents to isolate microorganisms with a desired activity. In general, defined media contains mineral salts, trace mineral elements, phosphorus, nitrogen, and carbon source, nutrients, and reducing agents (Table 1). Among the nutrients, vitamin addition is an important consideration for the cultivation of anaerobic prokaryotes, in particular vitamin B [43]. This can be added from complex sources, such as yeast extract, or from a vitamin stock solution. It can be difficult to identify key nutrients for the growth of specific microorganisms from complex and rich environmental samples; a cell-free extract of the sample of origin may be added to the culture media to aid the microbial growth. Such examples are the addition of

Table 1 Reducing agents in anaerobic media[a]

Compound	E_0' (mV)[b]	Concentration in media
Dihydroascorbate/ascorbate	58	
Thioglycollate	−140	
Dithiothreitol (DTT)	−330	0.05%
Cysteine HCl	−210	0.025%
Glutathione[c]	−0.206	
H_2 + palladium chloride	−420	
Na_2S*9H_2O	−571	0.025%
S^0/H_2S	−250	
S^0/HS	−270	
$2SO_3^{2-}/S_2O_4^{2-}$	−574	
Titanium(IV) citrate/titanium(III) citrate	−480	0.2–2 mM
Titanium(III) nitrilotriacetate		>30 μM
Resazurin (pink)/dihydroresofurin (colorless)[d]	−80	0.1 g/L

[a]Adapted from [31, 43]
[b]Reduction potential defined at pH 7 and 298°K
[c]Millis et al. [49]
[d]Used as color indicator in anaerobic media

clarified rumen fluid or sludge supernatant to anaerobic media [43, 44], which occasionally can further be replaced by defined compounds to cultivate specific isolated strains. Another example of a compound used to provide growth-promoting conditions associated with the sample origin is the use of hemin to cultivate strains of animal origin. Hemin has also been associated as a key factor for the growth of methanogens and their tolerance to oxidative stress [45].

Cysteine hydrochloride is one of the most common reducing agents employed in anaerobic media. It is also common to combine more than one reducing agent, such as sodium sulfide, to reduce the reduction potential even more. The different compounds used as reducing agents in anaerobic media are listed in Table 1. Modifications on the choice of reducing agents have improved the isolation of strains belonging to taxa with few described species [46, 47]. It has been seen that using titanium(III) citrate instead of cysteine hydrochloride and sulfide could lead to more methanogenic isolates as it was shown to be less toxic for the cells [46]. The use of ascorbate and glutathione in high doses in culture medium has helped to cultivate few strict anaerobic microorganisms under aerobic conditions [48] and could be an alternative for cultivation of anaerobes when specialized equipment for preparation of anoxic media is lacking.

Antibiotics are used as selection agent for the isolation and cultivation of targeted taxa. They are used for the cultivation of archaea [47], anaerobic fungi [50], and the enumeration of protozoa [43]. Combinations of different antibiotics such as norfloxacin (200 mg/L) and mupirocin (100 mg/L) were recently used to facilitate the isolation and cultivation of bifidobacteria from fecal samples [51]. A tailored combination of antibiotics has also been used to isolate previously uncultured species from the Human Gut Microbiome Project's most wanted list

[27]. It demonstrates that carefully designed selective agents can be used to get the "non-cultivable" strains to grow.

Selection of microorganisms depending on ability to degrade complex carbon sources has been used extensively in industrial and environmental biotechnology. Such examples are the selection of cellulolytic and fiber-degrading microorganisms from rumen [43, 50] and the isolation of bacteria able to decolorize [52] and degrade [53] azo-dyes. Difficulties are encountered when the substrate is insoluble in water, such as plant biomass. This can be solved by homogenization of the substrate with the anoxic medium before autoclaving, and the procedure can be performed inside the anaerobic chamber.

Agar is the most common gelling agent for preparation of solid media for the isolation of anaerobic microorganisms. Nevertheless, other gelling agents, such as the nitrogen free polysaccharide gellan gum (e.g., Gelrite, Phytagel), conventionally used for the culture of thermophilic anaerobes, have gained support for use in isolation of mesophilic aerobes [54, 55] and anaerobes [27, 47, 56, 57]. This is believed to be because of the diminished content of phenolic compounds in the gelling agent [58] which can potentially inhibit certain microbial species.

5.3 Environmental Factors

Anaerobic microorganisms are found in a wide range of environments, and thus environmental conditions for their cultivation vary according to their original habitat. Cultivation factors such as temperature, pH, and osmosis vary from "normal" to extreme conditions. Anaerobic extremophile microorganisms are also an attractive source for biotechnological applications and increased efforts on cultivating such microorganisms should also be pursued.

Extremophiles have long been considered to be of high interest in biotechnology as a source of enzymes. Recent years have seen increased interest in anaerobic extremophiles, in particular thermophiles (growth range 60–100°C), for the production of chemicals [59, 60] and fuels [61, 62] and as metabolic engineering platforms [63]. This gives a bioprocessing advantage of simultaneous product recovery during fermentation at high temperatures of more volatile compounds, such as acetone or ethanol, as well as low contamination risk. Moreover, in combination with the native metabolic capabilities of these microorganisms, e.g., cellulose degradation, it makes them an interesting cellular platform for the biorefinery sector. At the other extreme, anaerobic psychrophilic microorganisms are gaining interest for their participation in bioprocesses such as the psychrophilic (~15°C) anaerobic digestion. This process is of increasing interest as an alternative to mesophilic waste water treatment process with the surplus of energy generation in the form of biogas under low energy costs [64]. To isolate and cultivate microorganisms from such environmental processes at low temperature can bring valuable information for its establishment and control.

Another example of extremophilic anaerobes with relevance to biotechnology are halo-alkaliphilic microorganisms for their application in production of methane-rich biogas. Recently a community from a soda lake was used in anaerobic digestion using an alkaline medium which acts as a CO_2 scrubber, resulting in 96% pure methane [65]. Acidophilic anaerobes, on the other hand, have long been important players in the recovery of heavy metals, especially anaerobic sulfur- and sulfate-reducing bacteria [66]. For cultivation of pH extreme microorganisms it is important to consider other media supplements, e.g., carbonates, sulfur, and iron sources and corresponding buffer systems for pH control of the media. Special adaptations must also be considered for preparation of solid media, for example by addition of extra Ca^+ and Mg^+ sources for preparation of agar or gellan gum at low pH and incubation at high temperature.

Another important environmental condition to consider when cultivating anaerobes is the atmospheric gas composition. The gases most commonly used for anaerobic media preparation are CO_2 and N_2. A combination of both gases (e.g., 85% N_2 and 15% CO_2) is also commonly encountered in anaerobe labs nowadays. Addition of H_2 to the gas mixture is of great importance for cultivating archaea, especially methanogens [67]. Hydrogen gas can also be present in gas mixtures (e.g., 85% N_2, 10% CO_2, and 5% H_2). Other gases applicable to the cultivation of anaerobe microorganisms are carbon oxide for acetogens [68] or methane for anaerobic methanotrophs [69].

As with the media composition, the choice of environmental conditions employed during the cultivation of the microorganisms acts as a selection pressure factor for their isolation. When deciding on the conditions, it is important to consider the goal of the study, taking into account the potential applications of the microorganisms.

6 New Methods for Isolation and Cultivation of Anaerobes

Progress has been made in developing novel cultivation strategies that allow improved growth of bacteria. However, few examples have focused exclusively on anaerobic microorganisms. Alternative methods to the conventional cultivation techniques are primarily based on simulation of the natural environment and separation and cultivation of single cells.

6.1 Simulating the Natural Environment

It is difficult to know in advance the growth requirements of the microorganisms present in an environmental sample or to replicate its conditions in the laboratory. One option to circumvent this obstacle is to set up anaerobic microcosm experiments with the environmental samples. With a longer time set up, it might be possible to maintain key microorganisms in the laboratory which would not have

been initially isolated if conventional cultivation techniques were used for the original sample. Following this strategy, novel anaerobic or facultative anaerobic bacteria were isolated with properties of interest for the environmental biotechnology field for the degradation of chlorinated compounds [70]. A good description of how to set up an anaerobic microcosm was described by Löffler et al. [70]. The experimental set-up consists of a sealed glass vessel which is closed with butyl rubber or Teflon septa. The sample and content of the microcosms is added before the vessel is sealed and later purged with sterile anoxic gases (N_2/CO_2 mixture). A broad range of sample composition and characteristics as well as set environmental conditions can be varied. The challenge in this strategy is still to be able to separate and isolate the microorganisms from the microcosm's community.

New devices have been developed to aid in the isolation and cultivation of anaerobic microorganisms. Many of these devices follow the strategy to simulate the natural environment or the co-culture with other microorganisms present in the sample of origin (Table 2). These devices have shown improvement in the isolation

Table 2 Devices developed for the cultivation and isolation of anaerobic microorganisms

Name of the device	Principle	Sample of origin	Example of taxonomic groups from the isolated strains	References
Calgary biofilm	In vitro biofilm model	Oral plaque	*Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*	[72]
Constant depth film fermenters (CDFF)	In vitro biofilm model	Human saliva	*Proteobacteria*, *Firmicutes*, *Bacteroidetes*	[73]
Diffusion growth chamber	In situ cultivation – the cambers are sealed and placed in an aquarium with marine/fresh water	Marine sediment, Fresh water sediment	*Bacteroidetes*, *Proteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Deltaproteobacteria*, *Verrumicrobia*, *Spirochaetas*, *Acidobacteria*	[71, 74]
Hollow-fiber membrane chamber (HFMC)	In situ cultivation – the membrane is placed in a liquid natural or engineered environment	Tidal flat sediment, activated sludge from sewage wastewater treatment plant, activated sludge from a laboratory scale enhanced biological phosphorus removal process	*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Spirochaetes*	[75]

rate of anaerobic bacteria and have resulted in the isolation of many fastidious bacteria. In some cases, the isolates still needed co-culture with other microorganisms or their culture supernatant [71, 72], indicating the requirement of unknown growth factors associated with the community dynamics. Extended efforts to design such devices and experimental set-ups should be promoted for gaining access to anaerobic diversity in cultivable form.

6.2 *Separating and Cultivating Single Cells*

Very promising methods for obtaining anaerobic cultures and coupling these with a high-throughput screening method are based on the use of single cells. These methods can be performed starting with free or entrapped cells in a polymer matrix (Fig. 1). Cells can be sorted and isolated as initial step or later on after a first co-culture stage. It is relevant to consider the nature and origin of the sample and how to process it for achieving access and separation of the single cells. For example, sediment samples might need a sonication step to separate the microbial cells from sand or plant debris. The most important consideration is to perform the whole process under anaerobic conditions to maintain the viability of the microbes for further cultivation.

6.2.1 Starting from Free Single Cells

Separation of single anaerobic cells can be performed manually by using a microscope equipped with a micromanipulator. Selected cells can be used directly for DNA extraction, for their identification or genome sequencing [19], or for further cultivation when microscope and micromanipulator set-ups are in an anaerobic chamber [32]. Although the process is low-throughput, it allows a high certainty that a single cell was captured and inoculated, and even direct observation of cell morphology and image documentation.

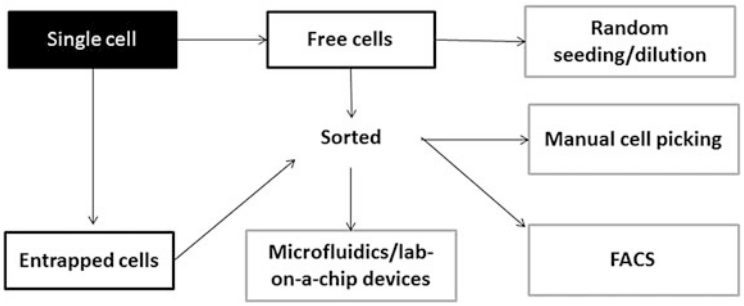


Fig. 1 Strategies for isolation and cultivation starting from a single cell

Separation of anaerobic microbial cells by dilution extinction has not yet been performed, but it could be an alternative when no special equipment (microscope with micro-tweezers or flow cytometer) is available. A series of dilutions of the sample should be inoculated into a microtiter plate where the goal is to obtain one cell per well [76]. Error possibility is high as the method is based on a Poisson probability, and hence wells need to be verified by direct observation.

When the starting point is free cells, flow cytometry (FCM) can be used to analyze and separate the cells in a high-throughput manner. Flow cytometry and sorting techniques have been applied to the isolation and cultivation of uncharacterized or slow-growing microorganisms from the environment, including anaerobic systems [77, 78]. Anaerobic single cells can be differentiated from debris because of their light-scattering properties in combination with the use of fluorescent markers [77]. However, the use of fluorescent markers that bind to DNA makes the further cultivation of these cells difficult if sorting is employed. As an alternative, Hamilton-Brehm et al. [78] have used light-scattering properties of the cells for their analysis and have adapted the atmospheric conditions in the FCM for maintaining it oxygen-free. It was done by connecting the compressed air valve of the FCM with a pressurized nitrogen gas and previously boiling the sheath fluid for easier gas replacement. This has allowed the isolation and cultivation of anaerobic thermophiles with plant-biomass degradation capabilities.

An alternative to using FCM is the adoption of microfluidic systems or lab-on-a-chip. With a rise in the development of these systems for different applications using single cells, an increase in its application within anaerobic microbiology is also expected. So far it has been used to isolate gut microbiome representatives of the "most wanted list" [28] at the same time as providing enough material for parallel genetic characterization. The possibility of having a miniaturized system containing cultivation chambers combined with different types of detectors in different stages could allow the detection of microcolony formation and the production of targeted metabolites. This strategy may allow for a great leap in the discovery of new microorganisms with biotechnological applications.

6.2.2 Starting from Entrapped Cells

Single cells entrapped in a polymer matrix (agarose or alginate) can be further cultivated in liquid medium to allow microcolony formation (Fig. 2a). This strategy allows a 3D structure for cell cultivation, offering support and mechanical stability for easier handling. When single cells of two or more bacterial species entrapped in the microbeads are cultivated together in liquid medium, community conditions can be simulated. In this case, the polymer beads provide protection from competitors and toxic compounds in the environment. Nevertheless, because of the porosity of the material, nutrient exchange with the medium and other metabolites and compounds necessary for cell-to-cell communication in cultivated communities can occur. Entrapped and free cells can easily be sorted afterwards with the help of a flow cytometer equipped with a sorter system. Beads containing microcolonies can

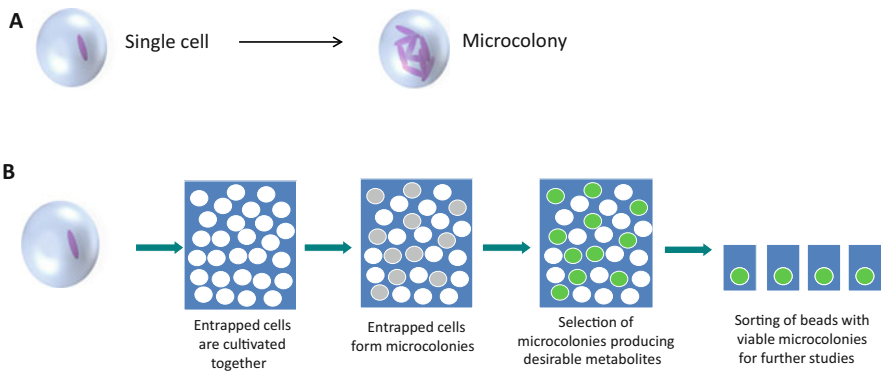


Fig. 2 Schematic representation of cultivation strategy using single cells entrapped in polymer microbeads. (**a**) Expected microcolony formation inside the polymer beads. (**b**) Scheme for isolation, cultivation and screening of microorganisms for biotechnological applications

then be passed onto single microtiter wells with fresh medium and further cultivated. For the application of this strategy on anaerobic microorganisms, it is advisable to perform modifications in the flow cytometer for assuring anoxic conditions during analysis and sorting [78].

This strategy has proved to be successful with slow-growing bacteria such as *Mycobacterium* strains [79–81]. However, the most interesting results were obtained by Zengler et al. [82], who developed a high-throughput method for single cell entrapment and cultivation of bacterial cells from sea and soil samples. This allowed the detection of strains that were previously considered "non-cultivable." A similar concept was used to entrap anaerobic microorganisms in alginate beads, allowing for cultivation of anaerobic bacteria starting from a single cell [33]. It has also been shown that gel microbeads can be used to enrich single cells from an environmental sample, such as the human microbiome, for whole-genome sequencing [83].

A great potential of this strategy would be its coupling to a screening system. The possibility to exploit the confinement of the cells in the polymeric material with substrates or indicators of specific metabolic activities could have a significant impact in a combined high-throughput isolation/cultivation/screening system (Fig. 2b).

7 Future Perspectives

The importance of anaerobic microorganisms in biotechnology is unquestionable and it is clear that an increase in the efforts for their isolation and cultivation could provide new sources for applications in different areas. The applications may be in

the health sector by understanding the human microbiome function and developing targeted therapies, finding and developing new production systems for a sustainable industry, or advancing environmental processes for treatment of waste streams.

There are plenty of unexplored niches on the planet where anaerobic microorganisms can be found and increasing efforts for the study of anaerobes from these systems could provide new biotechnological sources. Application of new methods for isolation and cultivation of anaerobes from already studied sites might also bring access to new strains. However, these efforts should not be limited to prokaryotes. Anaerobic eukaryotes are certainly underrepresented and deserve more attention.

Development of new methods in general for cultivation of anaerobes is a positive advance in the field. Nevertheless, special attention should be given to new methods and strategies for collection, transport, and processing of the anaerobic samples as well, as these initial steps are critical for the survival of the microorganisms. Pursuing cultivations in situ is also a promising venture as reports have shown that there is nothing better than to use the environment itself to select cultivation conditions.

Finally, high-throughput methods combining cultivation, isolation, and screening are most desirable for biotechnological purpose. Anaerobic microorganisms have the disadvantage of being slow growers and methods that facilitate their detection and metabolic potentialities while maintaining their viability are highly desired. Anaerobes are a great source for biotechnology and employing and developing new cultivation strategies can promote and widen the scope of the investigations and applications of these microorganisms.

References

1. Goldstein EJ (1995) Anaerobes under assault: from cottage industry to industrialization of medicine and microbiology. Clin Infect Dis 20:S112–S116
2. Antoni D, Zverlov VV, Schwarz WH (2007) Biofuels from microbes. Appl Microbiol Biotechnol 77:23–35
3. Kumar M, Gayen K (2011) Developments in biobutanol production: new insights. Appl Energy 88:1999–2012
4. Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET (2012) Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr Opin Biotechnol 23:364–381
5. Khanal SK (2008) Anaerobic biotechnology for bioenergy production. Principles and applications. Wiley-Blackwell, USA
6. Brulc JM, Antonopoulos DA, Miller MEB, Wilson MK, Yannarell AC, Dinsdale EA, Edwards RE, Frank ED, Emerson JB, Wacklin P, Coutinho PM, Henrissat B, Nelson KE, White BA (2009) Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. Proc Nat Acad Sci 106:1948–1953
7. Buerger S, Spoering A, Gavrish E, Leslin C, Ling L, Epstein SS (2012) Microbial scout hypothesis and microbial discovery. Appl Environ Microbiol 78:3229–3233
8. Epstein SS (2013) The phenomenon of microbial uncultivability. Curr Opin Microbiol 16:636–642

9. Zehnder AJB, Stumm W (1988) Geochemistry and biogeochemistry of anaerobic habitats. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. John Wiley, New York, pp 1–38
10. Tamaru Y, Miyake H, Kuroda K, Ueda M, Doi RH (2010) Comparative genomics of the mesophilic cellulosome-producing *Clostridium cellulovorans* and its application to biofuel production via consolidated bioprocessing. Environ Technol 31:889–903
11. Gosalbes MJ, Durbán A, Pignatelli M, Abellan JJ, Jiménez-Hernández N, Pérez-Cobas AE, Latorre A, Moya A (2011) Metatranscriptomic approach to analyze the functional human gut microbiota. PLoS One 6, e17447. doi:10.1371/journal.pone.0017447
12. Vanwonterghem I, Jensen PD, Ho DP, Batstone DJ, Tyson GW (2014) Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr Opin Biotechnol 27:55–64
13. Qi M, Wang P, O'Toole N, Barboza PS, Ungerfeld E, Leigh MB, Selinger LB, Butler G, Tsang A, McAllister TA, Forster RJ (2011) Snapshot of the eukaryotic gene expression in muskoxen rumen—a metatranscriptomic approach. PLoS One 6, e20521. doi:10.1371/journal.pone.0020521
14. Lü F, Bize A, Guillot A, Monnet V, Madigou C, Chapleur O, Mazéus L, He P, Bouchez T (2014) Metaproteomics of cellulose methanisation under thermophilic conditions reveals a surprisingly high proteolytic activity. ISME J 8:88–102
15. Busk PK, Lange L (2013) Function-based classification of carbohydrate-active enzymes by recognition of short, conserved peptide motifs. Appl Environ Microbiol 79:3380–3391
16. Nielsen HB, Almeida M, Juncker AS, Rasmussen S, Li J, Sunagawa S, Ehrlich SD (2014) Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nat Biotechnol 32:822–828
17. Aßhauer KP, Wemheuer B, Daniel R, Meinicke P (2015) Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. Bioinformatics 31:2882–2884
18. Lagier JC, Hugon P, Khelaifia S, Fournier PE, La Scola B, Raoult D (2015) The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. Clin Microbiol Rev 28:237–264
19. Ishoey T, Woyke T, Stepanauskas R, Novotny M, Lasken RS (2008) Genomic sequencing of single microbial cells from environmental samples. Curr Opin Microbiol 11:198–204
20. Stepanauskas R (2012) Single cell genomics: an individual look at microbes. Curr Opin Microbiol 15:613–620
21. Vasdekis AE, Stephanopoulos G (2015) Review of methods to probe single cell metabolism and bioenergetics. Metab Eng 27:115–135
22. Li K, Bihan M, Methé BA (2013) Analyses of the stability and core taxonomic memberships of the human microbiome. PLoS One 8, e63139
23. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, Clemente JC, Knight R, Heath AC, Leibel RL, Rosenbaum M, Gordon JI (2013) The long-term stability of the human gut microbiota. Science 341:1237439
24. Caron DA, Worden AZ, Countway PD, Demir E, Heidelberg KB (2009) Protists are microbes too: a perspective. ISME J 3:4–12
25. Lange L (2014) The importance of fungi and mycology for addressing major global challenges. IMA Fungus 5:463–471
26. Hugerth LW, Muller EE, Hu YO, Lebrun LA, Roume H, Lundin D, Wilmes P, Andersson AF (2014) Systematic design of 18S rRNA gene primers for determining eukaryotic diversity in microbial consortia. PloS One 9, e95567
27. Rettedal EA, Gumpert H, Sommer MO (2014) Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. Nat Commun 5:4714
28. Ma L, Kim J, Hatzenpichler R, Karymov MA, Hubert N, Hanan IM, Chang EB, Ismagilov RF (2014) Gene-targeted microfluidic cultivation validated by isolation of a gut bacterium listed in Human Microbiome Project's most wanted taxa. Proc Nat Acad Sci 111:9768–9773

29. Hentges DJ (1996) Anaerobes: general characteristics. In: Baron S (ed) Medical microbiology, 4th edn. University of Texas Press, Galvestone
30. Speers AM, Cologgi DL, Reguera G (2009) Anaerobic cell culture. Curr Protoc Microbiol 12:4F:A.4F.1–A.4F.16
31. Plugge CM (2005) Anoxic media design, preparation, and considerations. Methods Enzymol 397:3–16
32. Fröhlich J, König H (2000) New techniques for isolation of single prokaryotic cells. FEMS Microbiol Rev 24:567–572
33. Börner RA, Aliaga MTA, Mattiasson B (2013) Microcultivation of anaerobic bacteria single cells entrapped in alginate microbeads. Biotechnol Lett 35:397–405
34. Eini A, Sol A, Coppenhagen-Glazer S, Skvirsky Y, Zini A, Bachrach G (2013) Oxygen deprivation affects the antimicrobial action of LL-37 as determined by microplate real-time kinetic measurements under anaerobic conditions. Anaerobe 22:20–24
35. Zeidan AA, Van Niel EW (2010) A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensensis* OL T. Int J Hydrogen Energ 35:1128–1137
36. Lehmann-Richter S, Grosskopf R, Liesack W, Frenzel P, Conrad R (1999) Methanogenic archaea and CO_2-dependent methanogenesis on washed rice roots. Environ Microbiol 1:159–166
37. Edwards AN, Suárez JM, McBride SM (2013) Culturing and maintaining *Clostridium difficile* in an anaerobic environment. J Vis Exp 79, e50787. doi:10.3791/50787
38. Hungate RE (1969) A roll tube method for cultivation of strict anaerobes. Methods Microbiol 3:117–132
39. Bryant MP (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. Am J Clin Nutr 25:1324–1328
40. Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressureized atmosphere. Appl Environ Microbiol 32:781–791
41. Zengler K (2013) To grow or not to grow: isolation and cultivation procedures in the genomic age. In: Fredricks DN (ed) The human microbiota: how microbial communities affect health and disease. Wiley, Hoboken. doi:10.1002/9781118409855.ch12
42. Lagier JC, Edouard S, Pagnier I, Mediannikov O, Drancourt M, Raoult D (2015) Current and past strategies for bacterial culture in clinical microbiology. Clin Microbiol Rev 28:208–236
43. McSweeney CS, Denman SE, Mackie RI (2005) Rumen bacteria. Methods in gut microbial ecology for ruminants. Springer, The Netherlands, pp 23–37
44. Khelaifia S, Raoult D, Drancourt M (2013) A versatile medium for cultivating methanogenic archaea. PLos One. doi:10.1371/journal.pone.0061563
45. Brioukhanov AL, Netrusov AI (2012) The positive effect of exogenous hemin on a resistance of strict anaerobic archaeon *Methanobrevibacter arboriphilus* to oxidative stresses. Curr Microbiol 65:375–383
46. Bräuer SL, Yashiro E, Ueno NG, Yavitt JB, Zinder SH (2006) Characterization of acid-tolerant H_2/CO_2-utilizing methanogenic enrichment cultures from an acidic peat bog in New York State. FEMS Microbiol Ecol 57:206–216
47. Carbonero F, Oakley BB, Purdy KJ (2010) Improving the isolation of anaerobes on solid media: the example of the fastidious Methanosaeta. J Microbiol Methods 80:203–220
48. La Scola B, Khelaifia S, Lagier JC, Raoult D (2014) Aerobic culture of anaerobic bacteria using antioxidants: a preliminary report. Eur J Clin Microbiol Infect Dis 33:1781–1783
49. Millis KK, Weaver KH, Rabenstein DL (1993) Oxidation/reduction potential of glutathione. J Org Chem 58:4144–4146
50. Haitjema CH, Solomon KV, Henske JK, Theodorou MK, O'Malley MA (2014) Anaerobic gut fungi: advances in isolation, culture, and cellulolytic enzyme discovery for biofuel production. Biotechnol Bioeng 111:1471–1482

51. Vlková E, Salmonová H, Bunešová V, Geigerová M, Rada V, Musilová Š (2015) A new medium containing mupirocin, acetic acid, and norfloxacin for the selective cultivation of bifidobacteria. Anaerobe 34:27–33
52. Khalid A, Kausar F, Arshad M, Mahmood T, Ahmed I (2012) Accelerated decolorization of reactive azo dyes under saline conditions by bacteria isolated from Arabian seawater sediment. Appl Microbiol Biotechnol 96:1599–1606
53. Razo-Flores E, Luijten M, Donlon B, Lettinga G, Field J (1997) Biodegradation of selected azo dyes under methanogenic conditions. Water Sci Technol 36:65–72
54. Hara S, Hashidoko Y, Desyatkin RV, Hatano R, Tahara S (2009) High rate of N_2 fixation by East Siberian cryophilic soil bacteria as determined by measuring acetylene reduction in nitrogen-poor medium solidified with gellan gum. Appl Environ Microbiol 75:2811–2819
55. Tamaki H, Hanada S, Sekiguchi Y, Tanaka Y, Kamagata Y (2009) Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. Environ Microbiol 11:1827–1834
56. Nakamura K, Tamaki H, Kang MS, Mochimaru H, Lee ST, Nakamura K, Kamagata Y (2011) A six-well plate method: less laborious and effective method for cultivation of obligate anaerobic microorganisms. Microbes Environ 26:301–306
57. Nyonyo T, Shinkai T, Tajima A, Mitsumori M (2013) Effect of media composition, including gelling agents, on isolation of previously uncultured rumen bacteria. Lett Appl Microbiol 56:63–70
58. Scherer PA, Müller E, Lippert H, Wolff G (1988) Multielement analysis of agar and gelrite impurities investigated by inductively coupled plasma emission spectrometry as well as physical properties of tissue culture media prepared with agar on the gellan gum gelrite. Acta Horticult 226:655–658
59. Zhang F, Zhang Y, Chen Y, Dai K, van Loosdrecht MC, Zeng RJ (2015) Simultaneous production of acetate and methane from glycerol by selective enrichment of hydrogenotrophic methanogens in extreme-thermophilic (70°C) mixed culture fermentation. Appl Energy 148:326–333
60. Schiel-Bengelsdorf B, Dürre P (2012) Pathway engineering and synthetic biology using acetogens. FEBS Lett 586:2191–2198
61. Olson DG, Sparling R, Lynd LR (2015) Ethanol production by engineered thermophiles. Curr Opin Biotechnol 33:130–141
62. Panagiotopoulos IA (2015) Dark fermentative hydrogen production from lignocellulosic biomass. Production of hydrogen from renewable resources. Springer, The Netherlands, pp 3–40
63. Zeldes BM, Keller MW, Loder AJ, Straub CT, Adams MW, Kelly RM (2015) Extremely thermophilic microorganisms as metabolic engineering platforms for production of fuels and industrial chemicals. Front Microbiol 6:1209
64. Gunnigle E, Nielsen JL, Fuszard M, Botting CH, Sheahan J, O'Flaherty V, Abram F (2015) Functional responses and adaptation of mesophilic microbial communities to psychrophilic anaerobic digestion. FEMS Microbiol Ecol 91:132
65. Nolla-Ardèvol V, Strous M, Tegetmeyer HE (2015) Anaerobic digestion of the microalga Spirulina at extreme alkaline conditions: biogas production, metagenome, and metatranscriptome. Front Microbiol 6:597
66. Johnson DB (2014) Biomining—biotechnologies for extracting and recovering metals from ores and waste materials. Curr Opin Biotechnol 30:24–31
67. Joblin KN (2005) Methanogenic archaea. In: Methods in gut microbial ecology for ruminants. Springer, The Netherlands, pp 47–53
68. Latif H, Zeidan AA, Nielsen AT, Zengler K (2014) Trash to treasure: production of biofuels and commodity chemicals via syngas fermenting microorganisms. Curr Opin Biotechnol 27:79–87

69. Dianou D, Ueno C, Ogiso T, Kimura M, Asakawa S (2012) Diversity of cultivable methane-oxidizing bacteria in microsites of a rice paddy field: investigation by cultivation method and fluorescence in situ hybridization (FISH). Microbes Environ 27:278–287
70. Löffler FE, Sanford RA, Ritalahti KM (2005) Enrichment, cultivation, and detection of reductively dechlorinating bacteria. Methods Enzymol 397:77–111
71. Kaeberlein T, Lewis K, Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296:1127–1129
72. Thompson H, Rybalka A, Moazzez R, Dewhirst FE, Wade WG (2015) In vitro culture of previously uncultured oral bacterial phylotypes. Appl Environ Microbiol 81:8307–8314
73. McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Gilbert P (2003) Growth and molecular characterization of dental plaque microcosms. J Appl Microbiol 94:655–664
74. Bollmann A, Lewis K, Epstein SS (2007) Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. Appl Environ Microbiol 73:6386–6390
75. Aoi Y, Kinoshita T, Hata T, Ohta H, Obokata H, Tsuneda S (2009) Hollow-fiber membrane chamber as a device for in situ environmental cultivation. Appl Environ Microbiol 75:3826–3833
76. Gross A, Schoendube J, Zimmermann S, Steeb M, Zengerle R, Koltay P (2015) Technologies for single-cell isolation. Int J Mol Sci 16:16897–16919
77. Müller S, Hübschmann T, Kleinsteuber S, Vogt C (2012) High resolution single cell analytics to follow microbial community dynamics in anaerobic ecosystems. Methods 57:338–349
78. Hamilton-Brehm SD, Vishnivetskaya TA, Allman SL, Mielenz JR, Elkins JG (2012) Anaerobic high-throughput cultivation method for isolation of thermophiles using biomass-derived substrates. Methods Mol Biol 908:153–168
79. Weaver JC, Williams GB, Klibanov A, Demain AL (1988) Gel microdroplets: rapid detection and enumeration of individual microorganisms by their metabolic activity. Nat Biotech 6:1084–1089
80. Manome A, Zhang H, Tani Y, Katsuragi T, Kurane R, Tsuchida T (2001) Application of gel microdroplet and flow cytometry techniques to selective enrichment of non-growing bacterial cells. FEMS Microbiol Lett 197:29–33
81. Akselband Y, Cabral C, Castor TP, Chikarmane HM, McGrath P (2006) Enrichment of slow-growing marine microorganisms from mixed cultures using gel microdrop (GMD) growth assay and fluorescence-activated cell sorting. J Exp Mar Biol Ecol 329:196–205
82. Zengler K, Toledo G, Rappé M, Elkins J, Mathur EJ, Short JM, Keller M (2002) Cultivating the uncultured. Proc Natl Acad Sci 99:15681–15686
83. Fitzsimons MS, Novotny M, Lo CC, Dichosa AE, Yee-Greenbaum JL, Snook JP, Gu W, Chertkov O, Davenport KW, McMurry K, Reitenga KG, Daughton AR, He J, Johnson SL, Gleasner CD, Wills PL, Parson-Quintana B, Chain PC, Detter JC, Lasken RG, Han CS (2013) Nearly finished genomes produced using gel microdroplet culturing reveal substantial intra-species genomic diversity within the human microbiome. Genome Res 23:878–888

Adv Biochem Eng Biotechnol (2016) 156: 55–78
DOI: 10.1007/10_2015_5003

Published online: 24 February 2016

Glycolysis as the Central Core of Fermentation

M. Taillefer and R. Sparling

Abstract The increasing concerns of greenhouse gas emissions have increased the interest in dark fermentation as a means of productions for industrial chemicals, especially from renewable cellulosic biomass. However, the metabolism, including glycolysis, of many candidate organisms for cellulosic biomass conversion through consolidated bioprocessing is still poorly understood and the genomes have only recently been sequenced. Because a variety of industrial chemicals are produced directly from sugar metabolism, the careful understanding of glycolysis from a genomic and biochemical point of view is essential in the development of strategies for increasing product yields and therefore increasing industrial potential. The current review discusses the different pathways available for glycolysis along with unexpected variations from traditional models, especially in the utilization of alternate energy intermediates (GTP, pyrophosphate). This reinforces the need for a careful description of interactions between energy metabolites and glycolysis enzymes for understanding carbon and electron flux regulation.

Keywords ATP, Dark fermentation, Embden–Meyerhof–Parnas pathway, Energy conservation, Fermentation, Glycolysis, GTP, Pyrophosphate, Substrate level phosphorylation

Contents

M. Taillefer and R. Sparling (✉)
Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada, R3T 2N2
e-mail: Richard.Sparling@umanitoba.ca

Abbreviations

ABE	Acetone–Butanol–Ethanol process
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ED	Entner–Doudoroff pathway
EMP	Embden–Meyerhof–Parnas pathway
GTP	Guanosine triphosphate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PPi	Pyrophosphate
PPP	Pentose phosphate pathway
TCA	Tricarboxylic acid cycle

1 Introduction

Microbial fermentations have been used in the preparations of various forms of foods and beverages for thousands of years, with evidence of fermented rice, honey, and fruit found as far back as 8,000 BC [1]. Although beverages draw flavors from the chemicals produced during fermentation, such as lactic acid, acetic acid, ethanol, and CO_2, these chemicals have also become of direct interest to the chemical industries. Although many of these chemicals can be synthesized via various chemical processes, such as the carbonylation of methanol for acetic acid production, many of these processes rely on petroleum-based precursors for synthesis, rendering them unsustainable and reliant on increasingly hard to access fossil fuels sources [2]. Anaerobic organisms can naturally produce many valuable chemicals using renewable biomass sources.

One of the first non-food industrial applications of biological fermentation processes was initiated by the greatly increased demand for acetone during World War I by the British Government for the production of cordite. This led to the development of alternative means for acetone synthesis [3, 4]. *Clostridium acetobutylicum,* also known as the Weizmann organism, produces acetone through the Acetone–Butanol–Ethanol fermentation process (ABE) and was widely used for acetone production starting in 1916 [3–5]. *C. acetobutylicum* produces acetone, butanol, and ethanol in a 6:3:1 ratio using various sugars as biomass feedstock. The ABE process relies on the two phases of *C. acetobutylicum growth*. Phase 1 is the

acidogenic phase in which *C. acetobutylicum* consumes sugars, producing acetic acid and butyric acid. The acids produced in the acidogenic phase cause a drop in pH, which causes a metabolic shift to Phase 2, the solventogenic phase. In the solventogenic phase, the organism continues to utilize sugars but also takes up the acetic acid and butyric acid which are then converted into acetone, butanol, and ethanol [4, 6–8]. The production of acetone and butanol via the ABE process remained widely used in industry until the 1950s when the production of acetone and butanol from fossil fuels became more economically viable when compared to fermentation [3, 4]. The branched nature of the ABE process limits the yields of acetone or butanol, thereby limiting its industrial potential.

Nevertheless, the current issues surrounding the potential for petroleum shortages as well as greenhouse gases from our extensive combustion of petroleum productions has revived interest in biological alternatives to petrochemicals [9]. This revival has been taking full advantage of recent advances in our understanding of bacterial fermentation through genomic and other high throughput techniques [10, 11]. The possibility of genetic manipulation [12] has also caused researchers to revisit dark fermentation processes for biofuel production. There is special interest in the direct fermentation of the sugar content from inexpensive but complex carbon feedstocks such as lignocellulose [13–15], because many industrially relevant anaerobic bacteria have the capabilities of utilizing various carbon sources and also harbor the capability of degrading complex materials such as lignocellulosic biomass (Table 1) [30–33]. Although many anaerobic organisms have the potential to be utilized for various industrial processes, a large number of organisms have branched metabolic pathways, using pyrvuate as a major metabolic intermediate, leading to the simultaneous production of multiple chemicals of greater or lesser value (Fig. 1), which leads to the need for increased downstream processing.

Because most of the fermentative products of interest are the direct products of central metabolism associated with sugar fermentation (both hexoses and pentoses) for the conservation of usable energy, an understanding of the core metabolism of these organisms is important to exploit better the wide range of fermentative organisms available. This is the primary purpose of the current review, which is carried out in light of the most recent knowledge of genomic approaches.

2 Fermentation: Variations on Glycolytic Pathways to Pyruvate

The conservation of chemical energy, generally in the form of ATP, is essential for growth of both aerobic and anaerobic bacteria [34, 35]. Both aerobic and anaerobic organisms oxidize sugars to pyruvate and NADH, but whereas aerobic organisms can utilize the TCA cycle to oxidize pyruvate completely to CO_2 and utilize the NADH and FADH for oxidative phosphorylation via the electron transport chain, anaerobic organisms rely on substrate level phosphorylation, or direct chemical

Table 1 Industrially relevant Firmicutes producing bioenergy products from inexpensive renewable sources

Organism	Growth temperature	Bioenergy products	Carbon source	Reference
Ruminiclostridium thermocellum	60	Ethanol H_2	Lignocellulosic	[16]
Ruminiclostridium termitidis	37	Ethanol H_2	Lignocellulosic	[17]
Ruminiclostridium cellulolyticum	37	Ethanol H_2	Lignocellulosic	[18]
Ruminiclostridium stercorarium	60	Ethanol	Lignocellulosic	[19]
Clostridium ljungdahlii	37	Ethanol	CO_2,CO, H_2 (Syngas)	[20]
Clostridium acetobutylicum	37	Acetone Butanol Ethanol	Lignocellulosic, Starch	[3, 5]
Clostridium tyrobutyricum	37	Butanol	Maltose Starch	[21]
Clostridium beijerinckii	35	Acetone Butanol Ethanol	Lignocellulosic	[22]
Lacnoclostridium phytofermentans	37	Ethanol	Lignocellulosic	[23]
Thermoanaerobacter ethanolicus	70	Ethanol	Glucose Xylose	[24]
Thermoanaerobacter pseudethanolicus	65	Ethanol	Glucose Xylose	[25]
Thermoanaerobacterium saccharolyticum	55	Ethanol Butanol	Xylose	[26, 27]
Caldicellulosiruptor bescii	75	Ethanol	Lignocellulosic	[28]
Caldicellulosiruptor saccharolyticus	70	H_2	Lignocellulosic	[29]

phosphorylation, for ATP generation during oxidation of sugars to pyruvate, and NADH and pyruvate are converted into various end products such as lactate, acetate, and ethanol [36], which may not necessarily be associated with further energy conservation.

In general, anaerobic bacteria employ the Embden–Meyerhof–Parnas (EMP) pathway for sugar utilization [6]. The traditional EMP pathway can be divided into two sections. The upper section or investment phase utilizes ATP as a phosphate group donor to phosphorylate glucose and fructose-6-phosphate. The lower section or pay-off phase produces ATP through the phosphoglycerate kinase and pyruvate kinase. Therefore, through the EMP pathway, it is possible to produce a net of 2 ATP molecules and 2 NADH per glucose [36].

An alternative for the EMP pathway for growth on hexoses would be the Entner–Doudoroff (ED) pathway. The traditional ED pathway, originally characterized in *Pseudomonas*, is found generally in Gram-negative facultative anaerobes [37, 38] including the ethanol producer *Zymomonas* [39, 40]. However, slight alterations to

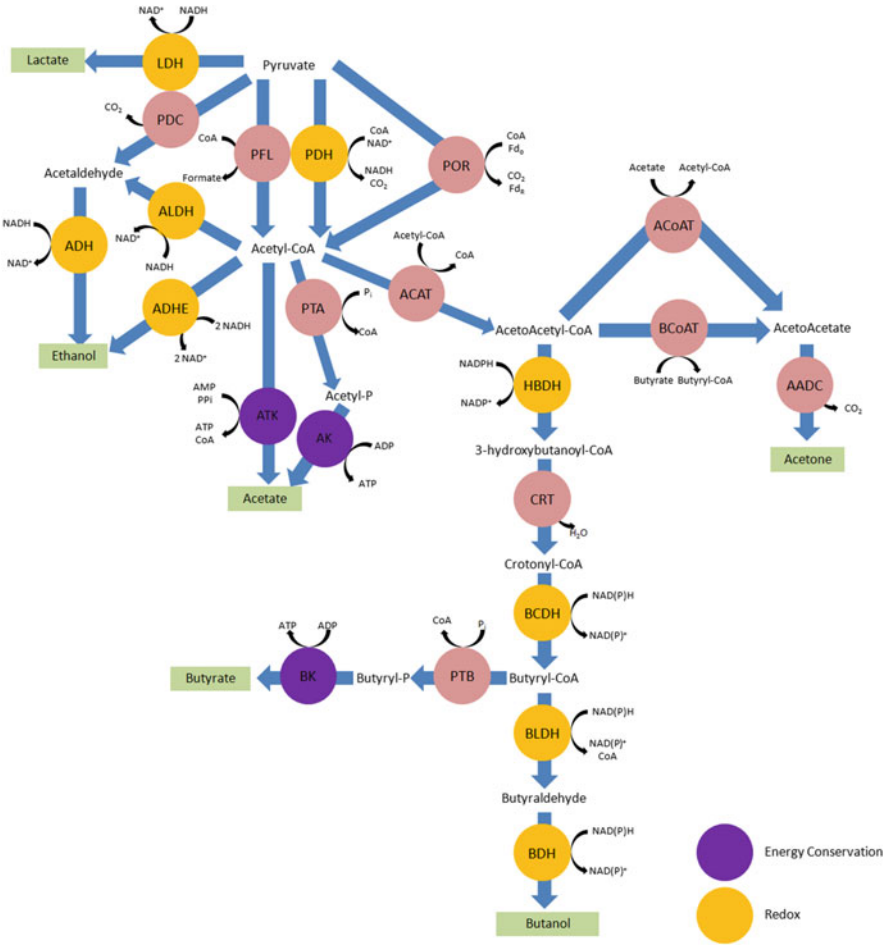


Fig. 1 Various end product synthesis pathways. *LDH* lactate dehydrogenase, *PDC* pyruvate decarboxylase, *PFL* pyruvate formate lyase, *PDH* pyruvate dehydrogenase, *POR* pyruvate ferredoxin oxidoreductase, *ALDH* acetaldehyde dehydrogenase, *ADH* alcohol dehydrogenase, *ADHE* bifunctional acetaldehyde alcohol dehydrogenase, *PTA* phosphotransacetylase, *AK* acetate kinase, *ATK* acetate thiokinase, *ACAT* acetyl-CoA acetyltransferase, *BCoAT* butyrate-acetoacetate CoA-transferase, *ACoAT* acetate-acetoacetate CoA-transferase, *AADC* acetoacetate decarboxylase, *HBDH* hydroxybutyryl-CoA dehydrogenase, *CRT* crotonase, *BCDH* butyryl-CoA dehydrogenase, *BLDH* butyraldehyde dehydrogenase, *BDH* butanol dehydrogenase, *PTB* phosphobutyrylase, *BK* butyrate kinase

the ED have been identified in all three domains of life: Bacteria, Eukarya, and Archaea [38, 41, 42]. Some *Clostridia* can utilize a semi-phosphorylative ED pathway in which glucose is converted to gluconate by gluconate dehydrogenase. The gluconate is converted to 2-keto-3-deoxy-gluconate which is then phosphorylated into 2-keto-3-deoxy-6-phosphogluconate via gluconate dehydratase and 2-keto-3-deoxy-gluconate kinase [38, 43]. Some hyper-thermophilic archaea utilize

another alternative to the ED pathway known as the non-phosphorylated ED pathway [38, 44]. The traditional, semi-phosphorylated, and non-phosphorylated ED pathways lead to a net production of only one ATP and 2 NAD(P)H per glucose [37, 38, 41, 44].

The pentose phosphate pathway (PPP) can utilize both hexoses and pentoses. The PPP also allows for the interconversion of hexoses and pentoses. The PPP can be separated into two branches, the oxidative branch and the non-oxidative branch. In the oxidative phase, glucose-6-phosphate is converted to ribulose-5-phosphate through various steps producing NADPH. This oxidative branch is viewed as a very important contributor to the production of the biosynthetic molecule NADPH [36, 45]. The non-oxidative phase produces various biosynthetic precursors such as ribose-5-phosphate and erythrose-4-phosphate. For all the pathways above, pyruvate is produced and electrons are transferred to an electron carrier, typically nicotinamide. These various pathways are contrasted in Fig. 2.

During fermentation, various compounds, primarily derived from pyruvate, can be utilized as terminal electron acceptors to produce various chemicals such as lactic acid and butanol [6, 36]. The reoxidation of electron carriers, generally NADH, is essential for glycolysis to continue, specifically phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, and therefore a careful balance is achieved between the reduction of NAD^+ and the oxidation of NADH during anaerobic growth.

3 Variations in Cofactor Specificity in Embden–Meyerhof–Parnas Pathway

Focusing on the EMP pathway, the variations in pathways that hexoses can take are compounded by the differences in cofactor specificity of key enzymes in glycolysis as used by different organisms.

3.1 Glucokinase

Glucokinase (GK) catalyzes the phosphorylation of glucose into glucose-6-phosphate, generally using ATP as a phosphate group donor. Bacterial GK, however, shares no homologies with the less specific hexokinases found throughout the domain Eukaryota and also differs from hexokinases by being specific for glucose. Nevertheless, despite the lack of homology, the affinity for glucose binding (Km) of bacterial GK is similar to that of eukaryotic hexokinases [46, 47]. GK in prokaryotes can be further divided into three major groups – ATP-dependent, ADP-dependent, and polyphosphate (polyP)-dependent [46, 48–52]. ATP-dependent GKs are found in all domains of life ranging from human, yeast, Bacteria, and Archaea, and have a very

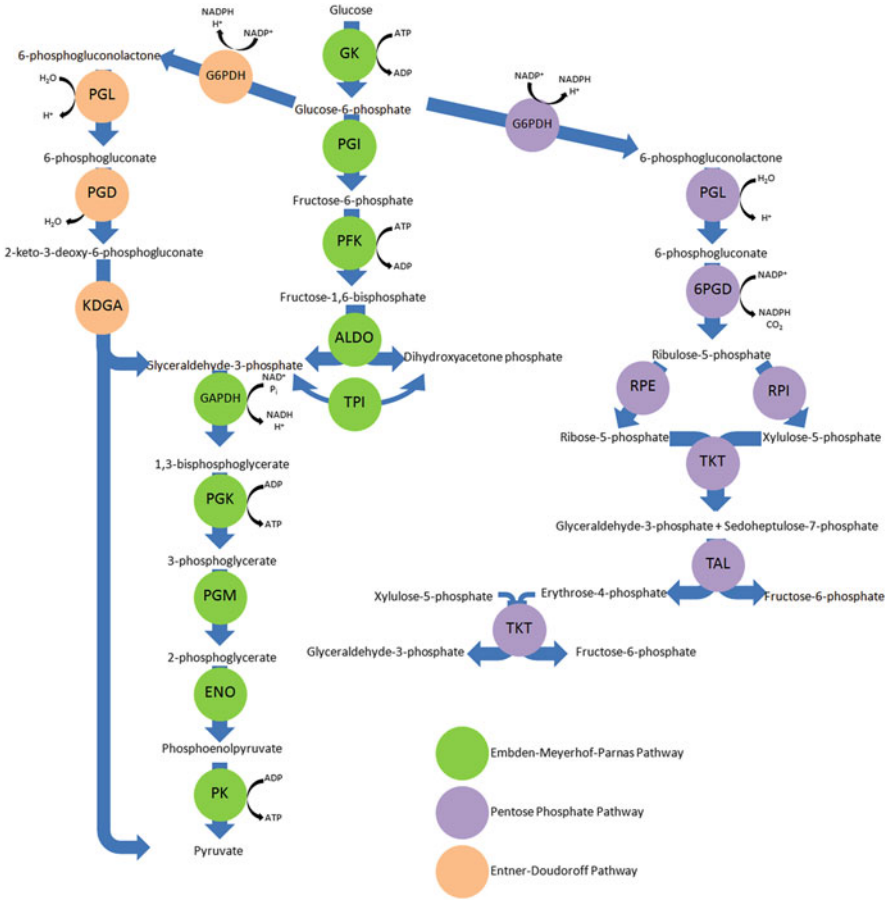


Fig. 2 Conventional pathways for glycolysis. *GK* glucokinase, *PGI* phosphate glucose isomerase, *PFK* phosphofructokinase, *ALDO* fructose-1,6-bisphosphate aldolase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *PGK* phosphoglycerate kinase, *PGM* phosphoglycerate mutase, *ENO* enolase, *PK* pyruvate kinase, *G6PDH* glucose-6-phosphate dehydrogenase, *PGL* 6-phosphogluconolactonase, *PGD* phosphogluconate dehydratase, *KDGA* 2-keto-3-deoxygluconate-6-phosphate aldolase, *6PGD* 6-phosphogluconate dehydrogenase, *RPE* ribulose-5-phosphate 3-epimerase, *RPI* ribose-5-phosphate isomerase, *TKT* transketolase, *TAL* transaldolase

conserved structure [53–55]. ADP-dependent GKs are found extensively in thermophilic archaea, especially in the phylum *Euryachaeota* [56, 57]. The prevalence of ADP-dependent GK in thermophilic archaea is believed to be a result of the increased thermostability of ADP at higher temperatures. This, along with the possibility of recycling ADP generated from ATP hydrolysis used in anabolic reactions as a high energy phosphate donor, can lead to more efficient energy conservation during growth. However, despite the increased thermostability and the possible increase in

net ATP generation, no functional ADP-dependent GKs have been identified in thermophiles within the domain Bacteria. PolyPhosphate (polyP)-dependent GKs were believed to have evolved early in the evolution of life because is believed that polyP was already available in prebiotic times [58]. Therefore, it is believed that the primitive polyP-dependent GK evolved through time to utilize nucleotide triphosphates or nucleotide diphosphates because many polyP-GKs are not specific for polyP but can often utilize ATP, CTP, UTP, or GTP as phosphate group donors as well as PolyP [59, 60].

3.2 Phosphofructokinase

Phosphofructokinase (PFK) catalyzes the phosphorylation of fructose-6-phosphate into fructose-1,6-bisphosphate generally using ATP as a phosphate donor in organisms ranging from *Escherichia coli* to mammals. ATP-dependent PFK plays a pivotal role in the regulation of carbon flux through glycolysis as the first irreversible reaction of glycolysis [61]. Therefore, it was believed that ATP-dependent PFK is conserved throughout the tree of life and is essential for glycolysis. However, pyrophosphate (PPi)-dependent PFKs have been found in lower eukaryotes such as *Entamoeba histolytica* and *Toxoplasma gondii*, in plants, in Archaea, and in some Bacteria [62–65]. PFKs can be organized into three main phylogenetically distinct-but-related families. Family A PFK includes the ATP-dependent PFK from higher eukaryotes, ATP- and PPi-dependent PFK from bacteria, PPi-dependent from some Archaea, and PPi-dependent from plants [63, 66–69]. Family A PPi-dependent PFK can further be divided into Type I, which are not regulated by fructose-2,6-bisphosphate and found predominantly in anaerobic bacteria , and Type II which are activated by the presence of fructose -2,6-bisphosphate similar to ATP-dependent PFK found in higher eukaryotes, and are generally only found in plants [46, 63]. PPi-dependent PFK, unlike ATP-dependent PFK, is reversible and can also be utilized for gluconeogenesis, essentially replacing fructose-1,6-bisphosphatase. Family B PFKs are ATP-dependent and found generally in Enterobacteria such as *E. coli* [46, 70, 71]. Family B PFKs have demonstrated the potential of phosphorylating various substrates such as fructose, fructose-1-phosphate, adenosine, and ribose [72]. Family C are ADP-dependent PFKs that have only been identified in thermophilic Archaea and some mesophilic methanogenic Archaea [71, 73, 74].

3.3 Glyceraldehyde-3-Phosphate Dehydrogenase and Phosphoglycerate Kinase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the reversible phosphorylation of glyceraldehyde-3-phosphate utilizing NAD^+ and inorganic phosphate (P_i) into 1,3-bisphosphoglycerate and NADH. Some plants and photosynthetic cyanobacteria have NADP-dependent GAPDH which is utilized for gluconeogenesis involved in CO_2 assimilation [75–77]. Phosphoglycerate kinase (PGK) catalyzes the transfer of a phosphate group from 1,3-bisphosphoglycerate onto ADP forming ATP and 3-phosphoglycerate. Most PGKs can utilize other purine nucleotides such as GDP/GTP with similar affinities as ADP/ATP but with much lower catalytic (<50%) rates, making ATP/ADP the preferred substrates [78–80].

At this point in the glycolytic pathway, many thermophilic Archaea utilize variants of the typical EMP pathway such as a tungsten dependent glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR). This bypasses the transfer of the phosphate group onto ADP catalyzed by PGK yielding a lower net ATP gained directly from glycolysis. GAPOR catalyzes the direct irreversible conversion of glyceraldehyde-3-phosphate into 3-phosphoglycerate using ferredoxin as an electron acceptor, bypassing the need for GAPDH and PGK [46, 81, 82]. There has been no GAPOR orthologs found in Bacteria. However, some Bacteria have an enzyme similar to GAPOR in the form of a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDHN). GAPDHN, similar to GAPOR, directly and irreversibly catalyzes the conversion of glyceraldehyde-3-phosphate into 3-phosphoglycerate, but using $NADP^+$ as an electron acceptor rather than ferredoxin [83, 84]. Once believed to be found exclusively in plants, GAPDHN has been identified in many bacteria including some Firmicutes such as *Clostridium acetobutylicum* [85]. However, in *C. acetobutylicum* the activity of the GAPDHN was roughly 100-fold lower than the GAPDH activity under the conditions tested and therefore its role in metabolism remains unclear [86].

3.4 Pyruvate Kinase

The final step of the traditional EMP pathway is the conversion of phosphoenolpyruvate (PEP) into pyruvate. Generally, this final step is catalyzed by pyruvate kinase (PK) transferring the phosphate onto ADP creating ATP. PKs can be generally assembled into type I and type II based on their regulatory mechanisms. Type I PKs are activated by various sugar phosphates such as fructose-1,6-bisphosphate and are generally found in Bacteria and eukaryotes. Type II PKs are regulated by energy intermediates such as AMP and ATP rather than sugar phosphates, and are found throughout Bacteria and Archaea [46, 87]. The conversion of PEP into pyruvate can also be catalyzed by a pyruvate phosphate dikinase (PPDK)

or pyruvate water dikinase (PPS) rather than PK. PPDK utilizes PEP, AMP, and PPi to produce pyruvate, ATP, and P_i. PPS is similar to PPDK but utilizes PEP, AMP, and P_i, producing pyruvate and ATP. However, pyruvate production through PK is expected to be favored over PPDK and PPS because it is a more thermodynamically favorable reaction [7]. Despite PK being thermodynamically favorable, some organisms utilize PPDK or PPS over PK even if both are present in the genome. In the thermophilic Archaea *Thermococcus kodakarensis*, PK and PPS are utilized during glycolysis. The PPS is the main contributor of pyruvate flux with the PK being activated under high ADP concentrations in order to regulate the internal concentration of ADP [88]. This regulatory mechanism might be specific to Archaea, specifically the *Thermococales,* because many Archaea utilize ADP-dependent GK and PFK over ATP- or PPi-dependent versions employed in bacteria. Another Archaea, however, *Thermoproteus tenax*, utilizes ATP-dependent GK and PPi-dependent PFK rather than ADP-dependent versions and employs similar means of pyruvate kinase regulation. In the *T. tenax* genome, PK, PPDK, and PPS are all encoded with PK only being expressed during heterotrophic growth and PPS only being expressed during autotrophic growth, whereas the PPDK is expressed under all conditions. During heterotrophic growth, both PK and PPDK are utilized for glycolysis with the activity of the PPDK regulated by the ratio of ATP/AMP whereas the PK activity is regulated by the concentration of ADP [89]. A further example of regulation can be found in *Caldicellulosiruptor saccharolyticus*. During growth, *C. saccharolyticus* utilizes both PPDK and a type II PK regulated by the presence of PPi, AMP, and ADP during growth [90, 91]. All of these proposed regulatory mechanisms demonstrate the importance of energy carriers as direct regulators of pyruvate synthesis.

Taken together, alternative co-factor utilization can offer various advantages such as flexibility in cofactor utilization, allowing organisms to adapt to various conditions, and the recycling of byproducts from anabolic reactions for catabolic reactions to increase the net ATP gained from glycolysis. The utilization of alternative cofactors can alter the regulation of glycolytic flux by alteration of key regulatory steps such as PFK (PPi vs ATP). Increased energy efficiency in modified glycolysis pathways could offer advantages to strictly fermentative organisms such as bacteria from the class Clostridia. Studying these variations in greater depth is of importance, because several fermentative organisms with industrial potential show differences in co-factor utilization observed through both genomic studies and recent physiological studies.

4 Variant Glycolytic Pathway of *Ruminiclostridium thermocellum*

The genus *Clostridium* contains a wide variety of obligately anaerobic sporulating organisms, several of which have the potential to generate industrially relevant fermentative end-products. From a phylogenetic point of view, it encompasses

rather a wide range of distinct clades. A recent phylogenetic re-evaluation has broken up the genus redistributing many of the species into six new genera [92]. For example, *C. acetobutylicum* and several other acetone–butanol producers stay in the genus *Clostridium*, whereas several of the better studied ethanol-producing cellulose degrading clostridia have been moved to the *Ruminococcaceae*, a family which includes the cellulolytic ethanol producing *Ruminococcus albus,* into the genus *Ruminiclostridium*. The type species for this new genus is *Ruminiclostridium thermocellum* (formally *Clostridium thermocellum*), a well-studied thermophile capable of growth on crystalline cellulose and a candidate for commercial bioethanol production via consolidated bioprocessing of lignocellulosic biomass [9, 32, 92]. However, wild type *R. thermocellum* strains undergo mixed acid fermentations, producing lactate, formate, acetate, ethanol, H_2, and CO_2, which limits the production of bioethanol because of diversion of carbon and electrons by the branched metabolic pathways [93–98]. *R. thermocellum* has all of the genes required for a functional EMP pathway with the exception of a PK, and the required proteins are transcribed and translated during growth [99–102]. Therefore, it was believed that *R. thermocellum* had a classical glycolysis pathway, with the PK assumed to be present. Although PK activity was reported in *R. thermocellum* strain 651 in the 1970s, no gene could be linked to this activity [103]. None of the currently sequenced strains of *R. thermocellum* have any annotated PK gene. Explanations for the discrepancies are that the PK activity was linked to a specific strain of *R. thermocellum* strain 651, and subsequently this particular strain was lost, or that the *R. thermocellum* strain 651 described in the 1970s could have been contaminated with various saccharolytic organisms such as bacteria from the genus *Thermoanaerobacter* [104–106].

Looking at glycolysis in greater detail, cell extracts of *R. thermocellum* ATCC27405 and DSM1313 display very low or undetectable ATP-dependent GK activity [104, 107]. Instead, *R. thermocellum* displayed GK activity with a strong preference for GTP (50-fold higher than ATP) as a phosphate group donor. GTP-dependent GK has been identified in other organisms such as *Fibrobacter succinogenes*, *Fibrobacter intestinalis*, and *Ruminococcus albus* [108, 109]. Both *R. thermocellum* and *R. albus* fall under the family *Ruminococcaceae*. Interestingly, these organisms all utilize cellulosic materials as a carbon source during growth in their natural environments. The GTP-dependent GK activity from *F. intestinalis*, *R. albus,* and *R. thermocellum* were not specific for GTP but all displayed lower activity with ATP when measured in cell extracts similar to the partially purified GTP-dependent GK from *F. succinogenes* [104, 108, 109]. This indicates that GTP-dependent GK shares similarities with the polyP-dependent GK in that they have a preferred phosphate donor but are not specific to one phosphate donor. Although PPi was tested as a putative phosphate group donor for GK in the *R. thermocellum* cell extract, no activity was detectable [104].

The reaction catalyzed by ATP-dependent PFK is often viewed as the first committed step in glycolysis because it is essentially irreversible under biological conditions. However, *R. thermocellum* does not exhibit any detectable ATP-dependent PFK activity but instead utilizes a PPi-dependent PFK for

fructose-6-phosphate phosphorylation when grown on cellobiose [104]. The *R. thermocellum* genome contains a copy of both ATP- and PPi-dependent PFK genes and both are transcribed and translated. However, the levels of PPi-dependent PFK in the proteome and transcriptome is much greater than that of the ATP-dependent PFK [100–102]. Utilization of PPi-dependent PFK offers a net increase of 1 ATP per glucose during glycolysis by recycling PPi generated as a byproduct from various anabolic reactions in order to phosphorylate fructose-6-phosphate [63, 90, 110, 111].

In the absence of PK, *R. thermocellum* must utilize alternative means of pyruvate generation. Possible alternatives include PPDK, oxaloacetate decarboxylase (OOADC), or a malate shunt, all of which are present in the genome, transcribed and translated [100–102]. PPDKs are believed to play a role in gluconeogenesis rather than glycolysis in many organisms [112, 113]. However, *Trypanosoma cruzi* utilizes the internal concentrations of PPi to control phosphoenolpyruvate utilization. Under high internal concentrations of PPi, phosphoenolpyruvate utilization shifts from PK to PPDK for the production of pyruvate and ATP [114]. Although this provides precedence for PPDK use in *R. thermocellum*, it was observed that the deletion of the PPDK gene had little effect on the growth rate or final culture density [104]. Pyruvate may also be produced indirectly through a malate shunt. Phosphoenolpyruvate is converted to oxaloacetate via the GDP-dependent phosphoenolpyruvate carboxykinase (PEPCK). The oxaloacetate can then be converted to malate and finally pyruvate by NADH-dependent malate dehydrogenase (MDH) and $NADP^+$-dependent malic enzyme (MalE) (Fig. 3). MDH catalyzes the reduction of oxaloacetate into malate with NADH as a preferred cofactor. MalE catalyzes the decarboxylation of malate into pyruvate utilizing $NADP^+$ as cofactor [115, 116]. Overall, the indirect conversion of phosphoenolpyruvate into pyruvate via the malate shunt produces GTP rather than ATP and a transfer of electron between NADH and $NADP^+$. Conversely, oxaloacetate can be directly decarboxylated into pyruvate using a proton translocating, membrane-bound OAADC. Either way, GTP produced through PEPCK could be used to recharge GDP discharged from the GK, thus linking the malate shunt with the initiation of glycolysis.

PPDK is expected to be activated by PPi, while the MalE, and therefore the malate shunt, is inhibited by the presence of PPi [108]. High concentrations of PPi would be expected to direct carbon flux through PPDK whereas low concentrations of PPi redirect carbon through OAADC with the malate shunt ultimately regulating the production of pyruvate [116]. However, based on theoretical calculations, the amount of PPi generated as byproduct from various reactions would not be able to account for the PPi requirement for PPi-dependent PFK and PPDK during active growth [104, 117, 118]. Therefore, the active generation of PPi seems to be a requirement for glycolysis during growth using PPi-dependent PFK and PPDK. The active generation of PPi can possibly be done through multiple means in *R. thermocellum* such as the utilization of a membrane bound H^+-translocating V-type inorganic pyrophosphatase (V-type PPase), a modified pentose phosphate pathway via the PPi-dependent PFK (Fig. 3), and glycogen cycling [117, 119–121].

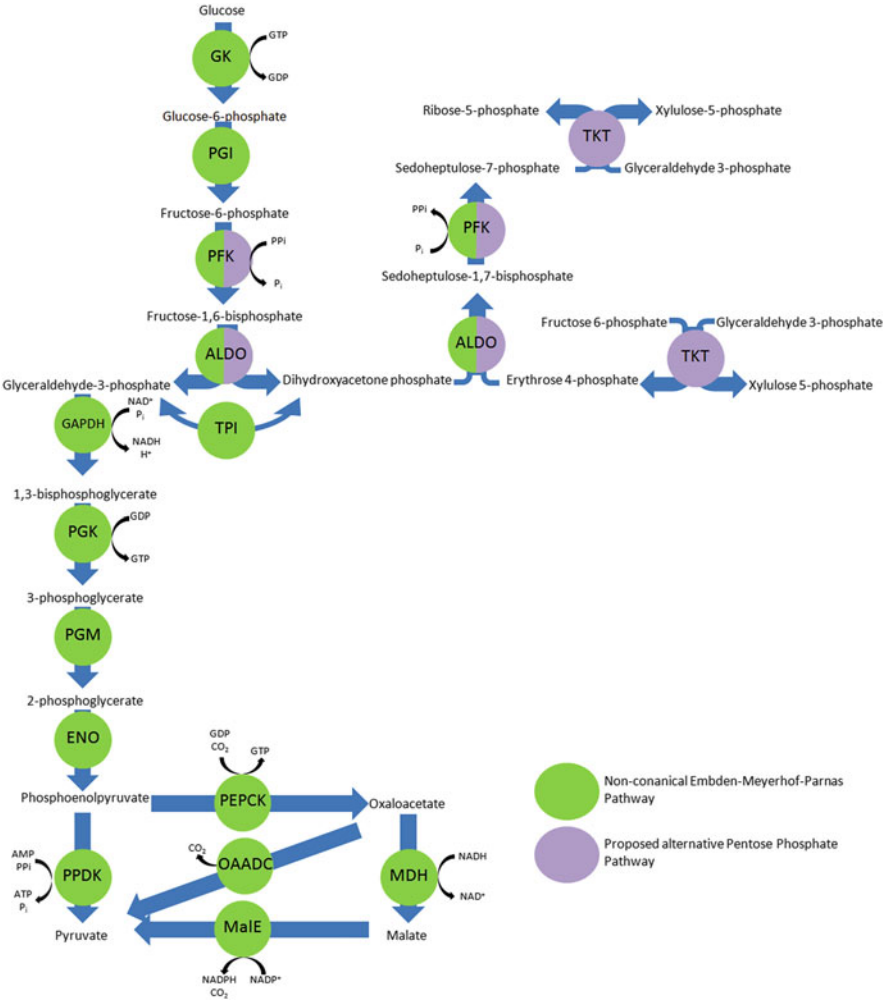


Fig. 3 Proposed *R. thermocellum* central catalysis pathways. *GK* glucokinase, *PGI* phosphate glucose isomerase, *PFK* phosphofructokinase, *ALDO* fructose-1,6-bisphosphate aldolase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *PGK* phosphoglycerate kinase, *PGM* phosphoglycerate mutase, *ENO* enolase, *PPDK* pyruvate phosphate dikinase, *PEPCK* phosphoenolpyruvate carboxykinase, *MDH* malate dehydrogenase, *MalE* malic enzyme, *OAADC* oxaloacetate decarboxylase, *TKT* transketolase

In *R. thermocellum*, the genome does not encode a soluble inorganic pyrophosphatase; rather it encodes for one V-type PPase that is transcribed and translated throughout the growth, indicating that it is active during growth [100–102]. Generally, V-type PPase utilizes the breakdown of PPi into P_i to pump H^+ across the membrane. Because of the need for active generation of PPi, however,

the V-type PPase could utilize proton motor force to synthesize PPi, similar to how ATP is generated through ATP synthase [117, 119].

R. thermocellum does not have a complete pentose phosphate pathway as it lacks the enzymes for the oxidative branch (glucose-6-phosphate dehydrogenase, gluconolactonase, and 6-phosphogluconate dehydrogenase) and also the transaldolase for the non-oxidative branch [101]. Rather, the pentose phosphate pathway of *R. thermocellum* is predicted to rely on intermediates produced by the EMP pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate, to feed into the pentose phosphate pathway for the production of pentose intermediates. In the absence of a transaldolase, *R. thermocellum* possibly employs a novel mechanism for pentose generation that has been demonstrated in several parasitic protists such as *Entamoeba histolytica* and *E. coli* [101, 122–125]. This mechanism involves the aldolase and PPi-dependent PFK from the EMP pathway along with the transketolase. The aldolase converts dihydroxyacetone phosphate and erythrose-4-phosphate into sedoheptulose-1,7-bisphosphate. The sedoheptuloase-1,7-bisphohate is then dephosphorylated by PPi-dependent PFK into sedoheptulose-7-phosphate, producing PPi. Finally, the sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate are converted into xylulose-5-phosphate and ribose-5-phosphate [101, 124]. Therefore, the conversion of hexoses to pentoses could help supply the PPi required for growth in *R. thermocellum*. The lack of an oxidative phase of the PPP also removes the NADPH-generating reactions of the PPP required for many biosynthetic reactions. However, this lack of NADPH generation could be alleviated by flux through the transhydrogenation reaction of the malate shunt or by the activity of an NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase (NfnAB) which simultaneously utilizes both NADH and reduced ferredoxin, producing NADPH [126, 127]. This also raises an interesting question with respect to cofactor regulation based on the production of a biosynthetic byproduct, PPi, and a biosynthetic requirement, NADPH.

PPi can also be produced during glycogen cycling, more specifically by glucose-1-phosphate adenyltransferase. The simultaneous production and consumption of glycogen has been observed in some related cellulolytic organisms such as *Ruminiclostridium cellulolyticum* and *R. albus* [120, 121]. The proteome of *R. thermocellum* supports simultaneous production and consumption of glycogen because both glycogen synthesis and breakdown proteins are detectable throughout growth [101]. However, uncontrolled cycling would result in futile cycles of production and consumption. However, it is possible that glycogen cycling could be regulated by the internal concentrations of PPi and ATP. At high concentrations of ATP, glucose-1-phosphate adenyltransferase would utilize ATP and glucose-1-phosphate to produce PPi and ADP-glucose, whereas at low ATP concentrations the flux through glucose-1-phosphate adenyltransferase would be greatly reduced, limiting PPi production.

Therefore, despite the requirement for *R. thermocellum* to produce PPi for glycolysis, utilization of a PPi-dependent PFK and PPDK leads to a net increase in the conservation of chemical energy because of the recycling of PPi from various biosynthetic reactions, such as the synthesis of nucleic acids, amino acids, fatty

acids, and lipids [118]. The utilization of PPi-dependent enzymes in glycolysis could reflect the strict fermentative lifestyle of *R. thermocellum* where efficient energy generation is a requirement for its growth and survival.

5 *Ruminiclostridium* Glycolysis a Widespread Model?

When compared to the traditional *E. coli* model, it would seem that the *R. thermocellum* central catabolism is not canonical in the sense that it may rely on enzymes (GK, PFK, PGK, PPDK) that utilize alternative cofactors during growth, and adapt an alternative strategy for a functional non-oxidative pentose phosphate pathway [101, 104]. An analysis of the genome of some Firmicutes and other phyla, especially thermophiles, revealed that many *Ruminiclostridium, Caldicellulosiruptor, Thermotoga, and Thermoanaerobacter* have a PPi-dependent PFK and an ATP-dependent PFK in their genomes [90, 128, 129]. Although both ATP- and PPi-dependent PFK have been found in *R. stercorarium* based on sequence homology with the *R. thermocellum* PFK, the proteome of *R. stercorarium* shows significantly higher expression of the PPi-dependent PFK vs the ATP-dependent PFK [19]. A further role of PPi-dependent PFK in *Ruminiclostridium* may be to compensate for the missing transaldolase gene for PPP in, for example, *R. stercorarium* [19] and *R. termitidis* (Munir and Levin, unpublished data) (Fig. 3).

Although *R. thermocellum* does not contain a PK gene, most other organisms do encode a PK in their genome. Despite the presence of a PK gene in their genomes, *C. saccharolyticus*, *R. termitidis,* and *R. stercorarium* seem to prefer the utilization of their PPDK based on expression levels and/or enzyme activities ([19, 90], Munir and Levin, unpublished data). The presence of both a PPDK and PK is expected to be regulated by the internal concentrations of PPi because PK activity is strongly inhibited by PPi [90, 114]. Because the internal concentrations of PPi were found to be relatively high (4 mM) during exponential growth in *C. saccharolyticus*, it would seem that PPDK is utilized over PK during active growth showing a strong preference for PPDK, even with an active PK present [90]. Therefore, the regulation of pyruvate generation seems to be similar to what is proposed in *R. thermocellum* in which the malate shunt is inhibited by the internal concentration of PPi, leading to PPDK utilization during high internal PPi concentrations [116].

With the absence of the oxidative branch of the PPP, production of NADPH can be done through the malate shunt or through the NfnAB. As with *R. thermocellum*, *R. stercorarium* and *R. termitidis* are lacking a complete oxidative branch and therefore would rely on alternative means to produce NADPH such as the malate shunt [116]; (Munir and Levin, unpublished data). This also correlates with the phylogenetic grouping of the *R. thermocellum*, *R. termitidis*, and *R. stercorarium* MalE, hinting at a similar regulation by PPi [116]. Taken together, the central catalysis of *R. thermocellum* is different from the "conventional" model. However, it seems to be similar to various other Firmicutes, especially within the genus

Ruminiclostridium. This also raises the importance of PPi for *Ruminiclostridium* not only as a putative energy-recycling source but also as a regulatory molecule regulating pyruvate synthesis and NADPH generation. Aspects of this pathway variant are likely to extend to other genera, for example in the *Caldicellulosiruptor*.

6 Conclusion

Utilization of inexpensive renewable biomass sources by anaerobic organisms is of particular interest because of their ability to utilize various and complex substrates without requiring extensive pre-treatments. However, the branched nature of the central catalysis of many anaerobic bacteria diminishes the industrial strength of this process.

As the bioenergy products are produced directly from glycolysis products and intermediates, a clear understanding of glycolysis is essential in the development of strategies for increasing product yields and therefore increasing industrial potential. Although *R. thermocellum* glycolysis differs in cofactor preference when compared to the 'traditional' model, it seems to be a representative model of the metabolism found in various Firmicutes, especially in the genus *Ruminiclostridium*. Alterations in cofactor utilization, such as the preference for PPi rather than ATP as the phosphate group donor for PFK, can render the reaction reversible, damping the regulatory role of PFK in controlling the glycolytic flux. Insights into the cofactor utilization of *R. thermocellum* have also revealed PPi as a very important putative regulator of many essential reactions, such as the control of pyruvate production and NADPH production. However, the importance of PPi as a putative regulator of catabolic and anabolic reactions is not strictly a phenomenon of *R. thermocellum* metabolism, but seems widespread among the genus *Ruminiclostridium* and other related Firmicutes. Therefore, *R. thermocellum* glycolysis appears as a valid alternative model for many industrially relevant anaerobic bacteria.

Furthermore, the diversity of potential variations in pathways described in the current review reemphasizes the importance of refining annotation on the basis of biochemical characterization of key enzymes in central metabolism in relevant organisms, especially with respect to cofactor specificity and allosteric regulation. The presence within the genome of multiple genes putatively coding for the same annotated function, and even their expression in the transcriptome and proteome, reminds us of the further importance of allostery in regulating flux of intermediates through these enzymes. There is a potential interplay of high energy phosphate carriers (ATP, GTP, PPi) and electron carriers (NADH, NADPH, ferredoxin), making the measurement of in vivo concentrations of these co-factors crucial in understanding not only central metabolism but also end product selection in industrially relevant fermentative organisms (Table 1).

Acknowledgements This work was funded through the Genome Canada-funded Microbial Genomics for Biofuels and Co-products Biorefineries project and through NSERC Discovery Grant (RGPIN-2014-06173).

References

1. McGovern PE, Zhang J, Tang J, Zhang Z, Hall GR, Moreau RA, Nuñez A, Butrym ED, Richards MP, Wang C-S, Cheng G, Zhao Z, Wang C (2004) Fermented beverages of pre- and proto-historic China. Proc Natl Acad Sci USA 101:17593–17598
2. Rogers P, Chen J, Zidwick MJO (2013) Organic acid and solvent production. Part I: acetic, lactic, gluconic, succinic and polyhydroxyalkanoic acids. Prokaryotes 511–755
3. Ross D (1961) The acetone-butanol fermentation. Prog Ind Microbiol 3:71–90
4. Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50:484–524
5. Gabriel CL (1928) Butanol fermentation process 1. Ind Eng Chem 20:1063–1067
6. Gottschalk G (1986) Bacterial metabolism, 2nd edn. Springer-Verlag, New York
7. Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180
8. Häggström L (1985) Acetone-butanol fermentation and its variants. Biotechnol Adv 3:13–28
9. Demain AL, Newcomb M, Wu JHD (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69:124–154
10. Bao G, Dong H, Zhu Y, Mao S, Zhang T, Zhang Y, Chen Z, Li Y (2014) Comparative genomic and proteomic analyses *of Clostridium acetobutylicum* Rh8 and its parent strain DSM 1731 revealed new understandings on butanol tolerance. Biochem Biophys Res Commun 450:1612–1618
11. Linville JL, Rodriguez M, Land M, Syed MH, Engle NL, Tschaplinski TJ, Mielenz JR, Cox CD (2013) Industrial robustness: understanding the mechanism of tolerance for the *Populus* hydrolysate-tolerant mutant strain of *Clostridium thermocellum*. PLoS One 8:1–16
12. Olson DG, Sparling R, Lynd LR (2015) Ethanol production by engineered thermophiles. Curr Opin Biotechnol 33:130–141
13. McKendry P (2002) Energy production from biomass (part 1): overview of biomass. Bioresour Technol 83:37–46
14. Hamelinck CN, Van Hooijdonk G, Faaij APC (2005) Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass Bioenergy 28:384–410
15. Carroll A, Somerville CR (2009) Cellulosic biofuels. Annu Rev Plant Biol 60:165–82
16. Lynd LR (1996) Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy. Annu Rev Energy Environ 21:403–465
17. Hethener P, Brauman A, Garcia J-L (1992) *Clostridium termitidis* sp. nov., a cellulolytic bacterium from the gut of the wood-feeding termite, *Nasutitermes lujae*. Syst Appl Microbiol 15:52–58
18. Ren Z, Ward TE, Logan BE, Regan JM (2007) Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* species. J Appl Microbiol 103:2258–2266
19. Schellenberg JJ, Verbeke TJ, McQueen P, Krokhin OV, Zhang X, Alvare G, Fristensky B, Thallinger GG, Henrissat B, Wilkins JA, Levin DB, Sparling R (2014) Enhanced whole genome sequence and annotation of *Clostridium stercorarium* DSM8532T using RNA-seq transcriptomics and high-throughput proteomics. BMC Genomics 15:567
20. Phillips JR, Klasson KT, Clausen EC, Gaddy JL (1993) Biological production of ethanol from coal synthesis gas. Appl Biochem Biotechnol 39–40:559–571

21. Yu M, Zhang Y, Tang IC, Yang ST (2011) Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production. Metab Eng 13:373–382
22. Bellido C, Infante C, Coca M, González-Benito G, García-Cubero MT (2015) Efficient acetone-butanol-ethanol production by *Clostridium beijerinckii* from sugar beet pulp. Bioresour Technol 190:332–338
23. Warnick TA, Methé BA, Leschine SB (2002) *Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil. Int J Syst Evol Microbiol 52:1155–1160
24. Wiegel J, Ljungdahl LG (1981) *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. Arch Microbiol 128:343–348
25. Hemme CL, Fields MW, He Q, Deng Y, Lin L, Tu Q, Mouttaki H, Zhou A, Feng X, Zuo Z, Ramsay BD, He Z, Wu L, Van Nostrand J, Xu J, Tang YJ, Wiegel J, Phelps TJ, Zhou J (2011) Correlation of genomic and physiological traits of *Thermoanaerobacter* species with biofuel yields. Appl Environ Microbiol 77:7998–8008
26. Shaw AJ, Jenney FE, Adams MWW, Lynd LR (2008) End-product pathways in the xylose fermenting bacterium, *Thermoanaerobacterium saccharolyticum*. Enzyme Microb Technol 42:453–458
27. Bhandiwad A, Shaw AJ, Guss A, Guseva A, Bahl H, Lynd LR (2014) Metabolic engineering of *Thermoanaerobacterium saccharolyticum* for n-butanol production. Metab Eng 21:17–25
28. Chung D, Cha M, Guss AM, Westpheling J (2014) Direct conversion of plant biomass to ethanol by engineered *Caldicellulosiruptor bescii*. Proc Natl Acad Sci USA 111:8931–6
29. Bielen AAM, Verhaart MRA (2013) Biohydrogen production by the thermophilic bacterium *Caldicellulosiruptor saccharolyticus*: current status and perspectives. Life 3:52–85
30. Lamed R, Zeikus JG (1980) Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. J Bacteriol 144:569–578
31. Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET (2012) Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr Opin Biotechnol 23:364–381
32. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577
33. Lynd LR, Van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583
34. Stouthamer AH (1973) A theoretical study on the amount of ATP required for synthesis of microbial cell material. Antonie Van Leeuwenhoek 39:545–565
35. Schneider DA, Gourse RL (2004) Relationship between growth rate and ATP concentration in *Escherichia coli*: a bioassay for available cellular ATP. J Biol Chem 279:8262–8268
36. Prescott LM, Harley JP, Klein DA (2005) Microbiology. McGraw-Hill, New York
37. Entner N, Doudoroff M (1952) Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. J Biol Chem 196:853–862
38. Conway T (1992) The Entner–Doudoroff pathway: history, physiology and molecular biology. FEMS Microbiol Rev 9:1–27
39. Barker BTP, Hillier VF (1912) Cider sickness. J Agric Sci 5(01):68–85
40. Swings J, De Ley J (1977) The biology of *Zymomonas*. Bacteriol Rev 41:1–46
41. Andreesen JR, Gottschalk G (1969) The occurrence of a modified Entner–Doudoroff pathway in *Clostridium aceticum*. Arch Microbiol 69:160–170
42. Romano AH, Conway T (1996) Evolution of carbohydrate metabolic pathways. Res Microbiol 147:448–455
43. Bender R, Andreesen JR, Gottschalk G (1971) 2-Keto-3-deoxygluconate, an intermediate in the fermentation of gluconate by Clostridia. J Bacteriol 107:570–573
44. Ahmed H, Ettema TJG, Tjaden B, Geerling ACM, van der Oost J, Siebers B (2005) The semi-phosphorylative Entner–Doudoroff pathway in hyperthermophilic archaea: a re-evaluation. Biochem J 390:529–540

45. Boyle J (2005) In: Nelson D, Cox M (eds) Lehninger principles of biochemistry, 4th edn. Biochem Mol Biol Educ 33:74–75
46. Ronimus RS, Morgan HW (2003) Distribution and phylogenies of enzymes of the Embden–Meyerhof–Parnas pathway from archaea and hyperthermophilic bacteria support a gluconeogenic origin of metabolism. Archaea 1:199–221
47. Cárdenas ML, Cornish-Bowden A, Ureta T (1998) Evolution and regulatory role of the hexokinases. Biochim Biophys Acta Mol Cell Res 1401:242–264
48. Ito S, Fushinobu S, Yoshioka I, Koga S, Matsuzawa H, Wakagi T (2001) Structural basis for the ADP-specificity of a novel glucokinase from a hyperthermophilic archaeon. Structure 9:205–214
49. Labes A, Schonheit P (2003) ADP-dependent glucokinase from the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus* strain 7324. Arch Microbiol 180:69–75
50. Holwerda EK (2014) The exometabolome of *Clostridium thermocellum* reveals overflow metabolism at high cellulose loading. Biothechnol Biofuels 7:1–11
51. Phillips NF, Horn PJ, Wood HG (1993) The polyphosphate- and ATP-dependent glucokinase from *Propionibacterium shermanii*: both activities are catalyzed by the same protein. Arch Biochem Biophys 300:309–319
52. Hsieh PC, Kowalczyk TH, Phillips NFB (1996) Kinetic mechanisms of polyphosphate glucokinase from *Mycobacterium tuberculosis*. Biochemistry 35:9772–9781
53. Anderson CM, Stenkamp RE, Steitz TA (1978) Sequencing a protein by X-ray crystallography. J Mol Biol 123:15–33
54. Rosano C, Sabini E, Rizzi M, Deriu D, Murshudov G, Bianchi M, Serafini G, Magnani M, Bolognesi M (1999) Binding of non-catalytic ATP to human hexokinase I highlights the structural components for enzyme-membrane association control. Structure 7:1427–1437
55. Aleshin AE, Zeng C, Bartunik HD, Fromm HJ, Honzatko RB (1998) Regulation of hexokinase I: crystal structure of recombinant human brain hexokinase complexed with glucose and phosphate. J Mol Biol 282:345–357
56. Koga S, Yoshioka I, Sakuraba H, Takahashi M, Sakasegawa S, Shimizu S, Ohshima T (2000) Biochemical characterization, cloning, and sequencing of ADP-dependent (AMP-forming) glucokinase from two hyperthermophilic archaea, *Pyrococcus furiosus* and *Thermococcus litoralis*. J Biochem 128:1079–1085
57. Dörr C, Zaparty M, Tjaden B, Brinkmann H, Siebers B (2003) The hexokinase of the hyperthermophile *Thermoproteus tenax*: ATP-dependent hexokinases and ADP-dependent glucokinases, two alternatives for glucose phosphorylation in Archaea. J Biol Chem 278:18744–18753
58. Kornberg A (1995) Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J Bacteriol 177:491–496
59. Szymona M, Widomski J (1974) A kinetic study on inorganic polyphosphate glucokinase from *Mycobacterium tuberculosis* H37RA. Physiol Chem Phys 6:393–404
60. Phillips NF, Hsieh PC, Kowalczyk TH (1999) Polyphosphate glucokinase. Prog Mol Subcell Biol 23:101–125
61. Moreno-Sánchez R, Encalada R, Marín-Hernández A, Saavedra E (2008) Experimental validation of metabolic pathway modeling. FEBS J 275:3454–3469
62. Mertens E (1991) Pyrophosphate-dependent phosphofructokinase, an anaerobic glycolytic enzyme? FEBS 285:1–5
63. Mertens E, Ladror US, Lee JA, Miretsky A, Morris A, Rozario C, Kemp RG, Müller M (1998) The pyrophosphate-dependent phosphofructokinase of the protist, *Trichomonas vaginalis*, and the evolutionary relationships of protist phosphofructokinases. J Mol Evol 47:739–750
64. Peng ZY, Mansour TE (1992) Purification and properties of a pyrophosphate-dependent phosphofructokinase from *Toxoplasma gondii*. Mol Biochem Parasitol 54:223–230
65. Wood HG, O'brien WE, Micheales G (1977) Properties of carboxytransphosphorylase; pyruvate, phosphate dikinase; pyrophosphate-phosphofructikinase and pyrophosphate-

acetate kinase and their roles in the metabolism of inorganic pyrophosphate. Adv Enzymol Relat Areas Mol Biol 45:85–155
66. Michels PA, Chevalier N, Opperdoes FR, Rider MH, Rigden DJ (1997) The glycosomal ATP-dependent phosphofructokinase of *Trypanosoma brucei* must have evolved from an ancestral pyrophosphate-dependent enzyme. Eur J Biochem 250:698–704
67. Siebers B, Klenk HP, Hensel R (1998) PP(i)-dependent phosphofructokinase from *Thermoproteus tenax*, an archaeal descendant of an ancient line in phosphofructokinase evolution. J Bacteriol 180:2137–2143
68. Wu LF, Reizer A, Reizer J, Cai B, Tomich JM, Saier MH (1991) Nucleotide sequence of the *Rhodobacter capsulatus* fruK gene, which encodes fructose-1-phosphate kinase: evidence for a kinase superfamily including both phosphofructokinases of *Escherichia coli*. J Bacteriol 173:3117–3127
69. Ding YHR, Ronimus RS, Morgan HW (2000) Sequencing, cloning, and high-level expression of the pfp gene, encoding a PP(i)-dependent phosphofructokinase from the extremely thermophilic eubacterium *Dictyoglomus thermophilum*. J Bacteriol 182:4661–4666
70. Kotlarz D, Buc H (1982) Phosphofructokinases from *Escherichia coli*. Methods Enzymol 90 Pt E:60–70
71. Ronimus RS, Morgan HW (2001) The biochemical properties and phylogenies of phosphofructokinases from extremophiles. Extremophiles 5:357–373
72. Sigrell JA, Cameron AD, Jones TA, Mowbray SL (1998) Structure of *Escherichia coli* ribokinase in complex with ribose and dinucleotide determined to 1.8 A resolution: insights into a new family of kinase structures. Structure 6:183–193
73. Verhees CH, Tuininga JE, Kengen SWM, Stams AJM, Van Der Oost J, De Vos WM (2001) ADP-dependent phosphofructokinases in mesophilic and thermophilic methanogenic archaea. J Bacteriol 183:7145–7153
74. Tuininga JE, Verhees CH, van der Oost J, Kengen SW, Stams AJ, de Vos WM (1999) Molecular and biochemical characterization of the ADP-dependent phosphofructokinase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Biol Chem 274:21023–21028
75. Fothergill-Gilmore LA, Michels PA (1993) Evolution of glycolysis. Prog Biophys Mol Biol 59:105–235
76. Brinkmann H, Cerff R, Salomon M, Soll J (1989) Cloning and sequence analysis of cDNAs encoding the cytosolic precursors of subunits GapA and GapB of chloroplast glyceraldehyde-3-phosphate dehydrogenase from pea and spinach. Plant Mol Biol 13:81–94
77. Valverde F, Losada M, Serrano A (1997) Functional complementation of an *Escherichia coli* gap mutant supports an amphibolic role for NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase of *Synechocystis* sp. Strain PCC 6803. J Bacteriol 179:4513–4522
78. Krietsch WK, Bücher T (1970) 3-Phosphoglycerate kinase from rabbit skeletal muscle and yeast. Eur J Biochem 17:568–580
79. Kuntz GWK, Krietsch WKG (1982) Phosphoglycerate kinase from spinach, blue-green algae, and yeast. Methods Enzymol 90:110–114
80. Encalada R, Rojo-Domínguez A, Rodríguez-Zavala JS, Pardo JP, Quezada H, Moreno-Sánchez R, Saavedra E (2009) Molecular basis of the unusual catalytic preference for GDP/GTP in *Entamoeba histolytica* 3-phosphoglycerate kinase. FEBS J 276:2037–2047
81. Reher M, Gebhard S, Schönheit P (2007) Glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) and nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), key enzymes of the respective modified Embden-Meyerhof pathways in the hyperthermophilic crenarchaeota *Pyrobaculum aerophi*. FEMS Microbiol Lett 273:196–205
82. Van Der Oost J, Schut G, Kengen SWM, Hagen WR, Thomm M, De Vos WM (1998) The ferredoxin-dependent conversion of glyceraldehyde-3-phosphate in the hyperthermophilic archaeon *Pyrococcus furiosus* represents a novel site of glycolytic regulation. J Biol Chem 273:28149–28154
83. Boyd DA, Cvitkovitch DG, Hamilton IR (1995) Sequence, expression, and function of the gene for the phosphate dehydrogenase of *Streptococcus mutans*. J Bacteriol 177:2622–2627

84. Brown AT, Wittenberger CL (1971) Mechanism for regulating the distribution of glucose carbon between the Embden–Meyerhof and hexose-monophosphate pathways in *Streptococcus faecalis*. J Bacteriol 106:456–467
85. Iddar A, Valverde F, Assobhei O, Serrano A, The S (2005) Widespread occurrence of non-phosphorylating dehydrogenase among Gram-positive bacteria. Int Microbiol 8:251–258
86. Iddar A, Valverde F, Serrano A, Soukri A (2002) Expression, purification, and characterization of recombinant nonphosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum*. Protein Expr Purif 25:519–526
87. Schramm A, Siebers B, Tjaden B, Brinkmann H, Hensel R (2000) Pyruvate kinase of the hyperthermophilic crenarchaeote *Thermoproteus tenax*: physiological role and phylogenetic aspects. J Bacteriol 182:2001–2009
88. Imanaka H, Yamatsu A, Fukui T, Atomi H, Imanaka T (2006) Phosphoenolpyruvate synthase plays an essential role for glycolysis in the modified Embden–Meyerhof pathway in *Thermococcus kodakarensis*. Mol Microbiol 61:898–909
89. Tjaden B, Plagens A, Dörr C, Siebers B, Hensel R (2006) Phosphoenolpyruvate synthetase and pyruvate, phosphate dikinase of *Thermoproteus tenax*: key pieces in the puzzle of archaeal carbohydrate metabolism. Mol Microbiol 60:287–298
90. Bielen AAM, Willquist K, Engman J, van der Oost J, van Niel EWJ, Kengen SWM (2010) Pyrophosphate as a central energy carrier in the hydrogen-producing extremely thermophilic *Caldicellulosiruptor saccharolyticus*. FEMS Microbiol Lett 307:48–54
91. Feng XM, Cao LJ, Adam RD, Zhang XC, Lu SQ (2008) The catalyzing role of PPDK in *Giardia lamblia*. Biochem Biophys Res Commun 367:394–398
92. Yutin N, Galperin MY (2013) A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. Environ Microbiol 15:2631–2641
93. Rydzak T, Levin DB, Cicek N, Sparling R (2009) Growth phase-dependent enzyme profile of pyruvate catabolism and end-product formation in *Clostridium thermocellum* ATCC 27405. J Biotechnol 140:169–175
94. Rydzak T, Levin DB, Cicek N, Sparling R (2011) End-product induced metabolic shifts in *Clostridium thermocellum* ATCC 27405. Appl Microbiol Biotechnol 92:199–209
95. Carere CR, Kalia V, Sparling R, Cicek N, Levin DB (2008) Pyruvate catabolism and hydrogen synthesis pathway genes of *Clostridium thermocellum* ATCC 27405. Indian J Microbiol 48:252–266
96. Islam R, Cicek N, Sparling R, Levin D (2006) Effect of substrate loading on hydrogen production during anaerobic fermentation by *Clostridium thermocellum* 27405. Appl Microbiol Biotechnol 72:576–583
97. Lynd L, Grethlein H, Wolkin R (1989) Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. Appl Environ Microbiol 55:3131–3139
98. Ellis LD, Holwerda EK, Hogsett D, Rogers S, Shao X, Tschaplinski T, Thorne P, Lynd LR (2012) Closing the carbon balance for fermentation by *Clostridium thermocellum* (ATCC 27405). Bioresour Technol 103:293–299
99. Roberts SB, Gowen CM, Brooks JP, Fong SS (2010) Genome-scale metabolic analysis of *Clostridium thermocellum* for bioethanol production. BMC Syst Biol 4:31
100. Raman B, McKeown CK, Rodriguez M, Brown SD, Mielenz JR (2011) Transcriptomic analysis of *Clostridium thermocellum* ATCC 27405 cellulose fermentation. BMC Microbiol 11:134
101. Rydzak T, McQueen PD, Krokhin OV, Spicer V, Ezzati P, Dwivedi RC, Shamshurin D, Levin DB, Wilkins JA, Sparling R (2012) Proteomic analysis of *Clostridium thermocellum* core metabolism: relative protein expression profiles and growth phase-dependent changes in protein expression. BMC Microbiol 12:214
102. Burton E, Martin VJJ (2012) Proteomic analysis of *Clostridium thermocellum* ATCC 27405 reveals the upregulation of an alternative transhydrogenase-malate pathway and nitrogen assimilation in cells grown on cellulose. Can J Microbiol 58:1378–1388

103. Patni NJ, Alexander JK (1971) Utilization of glucose by *Clostridium thermocellum*: presence of glucokinase and other glycolytic enzymes in cell extracts. J Bacteriol 105:220–225
104. Zhou J, Olson DG, Argyros DA, Deng Y, van Gulik WM, van Dijken JP, Lynd LR (2013) Atypical glycolysis in *Clostridium thermocellum*. Appl Environ Microbiol 79:3000–3008
105. Golovchenko N, Chuvilskaya N, Akimenko V (1986) Regulation of biosynthesis of cellulolytic enzymes and enzymes catalyzing initial catabolism of glucose and cellobiose in *Clostridium thermocellum*. Microbiology 55:23–25
106. Erbeznik M, Jones CR, Dawson KA, Strobel HJ (1997) *Clostridium thermocellum* JW20 (ATCC 31549) is a coculture with *Thermoanaerobacter ethanolicus*. Appl Environ Microbiol 63:2949–2951
107. Nochur SV, Jacobson GR, Roberts MF, Demain AL (1992) Mode of sugar phosphorylation in *Clostridium thermocellum*. Appl Biochem Biotechnol 33:33–41
108. Glass TL, Sherwood JS (1994) Phosphorylation of glucose by a guanosine-5′-triphosphate (GTP)-dependent glucokinase in *Fibrobacter succinogenes* subsp. succinogenes S85. Arch Microbiol 162:180–186
109. Lou J, Dawson KA, Strobel HJ (1997) Cellobiose and cellodextrin metabolism by the ruminal bacterium *Ruminococcus albus*. Curr Microbiol 35:221–227
110. Müller M (1992) Energy metabolism of ancestral eukaryotes: a hypothesis based on the biochemistry of a mitochondriate parasitic protists. Biosystems 28:33–40
111. Müller M, Lee JA, Gordon P, Sensen CW (2001) Presence of prokaryotic and eukaryotic species in all subgroups of the PP i -dependent group II phosphofructokinase protein family presence of prokaryotic and eukaryotic species in all subgroups of the PP i -dependent group II phosphofructokinase protein. J Bacteriol 183:6714–6716
112. Hutchins A, Holden J, Adams M (2001) Phosphoenolpyruvate synthetase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Bacteriol 183:709–715
113. Chao YP, Patnaik R, Roof WD, Young RF, Liao JC (1993) Control of gluconeogenic growth by pps and pck in *Escherichia coli*. J Bacteriol 175:6939–6944
114. Acosta H, Dubourdieu M, Quiñones W, Cáceres A, Bringaud F, Concepción JL (2004) Pyruvate phosphate dikinase and pyrophosphate metabolism in the glycosome of *Trypanosoma cruzi* epimastigotes. Comp Biochem Physiol B Biochem Mol Biol 138:347–356
115. Lamed R, Zeikus JG (1981) Thermostable, ammonium-activated malic enzyme of *Clostridium thermocellum*. Biochim Biophys Acta 660:251–255
116. Taillefer M, Rydzak T, Levin DB, Oresnik IJ, Sparling R (2015) Reassessment of the transhydrogenase/malate shunt pathway in *Clostridium thermocellum* ATCC 27405 through kinetic characterization of malic enzyme and malate dehydrogenase. Appl Environ Microbiol 81:2423–2432
117. Heinonen JK (2001) Biological role of inorganic pyrophosphate. Kluwer Academic Publishers, Boston
118. Willquist K, van Niel EWJ (2010) Lactate formation in *Caldicellulosiruptor saccharolyticus* is regulated by the energy carriers pyrophosphate and ATP. Metab Eng 12:282–290
119. Baltscheffsky M, Schultz A, Baltscheffsky H (1999) H+ -PPases: a tightly membrane-bound family. FEBS Lett 457:527–533
120. Hungate RE (1963) Polysaccharide storage and growth efficiency in *Ruminococcus albus*. J Bacteriol 86:848–854
121. Guedon E, Desvaux M, Petitdemange H (2000) Kinetic analysis of *Clostridium cellulolyticum* carbohydrate metabolism: importance of glucose 1-phosphate and glucose 6-phosphate branch points for distribution of carbon fluxes inside and outside cells as revealed by steady- state continuous culture. J Bacteriol 182:2010–2017
122. Mertens E (1993) ATP versus pyrophosphate: glycolysis revisited in parasitic protists. Parasitol Today 9:122–126

123. Mertens E, De Jonckheere J, Van Schaftingen E (1993) Pyrophosphate-dependent phosphofructokinase from the amoeba *Naegleria fowleri*, an AMP-sensitive enzyme. Biochem J 292 3:797–803
124. Susskind BM, Warren LG, Reeves RE (1982) A pathway for the interconversion of hexose and pentose in the parasitic amoeba *Entamoeba histolytica*. Biochem J 204:191–196
125. Nakahigashi K, Toya Y, Ishii N, Soga T, Hasegawa M, Watanabe H, Takai Y, Honma M, Mori H, Tomita M (2009) Systematic phenome analysis of *Escherichia coli* multiple-knockout mutants reveals hidden reactions in central carbon metabolism. Mol Syst Biol 5:306
126. Rydzak T, Grigoryan M, Cunningham ZJ, Krokhin OV, Ezzati P, Cicek N, Levin DB, Wilkins JA, Sparling R (2014) Insights into electron flux through manipulation of fermentation conditions and assessment of protein expression profiles in *Clostridium thermocellum*. Appl Microbiol Biotechnol 98:6497–6510
127. Wang S, Huang H, Moll J, Thauer RK (2010) $NADP^+$ reduction with reduced ferredoxin and $NADP^+$ reduction with NADH are coupled via an electron-bifurcating enzyme complex in *Clostridium kluyveri*. J Bacteriol 192:5115–5123
128. Carere CR, Rydzak T, Verbeke TJ, Cicek N, Levin DB, Sparling R (2012) Linking genome content to biofuel production yields: a meta-analysis of major catabolic pathways among select H_2 and ethanol-producing bacteria. BMC Microbiol 12:295
129. Bapteste E, Moreira D, Philippe H (2003) Rampant horizontal gene transfer and phosphodonor change in the evolution of the phosphofructokinase. Gene 318:185–191

Adv Biochem Eng Biotechnol (2016) 156: 79–112
DOI: 10.1007/10_2015_5007

Published online: 24 February 2016

Comparative Genomics of Core Metabolism Genes of Cellulolytic and Non-cellulolytic *Clostridium* Species

Sadhana Lal and David B. Levin

Abstract Microbial production of fuels such as ethanol, butanol, hydrogen (H_2), and methane (CH_4) from waste biomass has the potential to provide sustainable energy systems that can displace fossil fuel consumption. Screening for microbial diversity and genome sequencing of a wide-range of microorganisms can identify organisms with natural abilities to synthesize these alternative fuels and/or other biotechnological applications. *Clostridium* species are the most widely studied strict anaerobes capable of fermentative synthesis of ethanol, butanol, or hydrogen directly from waste biomass. *Clostridium termitidis* CT1112 is a mesophilic, cellulolytic species capable of direct cellulose fermentation to ethanol and organic acids, with concomitant synthesis of H_2 and CO_2. On the basis of 16S ribosomal RNA (*rRNA*) and chaperonin 60 (*cpn*60) gene sequence data, phylogenetic analyses revealed a close relationship between *C. termitidis* and *C. cellobioparum*. Comparative bioinformatic analyses of the *C. termitidis* genome with 18 cellulolytic and 10 non-cellulolytic *Clostridium* species confirmed this relationship, and further revealed that the majority of core metabolic pathway genes in *C. termitidis* and *C. cellobioparum* share more than 90% amino acid sequence identity. The gene loci and corresponding amino acid sequences of the encoded enzymes for each pathway were correlated by percentage identity, higher score (better alignment), and lowest e-value (most significant "hit"). In addition, the function of each enzyme was proposed by conserved domain analysis. In this chapter we discuss the comparative analysis of metabolic pathways involved in synthesis of various useful products by cellulolytic and non-cellulolytic biofuel and solvent producing *Clostridium* species. This study has generated valuable information concerning the core metabolism genes and pathways of *C. termitidis* CT1112, which is helpful in developing

S. Lal and D.B. Levin (✉)
Department of Biosystems Engineering, University of Manitoba, Winnipeg, MB, Canada R3T 5V6
e-mail: david.levin@umanitoba.ca

metabolic engineering strategies to enhance its natural capacity for better industrial applications.

Keywords Biomass, CAZymes, Cellulolytic bacteria, Cellulosomes, Genetic manipulation

Contents

Abbreviations

16S rRNA	16S ribosomal RNA
ACT	Artemis comparison tool
CAZymes	Carbohydrate-active enzymes
CDS	Coding sequence
COG/COGs	Clusters of orthologous groups
Cpn60	Chaperonin 60
cpnDB	Chaperonins database
GenePRIMP	Gene PRediction IMprovement Pipeline
IMG-ER	Integrated microbial genomes-expert review
JGI	Joint Genome Institute
KEGG	Kyoto encyclopedia of genes and genomes
NCBI	National Center for Biotechnology Information
PEP	Phosphoenolpyruvate
PPDK	Pyruvate phosphate dikinase
PPK	Pyruvate directly by pyruvate kinase
RDP	Ribosomal database project
UT	Universal target

1 Introduction

Low-cost, high-throughput sequencing is now being used extensively for investigating bacterial genomes. In silico screening of microbial diversity has enabled analyses of genetic variability and can help identify organisms with natural ability to synthesize fuels and bio products of interest. High throughput sequencing has also revealed how bacteria acquire and transmit genes via horizontal gene transfer over short periods of time. Such organisms can be exploited through genome shuffling for transgenic expression and efficient generation of clean fuel and other diverse biotechnological applications.

Clostridium is the second largest genus within the Phylum *Firmicutes*. *Clostridium* species are present in diverse environments and are known to utilize a wide-variety of substrates and to synthesize a great diversity of metabolites, such as ethanol, butanol, hydrogen, carbon dioxide, acetate, formate, lactate, propionate, butyrate, isobutyrate, and isovalerate [1, 2] (Table 1). The genus *Clostridium* consists of Gram-positive obligate anaerobes and contains common free-living bacteria and important pathogens. The great metabolic capabilities of *Clostridium* species have attracted researchers to investigate their diverse metabolisms for biofuel production and cellulose degradation. As of May 2015, the Integrated Microbial Genomes (IMG) system listed a total of 534 genome sequences for species in the genus *Clostridium* [40]. Of these, 56 genomes were listed as "finished genomes," 33 were listed as "draft genomes," and 410 were listed as "permanent drafts." IMG also lists 13 "finished genomes" of *Clostridium* plasmids and 22 "finished genomes" of bacteriophages which infect *Clostridium* species. Of the bacterial genome sequences listed, 247 sequences were from *C. difficile*, 39 were from *Clostridium* species, 34 were from *C. botulinum*, 25 were from *C. perfringens*, and 10 were from *C. clostridioforme*.

In this chapter we have discussed the draft genome sequence of *C. termitidis* and analyzed the genomes of 18 cellulolytic and 10 non-cellulolytic *Clostridium* species which synthesize fermentation end-products of interest as potential fuels [41] (Table 1). *C. termitidis* strain CT1112 (DSM 5398) was isolated from the gut of the wood-feeding termite *Nasutitermes lujae* from the Mayombe tropical rainforest, Congo, Central Africa [21]. *C. termitidis* was examined for H_2 and other end-products such as acetate, CO_2, formate, and ethanol formation on cellobiose, α-cellulose, xylan, xylose, and glucose by Ramachandran et al. [22] in our laboratory. Experimental data showed the produced amount of H_2, ethanol, acetate, formate, and CO_2 was comparable to other cellulolytic *Clostridium* species. Thus, *C. termitidis* could be used as a potential candidate for biofuel (H_2 and ethanol) production from biomass through consolidated bioprocessing [22, 42]. Comparative analysis of experimental data revealed that *C. termitidis* is distinct from *C. cellulolyticum*, which takes more time to grow on cellulose and produces less H_2 than ethanol during cellulose fermentation [22].

Identifying the core metabolic pathways using an experimental approach in any organisms is a major challenge and a time-consuming process. In this study we used

Table 1 Genome metadata for sequenced cellulolytic and non-cellulolytic *Clostridium* species

NCBI Ref Seq	Genome name	Fermentation product	Isolation	Gene size	GC %	Gene count	CDS count	16s rRNA count	COG count	References
Cellulolytic *Clostridium* species										
NC_014393.1[a]	*C. cellulovorans* 743B, ATCC 35296	H_2, CO_2, acetate, butyrate, formate, lactate, ethanol	Woody biomass digester	5.26	30	4,500	4,389	9	2,514	[3–5]
NC_011898.1[a]	*C. cellulolyticum* H10	H_2, CO_2, ethanol, acetate, lactate, formate	Decayed grass in compost pile (packaged for 3–4 months)	4.07	37	3,575	3,488	8	2,036	[6, 7]
NC_014376.1[a]	*C. saccharolyticum* WM1, DSM 2544	H_2, CO_2, ethanol	methanogenic cellulose-enrichment culture of sewage sludge	4.66	45	4,388	4,299	6	2,785	[8]
NC_010001.1[a]	*C. phytofermentans* ISDg	H_2, CO_2, ethanol, acetate, formate, lactate	Forest soil near the Quabbin Reservoir in Massachusetts	4.8	35	3,991	3,902	8	2,497	[9]
NC_009012.1[a]	*C. thermocellum* ATCC 27405[c]	Ethanol, organic acids	Yellowstone National Park	3.84	39	3,335	3,236	4	1,892	[10, 11]
NC_017304.1[a]	*C. thermocellum* LQ8, DSM 1313[c]	Ethanol, organic acids	Evolved mixed population	3.56	39	3,102	3,031	4	1,814	[12]
NC_016627.1[a]	*C. clariflavum* EBR 45, DSM 19732[c]	H_2, CO_2, lactate, acetate, ethanol, formate	Methanogenic sludge of a cellulose-degrading bioreactor	4.89	36	4,229	4,131	6	2,294	[13, 14]

NC_020134.1[a]	*Clostridium stercorarium* DSM 8532[c]	H_2, CO_2, ethanol, acetate, lactate, L-alanine	Compost	2.97	42	2,763	2,706	3	1,750	[15–17]
CP003259.1[a]	*Clostridium* species BNL1100	biofuel production	Corn stover enrichment culture	4.61	37	4,117	4,025	8	2,372	[18]
NZ_ACXX00000000.2[b]	*C. papyrosolvens* DSM 2782	H_2, CO_2, ethanol, acetate, lactate	Estuarine sediment, River Don, Aberdeenshire, Scotland	4.87	37	4,479	4,423	1	2,393	[19, 20]
NZ_AORV00000000.1[b]	*C. termitidis* CT1112, DSM 5398	H_2, CO_2, acetate, formate, lactate, ethanol	Gut of termite, *Nasutitermes lujae*, Congo, Africa	6.42	41	5,389	5,327	2	3,392	[21–23]
AUVG00000000.1[b]	*C. cellulosi* CS-4-4[c]	H_2, CO_2, ethanol, acetic acid		5.68	42	5,216	5,106	4	3,374	[24]
NZ_JHYD00000000.1[b]	*C. cellobioparum* ATCC 15832	H_2, CO_2, ethanol, acetic acid	Rumen of cattle, 1998, L. Ganqiu	6.13	41	5,220	5,134	4	3,224	[25]
2509887034[b] (from JGI/IMG)	*C. alkalicellulosi* Z-7026, DSM 17461	Cellulose degrader		5.31	32	4,473	4,390	2	2,214	[26]
2541046955[b] (from JGI/IMG)	*C. josui* JCM 17888[c]	H_2, CO_2, ethanol, acetate, butyrate	Thai compost	4.47	36	3,991	3,895	7	2,274	[27]
Genome and cpn60 not available	*C. chartatabidum*	H_2, ethanol, acetate, butyrate	Chloroform treated rumen contents	–	–	–	–	–	–	[28]
Genome not available	*C. aldrichii*	H_2, CO_2, acetate, propionate, butyrate, isobutyrate, isovalerate, lactate, succinate	Wood fermenting anaerobic digester	–	–	–	–	–	–	[29]

(continued)

Table 1 (continued)

NCBI Ref Seq	Genome name	Fermentation product	Isolation	Gene size	GC %	Gene count	CDS count	16s rRNA count	COG count	References
Genome and cpn60 not available	*C. celerecrescens* DSM 5628	H_2, CO_2, ethanol, acetate, formate, butyrate, isobutyrate, isovalerate, caproate, lactate, succinate	Methanogenic cellulose-enriched culture	–	–	–	–	–	–	[30]
Non-cellulolytic *Clostridium* species										
NC_003030.1[a]	*C. acetobutylicum* ATCC 824	Gelatin hydrolysis positive	Plant-derived foodstuff (corn meal)	4.13	31	4,022	3,848	11	2,589	[31]
NC_020291.1[a]	*C. saccharoperbutylacetonicum* N1-4(HMT)	Acetone, butanol, ethanol producer	Derived from existing strain (derived from ATCC 13564)	6.67	30	5,981	5,843	11	3,652	[32]
NC_009706.1[a]	*Clostridium kluyveri* DSM 555	H_2, butyrate, caproate	Mud of a canal in Delft, The Netherlands	4.02	32	4,073	3,913	7	2,261	[33]
NC_022571.1[a]	*C. saccharobutylicum* DSM 13864	Solvent producers such as acetone, butanol, ethanol	Soya bean field	5.11	29	4,552	4,430	12	2,667	[31]
NC_021182.1[a]	*C. pasteurianum* BC1	Nitrogen fixation	Coal-cleaning residues	5.04	31	4,966	4,851	9	2,914	[34]

NC_014328.1[a]	*C. ljungdahlii* PETC, DSM 13528	Acetogen, ethanol production	Chicken yard waste	4.63	31	4,283	4,184	9	2,662	[35]
NC_022592.1[a]	*C. autoethanogenum* DSM 10061	Ethanol and acetate	Enriched from rabbit feces and isolated using carbon monoxide as the sole source of energy and carbon	4.35	31	4,131	4,042	9	2,577	[36]
NC_021047.1[a]	*C.* cf. *saccharolyticum* K10	Metablolize a wide range of sugars	Mixed cellulolytic culture and relies on the cellulolytic microorganism to provide sugars for growth	3.77	50	3,124	3,073	1	1,675	–
NZ_ADEK00000000.1[b]	*C. carboxidivorans* P7, DSM 15243	Acetogenic, solvent producer	Agricultural settling lagoon at Oklahoma State University, Aquatic, USA	4.41	30	4,234	4,174	2	2,503	[37, 38]
NZ_APJA00000000.1[b]	*C. intestinale* URNW	Hydrogen, ethanol production	Contaminated cellobiose stock solution	4.67	30	4,660	4,549	10	2,941	[39]

[a]Sequencing status is "finished" (F)
[b]Sequencing status is "permanent draft" (PD)
[c]Thermophilic *Clostridium* species ; remaining *Clostridium* species are mesophilic

an in silico approach to explore the core metabolic pathways in a draft genome sequence of *C. termitidis* using *C. cellulolyticum* and *C. thermocellum* as reference organisms, as they are well studied for their metabolic pathways in comparison to other cellulolytic *Clostridium* species. We have selected six cellulolytic *Clostridium* species known for their ability to produce H_2, CO_2, acetate, ethanol, formate, lactate, or butyrate for comparative genomic analysis, which revealed the presence or absence of enzymes involved in glycolysis, pyruvate formation and catabolism, acetate, ethanol, and hydrogen synthesis. The information generated by the computational approach concerning the core metabolism genes and pathways of *C. termitidis* CT1112 could be helpful in developing metabolic engineering strategies to enhance the natural capacity of *C. termitidis* for better industrial applications. This study demonstrates that comparative genomics analysis is a very useful tool to generate large amount of highly informative data in less time, allowing quick functional prediction for many hypothetical or putative proteins in poorly studied organisms [43].

2 Genome Annotation and Phylogenetic Analysis of *Clostridium* Species

2.1 *Annotation and Manual Curation of Draft Genome of* C. termitidis *Strain CT1112*

The genome of *C. termitidis* CT1112 was sequenced by the Genome Québec/McGill University platform using a Roche/454s GS-FLX Titanium sequencer by a whole-genome shotgun strategy. A 454 standard flow-gram format (.sff) read file was assembled using Newbler v2.3. The assembled draft genome of *C. termitidis* strain CT1112 was deposited to the Joint Genome Institute's (JGI) Integrated Microbial Genomes-Expert Review (IMG-ER) platform for annotation using their annotation pipeline (http://img.jgi.doe.gov) [40] which generates protein coding genes (CDS) and assigns names to gene products. The annotated genome was subsequently submitted to the JGI's Gene Prediction Improvement pipeline (GenePRIMP) [44] which allows automated correction of genes, including insertion of missed genes, extension of "short" genes, and identification of putative pseudogenes [45]. GenePRIMP generated 360 anomalies of different types, including short genes, long genes, unique genes, dubious genes, split genes interrupted by frameshifts and stopcodons ("broken genes"), split genes interrupted by transposases "(interrupted genes"), missed genes, missed CrispR elements, and overlaps. Anomalies generated by the GenePRIMP pipeline were manually curated using Artemis Comparison Tool (ACT) [46]. Manual correction of 1,847 genes was accomplished using several lines of evidence, including BLAST searches of

nucleotides (BLAST-N), protein (BLAST-P), conserved domain analyses using the Conserved Domain Database (CDD), protein domains and motifs, Interpro (Pfam, prosite, SMART etc.), and characterized proteins in some cases (e.g., UNIPROT entries).

The manually curated draft genome has been deposited at DDBJ/EMBL/GenBank under the accession no. NZ_AORV00000000.1 [23]. The final draft genome of *C. termitidis* strain CT1112 has 78 contigs with a total size of 6,415,858 bp and G + C content of 41.18%. It was predicted to encode 5,389 genes, with 5,327 CDSs (98.85% of predicted genes). The coding region covers 87.94% of the genome sequence. Out of 5,327 CDSs, 4,403 were assigned with functions, although no functional prediction could be assigned to 924 CDSs. Among the 4,403 CDSs, 3,392 (62.94%) genes could be classified into COG families. A total of seven rRNA genes, including three 5S rRNAs, two 16S rRNAs (243 bp and 1,342 bp, of which 1,342 bp was used in phylogenetic tree), and two 23S rRNAs, were present on the *C. termitidis* chromosome. In addition, 55 tRNA genes that represent all 20 amino acids were identified.

2.2 *Genome Features of* C. termitidis *Strain CT1112 and Other* Clostridium *Species*

Comparative analyses of a range of genome features were conducted with 18 cellulolytic and 10 non-cellulolytic sequenced genomes of *Clostridium* species (Table 1). The genomic information included genome size, % G + C, total numbers of genes, numbers of coding sequences (CDS), numbers of Clusters of Orthologous Groups (COG), and 16S rRNAs derived from the IMG-ER platform (http://img.jgi.doe.gov; Table 1).

Comparative analyses of general features of *Clostridium* species revealed that the non-cellulolytic bacterium, *C. saccharoperbutylacetonicum* N1-4(HMT) had the largest genome size (6.67 Mb) with highest number of protein-encoding genes (5,843) and COG count (3,652), whereas the thermophilic, cellulolytic bacterium, *C. stercorarium*, had the smallest genome size (2.97 MB) with the lowest number of protein coding genes (2,706). *C. termitidis* had the largest genome size (6.42 Mb) of the cellulolytic *Clostridium* species [23, 42]. The finished genome of *C.* cf. *saccharolyticum* K10 had the highest G + C content (50%), with the lowest number of protein coding genes (3,073) and COG count (1,675). Interestingly, in this analysis we observed that the 16S rRNA count was higher in non-cellulolytic *Clostridium* species than in cellulolytic *Clostridium* species (Table 1). In addition, the average percentage G + C in non-cellulolytic *Clostridium* species (30%) was lower than the percentage G + C in cellulolytic *Clostridium* species (36%), except for *C. cellulovorans* 743B, which has 30% G + C [5].

2.3 *Phylogenetic Analyses of Cellulolytic and Non-cellulolytic* Clostridium *Species*

16S *rRNA* sequences have been used for phylogenetic studies and for sequence-based taxonomy for many years [47–50]. The dynamic nature of the genomes and the impact of lateral gene transfer on genomic evolution [51] have forced researchers to use more than one conserved target to understand the taxonomy and phylogenetics of bacterial diversity. The chaperonin-60 universal target (*cpn60* UT, also known as *GroEL* or *HSP60*) nucleotide sequence (549–567 bp), is highly conserved in bacteria. It can differentiate even more closely related isolates of the same bacterial species and thus find more reliable targets for phylogenetic studies, microbial identification, microbial ecology, and evolution [50, 52, 53] than 16S rRNA [54–56]. *Cpn60* UT sequence alignments have been shown to correlate to whole genome sequence alignments and resolve ambiguities associated with 16S rDNA gene phylogeny in bacteria [54].

To determine the evolutionary relationship between *C. termitidis* and sequenced 18 cellulolytic and 10 non-cellulolytic *Clostridium* species (Table 1), a phylogenetic tree was constructed based on 16S *rRNA* and *cpn60* universal target (UT) gene sequences. The 16S *rRNA* sequences chosen for this study were retrieved from the ribosomal database (RDP) (http://rdp.cme.msu.edu/) [57] and NCBI (http://www.ncbi.nlm.nih.gov/) [58]. The *cpn60* "universal target" (UT) sequences were retrieved from a Chaperonins database (cpnDB) (http://www.cpndb.ca/cpnDB/home.php) [50] and IMG database (http://img.jgi.doe.gov) [59]. The 16S rRNA and *cpn60* sequences of *Acidothermus cellulolyticus* was used as an out group for phylogenetic analysis.

16S rRNA and *cpn60* sequences of 28 cellulolytic and non-cellulolytic *Clostridium* species were aligned using the BioEdit v.7.0.9.0 program [60]. Phylogenetic trees were generated using the PHYLIP 3.69 package [61]. Evolutionary distances between all species were calculated with the DNADIST and the resultant distance matrix was then used to draw Neighbor Joining trees with the program NEIGHBOR [62]. The program SEQBOOT was used for statistical testing of the trees by resampling the dataset 1,000 times [62]. The trees were viewed through TreeView Version 1.6.6 [63]. Phylogenetic analyses with 16S *rRNAs* revealed that *C. termitidis* strain CT1112 is closely related to *C. cellobioparum* DSM 1351 (99%) and with other cellulolytic *Clostridium* species (Fig. 1). Moreover, the 16S *rRNA* phylogenetic tree showed that cellulolytic and non-cellulolytic species are very well separated, except for *C. cellulovorans* and *C. chartatabidum*. Interestingly, cellulolytic *C. cellulovorans* and *C. chartatabidum* clustered with non-cellulolytic species, such as *C. intestinale* URNW, *C. saccharoperbutylacetonicum* N1-4(HMT), and *C. saccharobutylicum* DSM 13864, in Clade 2. Thus, the 16S *rRNA* phylogeny suggests that *C. cellulovorans* and *C. chartatabidum* are different from other cellulolytic *Clostridium* species [2]. This observation is consistent with a previous study in

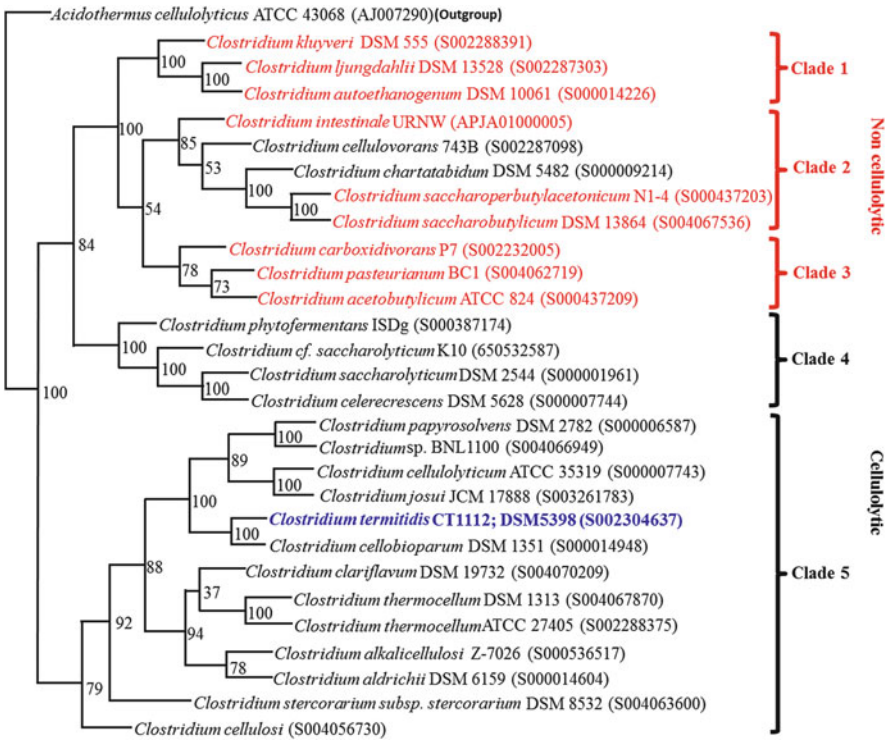


Fig. 1 Neighbor-joining tree based on 16S *rRNA* gene sequences, showing the phylogenetic position of *C. termitidis* with respect to species of the genus *Clostridium*. The non-cellulolytic *Clostridium* species are highlighted in *red* and the cellulolytic *Clostridium* species are indicated in *black*. The evolutionary history of the taxa analyzed was represented using 1,000 replicates obtained from the bootstrap consensus tree. Accession numbers are given in *parentheses*

which the genome sequence of *C. cellulovorans* 743B was compared with other *Clostridium* species [5] and revealed the G + C content of *C. cellulovorans* (30.0%) was very similar to the G + C content of the non-cellulolytic species, *C. acetobutylicum* ATCC 824 (30.9%).

Phylogenetic trees based on *cpn60* of 26 *Clostridium* species, clearly separated the cellulolytic and non-cellulolytic *Clostridium* species into two groups, except for the cellulolytic bacterium *C. cellulolvorans* 743B, which clustered with the non-cellulolytic species *C. acetobutylicum* ATCC 824 in Clade 3. Non-cellulolytic *Clostridium* species in the *cpn60* tree clustered into four Clades (1, 2, 3, and 4), whereas cellulolytic *Clostridium* species grouped into five Clades (5, 6, 7, 8, and 9; Fig. 2). The placement of *C. cellulovorans* with non-cellulolytic *Clostridium* species detected in the 16S *rRNA* tree is clearly supported by the *cpn60* tree (Fig. 2).

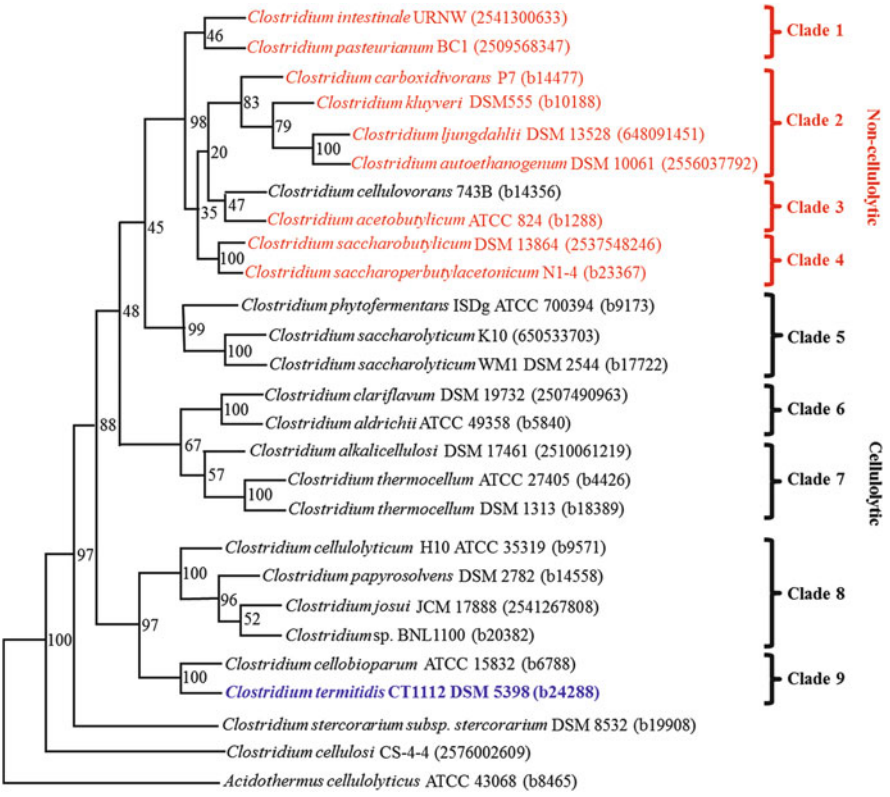


Fig. 2 Neighbor-joining tree based on UT region of the *cpn60* gene sequences, showing the phylogenetic position of *C. termitidis* with respect to species of the genus *Clostridium*. The non-cellulolytic *Clostridium* species are highlighted in *red* and the cellulolytic *Clostridium* species are indicated in *black*. The evolutionary history of the taxa analyzed was represented using 1,000 replicates obtained from the bootstrap consensus tree. Accession numbers are given in *parentheses*

3 Comparative Analysis of Core Metabolisms in *Clostridium* Species

The major fermentation and cellulose degradation end-products commonly observed in cellulolytic *Clostridium* species are H_2, CO_2, acetate, formate, and ethanol, whereas most of the non-cellulolytic *Clostridium* species are solvent producers that synthesize H_2, CO_2, butyrate, butanol, acetone, and ethanol (Table 1).

We have selected two cellulolytic *Clostridium* species – mesophilic *C. cellulolyticum* H10 and thermophilic *C. thermocellum* ATCC 27405 – as references to search for homologous genes of interest from glycolysis, pyruvate formation and catabolism, acetate, ethanol, and hydrogen synthesis in six *Clostridium* species. Some enzymes in certain pathways were found to be missing in *C. cellulolyticum* and, for these specific cases, we used *C. thermocellum* ATCC 27405 as the reference organism.

The corresponding gene loci and enzymes for each pathway in *C. termitidis*, *C. cellobioparum*, *C. phytofermentans*, *C. cellulovorans*, and *C. papyrosolvens* were identified using different tools from the IMG-ER platform. These tools included alignments against BLAST-P and RPS-BLAST using the conserved domain database [64] from NCBI and Pfams [65] and IMG Terms [40]. Amino acid sequences for each gene product were retrieved from the JGI genome portal (http://genome.jgi-psf.org/ [45]) and the NCBI database (http://www.ncbi.nlm.nih.gov/genomes). Confirmation of CDS features of *C. termitidis* was accomplished by comparing with six cellulolytic *Clostridium* species – *C. cellulolyticum* H10, *C. thermocellum* ATCC 27405, *C. phytofermentans* ISDg, *C. cellulovorans* 743B, *C. cellobioparum* ATCC 15832, and *C. papyrosolvens* DSM 2782.

The functions of predicted genes were manually assessed using different databases such as Clusters of Orthologous Groups (COG) [66], KEGG Orthology (KO) assignments [67], and TIGRFAMs [68]. Clusters of Orthologous Groups (COGs) analyses were conducted using "Abundance Profiles" tools from IMG, which provide a comprehensive examination of the functional components of genomes between strains. Genome clustering was done using cluster 3.0 analysis software [69] within the IMG-ER platform using the COG profile for each selected clostridial genome. In this analysis, genome sequences of cellulolytic mesophilic (*C. termitidis*, *C. cellulotylicum* H10, *C. cellobioparum* ATCC 15832, *C. cellulovorans* 743B, *C. phytofermentans* ISDg, *C. papyrosolvens* DSM 2782) and thermophilic (*C. thermocellum* ATCC 27405) *Clostridium* species were used for comparative analyses of core metabolic pathways. The corresponding protein sequences for enzymes were retrieved from the KEGG, JGI/IMG, and NCBI databases. Manual construction of metabolic pathways from the annotated *C. termitidis* CT1112 genome was accomplished using pathways from the KEGG database as a reference [70].

Genome sequence analysis of *C. termitidis* revealed the presence of all open reading frame (ORFs) encoding proteins for all metabolic pathways, except pyruvate phosphate dikinase (PPDK) when compared with *C. cellulolyticum* genome. However, a gene encoding PPDK was found in the *C. termitidis* genome when *C. thermocellum* was used as the reference organism. The absence of PPDK in *C. termitidis* with *C. cellulolyticum* was observed because of the sequence variation in functional domain. Amino acid sequence of PPDK from *C. termitidis* showed 85% identity when compared with the *C. thermocellum* PPDK gene. Some genes encoding phosphoenolpyruvate synthase, the γ- and δ-subunits of pyruvate ferredoxin oxidoreductase and aldehyde dehydrogenase enzymes, were absent in the *C. cellulolyticum* genome. In such cases, *C. thermocellum* was used as a reference to search for these enzymes in other *Clostridium* species (Table 2).

3.1 Pyruvate Metabolism and End-Product Synthesis

For in silico comparative analysis, it is very important to select more than one reference organism. The gene loci and sequences corresponding to each enzyme

Table 2 Comparative analysis of core metabolisms in cellulolytic *Clostridium* species

Metabolic enzymes for different metabolisms	EC no.	Locus tag							Amino acid identity (%)					
		Ccel_	CpapDRAFT_	T344DRAFT_	Cter_	Cthe_	Clocel_	Cphy_	Ccel/ CpapDRAFT	Ccel/ T344DRAFT	Ccel/ Cter	Ccel/ Cthe	Ccel/ Clocel	Ccel/ Cphy
Glycosis														
Cellobiose phosphorylase	2.4.1.20	2109	3781	03023	4494	0275	0032	0430	94	88	88	76	71	66
Glucose kinase/ROK domain containing protein	2.7.1.2	0700	3365	01639	3170	2938	3765	0329	86	75	74	47	32	29
ROK domain containing protein	2.7.1.2	3430	3726	3251	4330	0390	0594	3420	91	75	75	31	30	24
Glucose-6-phosphate isomerase	5.3.1.9	1445	2002	4897	3865	0217	1364	0419	96	90	90	79	68	66
6-Phosphofructokinase	2.7.1.11	2223	3751	00156	4719	0347	1603	3345	96	95	95	83	45	48
6-Phosphofructokinase	2.7.1.11	2612	4116	05203	0067	1261	0388	0336	96	90	90	67	54	56
Fructose-1,6-bisphosphate aldolase, class II	4.1.2.13	2222	3750	00157	4718	0349	0553	3683	97	93	93	85	36	38
Triose phosphate isomerase	5.3.1.1/ 2.7.2.3	2260	2776	00088	4786	0138	0720	2875	96	91	91	83	62	59
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	2275	4374	00072	4809	0137	0719	2876	96	96	95	84	49	45
Phosphoglycerate mutase	5.4.2.1	2259	2775	00089	4785	0140	0722	2868	95	88	87	76	61	63
Phosphoglycerate mutase	5.4.2.1	0619	0778	03891	2329	0946	3764	1900	87	74	74	67	33	30
Enolase	4.2.1.11	2254	2767	00096	4779	0143	0730	3001	96	93	93	85	70	67
Pyruvate formation														
Pyruvate kinase	2.7.1.40	2569	0002	03464	0649	–	0389	0741	90	83	83	–	53	56
Pyruvate phosphate dikinase[a]	2.7.9.1	2388	–	00787	–	–	–	–	–	29	–	–	–	–
		–	–	01049	0809	1308	1454	0651	–	86	85	–	74	68
Phosphoenolpyruvate synthase/pyruvate phosphate dikinase[a]	2.7.9.2	–	–	02136	5054	1253	–	–	–	46	44	–	–	–
Phosphoenolpyruvate carboxykinase [GTP]	4.1.1.32	0212	4012	01445	1146	2874	–	–	96	91	91	83	–	–
Oxaloacetate carboxykinase (α-subunit)	4.1.1.3	1736	0378	02498	0730	0701	2828	2433	92	86	87	84	35	67

Malate dehydrogenase	1.1.1.37	0137	4023	01233	0412	0345	2700	1117	94	91	90	65	45	40
Malic enzyme (NAD)	1.1.1.40	0138	4022	01232	0411	0344	0393	0409	95	95	89	77	57	60
Pyruvate catabolism														
L-Lactate dehydrogenase	1.1.1.27	2485	0740	00623	2504	1053	1533	1117	96	86	86	72	49	39
Pyruvate ferredoxin oxidoreductase, gamma subunit[a]	1.2.7.1	–	3734	02440	1025	2390	2840	–	32	88	88	–	26	–
Pyruvate ferredoxin oxidoreductase, delta subunit[a]	1.2.7.1	–	3735	02439	1024	2391	–	3558	46	74	74	–	–	37
Pyruvate ferredoxin oxidoreductase, alpha subunit[a]	1.2.7.1	1164	3736	02438	1023	2392	1684	0603	37	79	79	30	31	30
Pyruvate ferredoxin oxidoreductase, beta subunit[a]	1.2.7.1	1164	3737	02437	1022	2393	2840	3558	43	84	84	29	29	29
Pyruvate flavodoxin/ferredoxin oxidoreductase domain protein	1.2.1.51	1164	3182	03616	3589	3120	2840	0603	94	84	84	64	63	67
Pyruvate flavodoxin/ferredoxin oxidoreductase domain protein	1.2.1.51	0016	3065	03616	3589	3120	1684	3558	94	55	55	55	57	61
Pyruvate formate lyase	2.3.1.54	2582	0044	03479	0038	0505	1811	2823	95	90	90	80	61	67
Acetate and ethanol production														
Aldehyde dehydrogenase[a]	1.2.1.3	–	–	02407	0993	2238	0629	3041	–	43	43	–	26	46
Alcohol dehydrogenase	1.1.1.1	1083	2833	03763	1650	0423	3817	1029	91	82	82	39	48	48
Alcohol dehydrogenase	1.1.1.1	3337	1270	04004	2586	0101	1140	2463	92	49	49	54	42	43
Alcohol dehydrogenase	1.1.1.1	0894	3919	03683	3280	0394	4197	1029	95	83	86	30	34	31
Bifunctional acetaldehyde-alcohol dehydrogenase(adhE)	1.1.1.1/ 1.2.1.10	3198	2940	01310	5426	0423	2402	3925	96	88	88	75	67	76
Acetate kinase	2.7.2.1	2136	4304	03043	4518	1028	1892	1327	93	83	83	70	56	60
Phosphotransacetylase	2.3.1.8	2137	4305	03044	4519	1029	1891	1326	98	89	89	73	53	65
Acetate thiokinase	6.2.1.1	0494	2620	02662	5570	0551	0888	–	93	85	85	78	30	–

Ccel, *C. cellulolyticum* H10; CTER, *C. termitidis* CT1112, DSM 5398; Cthe27405, *C. thermocellum* ATCC 27405; Clocel, *C. cellulovorans* 743B, ATCC 35296; Cphy, *C. phytofermentans* ISDg; CpapDRAFT, *C. papyrosolvens* DSM 2782; T344DRAFT, *C. cellobioparum* ATCC 15832

[a]*C. thermocellum* ATCC 27405 used as a reference

obtained for each pathway were confirmed by the percent amino acids sequence identity, higher score (better alignment), and lowest e-value (most significant hit). In addition, the functionality of each enzyme was verified by conserved domain analysis. Here, we mainly discuss the comparative analysis of genes encoding proteins involved in pyruvate metabolism and end-product synthesis, xylose production, and hydrogen (H_2) production in seven cellulolytic *Clostridium* species.

Glucose is an important carbon source used by bacteria to synthesize a wide-range of metabolic intermediates used in many biosynthetic reactions. During metabolism, glucose can be stored as a polysaccharide, converted to sucrose, oxidized to pyruvate via glycolysis, or oxidized to pentose sugars via the pentose phosphate pathway (Fig. 4a–d). Pyruvic acid can be converted back to glucose via gluconeogenesis, or to acetyl-CoA which is a branch-point precursor for many biosynthesis pathways. Pyruvate can be converted to alanine or to citric acid in the presence of oxygen, whereas it ferments to produce lactic acid using the enzyme lactate dehydrogenase (CTER_2504) and NADH in the absence of oxygen. Alternatively, it is converted to acetaldehyde and then to ethanol in alcoholic fermentation.

Carbon and electrons are distributed between catabolic, anabolic, and energy-producing pathways in bacterial cells. In the glycolytic pathway, carbon and electrons flow from phosphoenolpyruvate (PEP) to the various end-products via different nodes, such as the Phosphoenolpyruvate/Oxaloacetate/Pyruvate node, the Acetyl-CoA/Acetate/Ethanol node, and the Ferredoxin/NAD(P)H/Hydrogen node [71, 72]. Several different enzymes involved in the conversion of intermediate metabolites and the presence of corresponding genes which encode these proteins in *C. termitidis* have been identified in the annotated genome (Fig. 3). Phosphoenolpyruvate (PEP) is converted to pyruvate directly by pyruvate kinase (PPK) or pyruvate phosphate dikinase (PPDK) (CTER_0809). PEP can also be converted to pyruvate via the "malate shunt" [73] using phosphoenolpyruvate carboxykinase (PEPCK; CTER_1146), malate dehydrogenase (MDH; CTER_0412), and malic enzyme (MalE; CTER_0411) or via other reactions using PEPCK and oxaloacetate decarboxylase (OAADC; CTER_0730). Conversion of pyruvate to lactate via NADH-dependent lactate dehydrogenase diverts reducing equivalents away from hydrogen and ethanol. The reducing equivalents NADH and reduced ferredoxin (Fdr) are generated by the oxidative decarboxylation of pyruvate to acetyl-CoA via pyruvate dehydrogenase (PDH) or pyruvate:ferreodoxin oxidoreductase (Pfor), respectively. NADH and Fdr are oxidized to NAD^+ and oxidized ferredoxin (Fdo) during hydrogen and ethanol synthesis. Another important enzyme in pyruvate catabolism is pyruvate formate lyase (PFL; CTER_0038), which converts pyruvate to acetyl-CoA and producing formate during the process.

The presence or absence of these enzymes was determined through comparative genomic analyses in *C. cellulolyticum*, *C. cellobioparum*, *C. phytofermentans*, C. *papyrosolvens*, *C. cellulovorans*, and *C. thermocellum* (summarized in Table 2). All genes associated with glycolytic pathways are observed in the *C. termitidis* genome. Seven copies of cellobiose phosphorylase, two copies of glucose kinase, three copies of 6-phosphofructokinase, and two copies of phosphoglycerate mutase were identified in the *C. termitidis* genome, and the functional domains of these

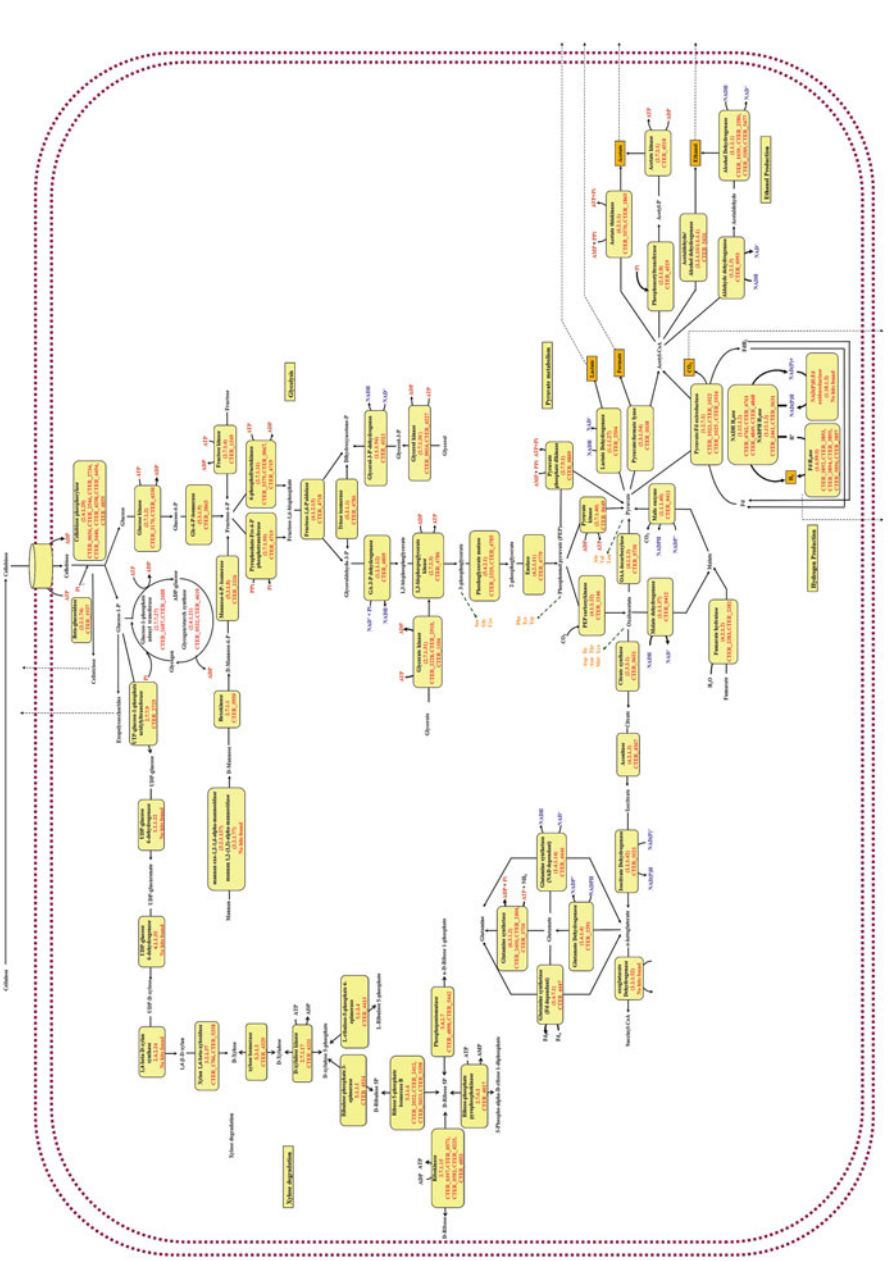


Fig. 3 Schematic representation of the core metabolic pathways in *Clostridium termitidis* indicating the flow of carbon from cellobiose degradation through glycolysis to pyruvate catabolism to synthesis of H_2 and ethanol

enzymes were confirmed by RPS-BLAST using the conserved domain database (CDD) [65].

3.2 Hydrogen Synthesis

Hydrogen (H_2) is an essential component in the metabolism of many microorganisms. *Clostridium* species are the most widely studied microorganisms for fermentative H_2 production [73–75]. Fermentative H_2 production is a process in which reversible reduction of proton to dihydrogen is catalyzed by hydrogenases [76, 77]. Hydrogenases have highly reactive and complex metallocenters and synthesize H_2 more efficiently than nitrogenases [78, 79]. On the basis of the metallocenter, hydrogenases are divided into three major classes: (1) [NiFe] hydrogenases which contain Ni and Fe atoms; (2) [FeFe] hydrogenases, which contain two Fe atoms bound to cysteine residues in their active sites; [FeFe] hydrogenases may be dimeric, trimeric, or even tetrameric enzymes, and size variations were observed because of the presence of additional domain accommodating different number of FeS cluster [80]; and (3) [Fe] hydrogenases with one Fe atom [81]. [FeFe] hydrogenases exist in multiple forms with different modular structures and are mostly observed in the genus *Clostridium*, whereas [NiFe] hydrogenases have been reported in many bacteria, archaea, and in a few *Clostridium* species. [Fe] hydrogenases are mainly reported in methanogenic archaea [82].

Sequences encoding [NiFe] and [FeFe] hydrogenases, which are homologous to *C. cellulolyticum* hydrogenases, have been searched in the genome sequences of seven cellulolytic *Clostridium* species. This analysis revealed the presence of a small subunit of genes encoding a [NiFe] hydrogenase and Cytochrome b5 only in *C. cellulolyticum*, *C. papyrosolvens*, and *C. cellulovorans*. The small subunit of [NiFe] hydrogenases contains FeS clusters in their active sites, which transfers electrons between the catalytic center of enzyme and the electron donors. The large subunit of [NiFe] hydrogenases contains the Ni-Fe cluster in the active site. Genes encoding the [NiFe] hydrogenase large subunit were observed in *C. cellulolyticum*, *C. termitidis*, *C. cellobioparum*, *C. cellulovorans*, *C. phytofermentans*, *C. papyrosolvens*, and *C. thermocellum*.

Cellulolytic *Clostridium* species were also investigated for Ech hydrogenases. The complex Ech hydrogenases have six membrane-bound subunits, and the genes encoding these subunits are organized into operons. Genes corresponding to these membrane-bound subunits were found in six of seven *Clostridium* species investigated and were absent in the genome of *C. cellulovorans*. Homologs of [FeFe] hydrogenases were also investigated in the seven *Clostridium* species. Amino acid sequences of hydrogenases from *C. cellulolyticum* showed highest percent identity with *C. papyrosolvens*, *C. termitidis*, and *C. cellobioparum* [83]. The locus tags of these predicted enzymes are included in Table 3.

This analysis showed that the genome of *C. termitidis* has all the hydrogenase and pyruvate-ferredoxin oxidoreductase encoding genes responsible for H_2 synthesis (Fig. 4c). Ramachandran et al. [22] reported that rate of H_2 synthesis by

Table 3 Comparative analysis of hydrogenases in cellulolytic *Clostridium* species

Remarks	EC no.	Predicted localization	Locus tag							Amino acid identity (%)					
			Ccel_	CpapDRAFT_	CTER_	T344DRAFT_	Cphy_	Cthe27405_	Clocel_	Ccel/ CpapDRAFT	Ccel/ Cter	Ccel/ T344DRAFT	Ccel/ cphy	Ccel/ Cthe	Ccel/ Clocel
[NiFe] HYDROGENASE															
Cytochrome b5	–	U	1069	2861	–	–	–	–	0269	74	–	–	–	–	50
Hydrogenase (NiFe) small SU HydA	1.12.99.6	CM	1070	2860	–	–	–	–	1155	88	–	–	–	–	52
Nickel-dependent hydrogenase large SU	1.6.99.5, 1.12.5.1	C	1071	2859	3893	02875	1734	3020	1156	84	31	32	33	30	51
4Fe-4S ferredoxin, iron-sulfur binding (EchF)	1.6.99.5	C	3366	1309	3892	02874	1735	3019	0104	92	82	82	46	50	40
Ech hydrogenase subunit E	1.6.99.5	C	3367	1310	3893	02875	1734	3020	–	96	90	89	62	62	–
Ech hydrogenase subunit D	1.6.99.5	C	3368	1311	3894	02876	1733	3021	–	91	79	78	37	44	–
Ech hydrogenase subunit C	1.6.99.5	C	3369	1312	3895	02877	1732	3022	–	97	92	92	72	73	–
Ech hydrogenase subunit B	1.6.99.5	CM (10 IH)	3370	1313	3896	02878	1731	3023	–	93	88	88	49	46	–
Ech hydrogenase subunit A	1.6.99.5	CM (18 IH)	3371	1314	3897	02879	1730	3024	–	93	85	84	41	41	–
NADH ubiquinone oxidoreductase 20 kDa SU	1.6.99.5	CM	1686	1634	0018	02571	1732	3022	–	88	73	73	47	47	–
NADH dehydrogenase (ubiquinone) 30 kDa SU	1.6.99.5	C	1687	1635	0657	02570	1734	3020	–	90	78	78	34	35	–
Hydrogenase, membrane SU 2-like protein	1.6.99.5	CM (13 IH)	1688	1636	0658	02569	1730	3024	–	93	78	78	25	26	–
Hydrogenase, membrane SU 2-like protein	1.6.99.5	CM (7 IH)	1689	1637	0659	02568	–	–	–	89	77	77	–	–	–
Respiratory-chain NADH dehydrogenase, SU 1	1.6.99.5	CM (8 IH)	1690	1638	0660	02567	1731	3023	–	88	74	74	24	26	–

(continued)

Table 3 (continued)

Remarks	EC no.	Predicted localization	Locus tag							Amino acid identity (%)					
			Ccel_	CpapDRAFT_	CTER_	T344DRAFT_	Cphy_	Cthe27405_	Clocel_	Ccel/ CpapDRAFT	Ccel/ Cter	Ccel/ T344DRAFT	Ccel/ cphy	Ccel/ Cthe	Ccel/ Clocel
NADH/Ubiquinone/plastoquinone (complex I)	1.6.99.5	CM (18 IH)	1691	1639	0661	02566	1730	3024	–	82	62	62	28	27	–
[FeFe] HYDROGENASE															
Putative PAS/PAC sensor protein	1.12.7.2	C	2300	3820	4846	00036	0092	0426	3813	94	79	79	41	42	26
Putative uncharacterized protein	–	C	2301	3821	4847	00035	0093	0431	2244	94	83	83	44	46	35
Hydrogenase, Fe-only	1.12.7.2	C	2303	3823	4848	00034	0087	0430	2243	94	85	85	57	56	44
NADH dehydrogenase (quinone)	1.6.99.5	C	2304	3824	4849	00033	0088	0429	2588	94	88	89	67	69	56
NADH dehydrogenase (ubiquinone), 24 kDa subunit	1.6.99.5	C	2305	3825	4850	00032	0089	0428	2583	92	85	86	65	68	60
Hydrogenase, Fe-only	1.12.7.2	C	2467	0711	2461	00655	3805	0342	2243	94	89	89	54	63	63
Hydrogenase, Fe-only	1.12.7.2	C	2232	2734	4761	00113	3805	0342	2243	95	87	87	60	79	56
Pyruvate ferredoxin oxidoreductase, delta subunit	1.6.99.5	C	2233	2735	4762	00112	3804	0341	2244	95	90	91	72	82	62
Ferredoxin	1.6.99.3, 1.4.1.13	C(IH)	2234	2736	4763	00111	3803	0340	2244	88	71	70	50	69	30
Ferredoxin	1.6.99.3, 1.4.1.13	C	2545	2719	0631	03446	2934	0372	2971	94	91	91	65	81	52

Ccel, *C. cellulolyticum* H10; CTER, *C. termitidis* CT1112, DSM 5398; Cthe27405, *C. thermocellum* ATCC 27405; Clo1313, *C. thermocellum* LQ8, DSM 1313; Cphy, *C. phytofermentans* ISDg; T344DRAFT, *C. cellobioparum* ATCC 15832; CpapDRAFT, *C. papyrosolvens* DSM 2782; Clocel, *C. cellulovorans* 743B, ATCC 35296; C, cytoplasmic; CM, cytoplasmic membrane (IH, number of internal membrane helices); U, unknown [83]

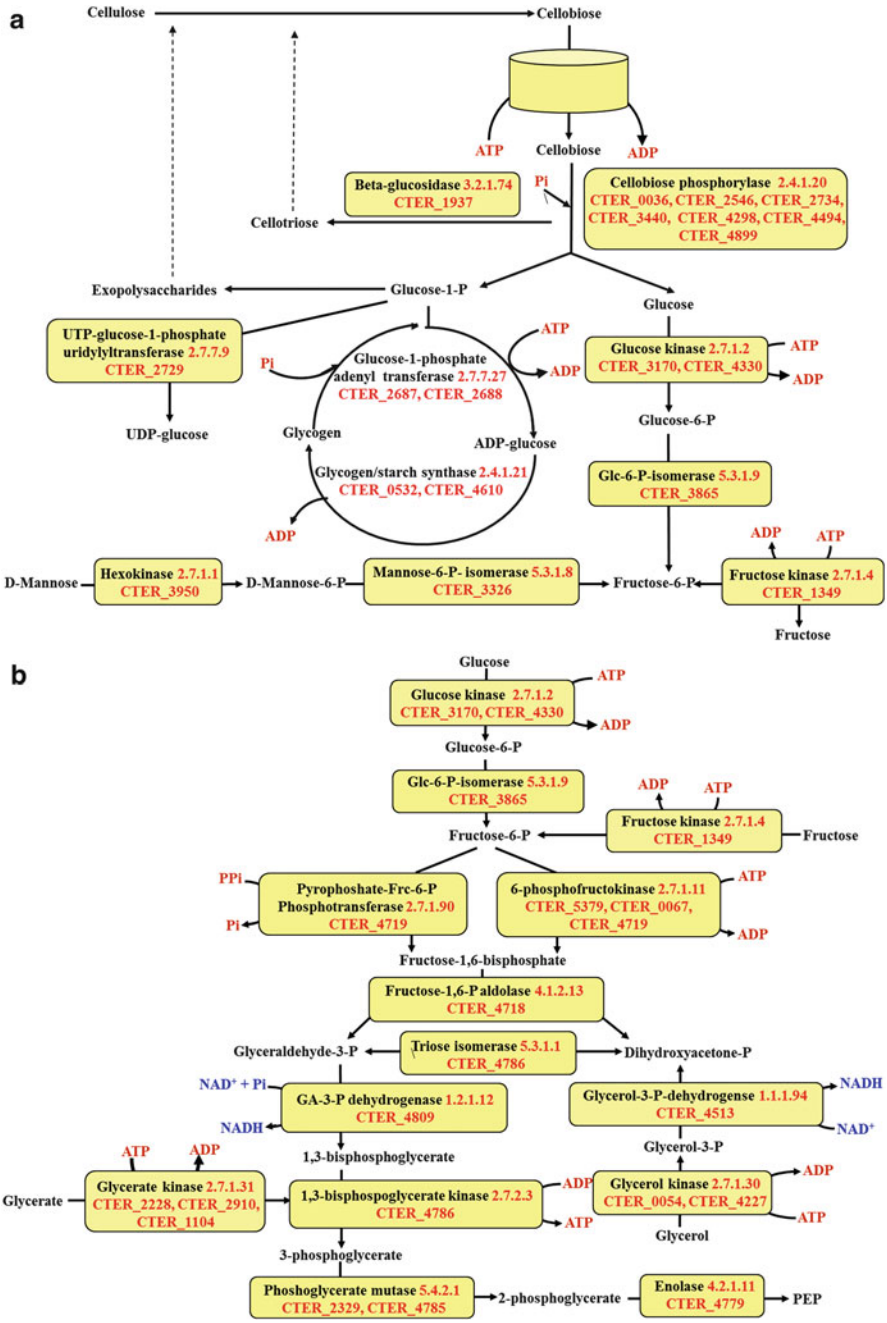


Fig. 4 Metabolic pathways in *Clostridium termitidis*. Detailed schematic representations of: (**a**) cellobiose degradation; (**b**) glycolysis; (**c**) pyruvate catabolism and H_2 synthesis; (**d**) ethanol synthesis. The KEGG database was used as the reference for construction of pathways. EC numbers and locus tags for each enzyme are written in *red*. "No hits found" means genes encoding these enzymes were not observed in *C. termitidis* genome

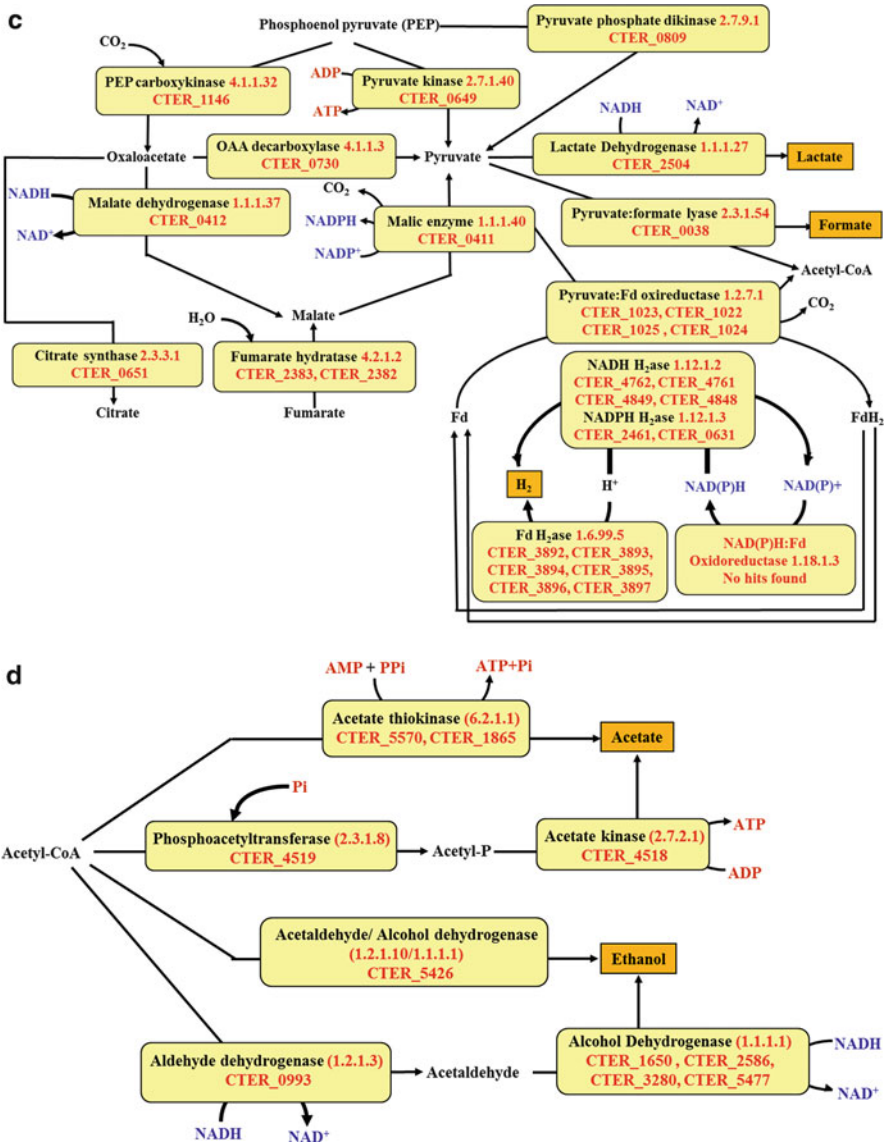


Fig. 4 (continued)

C. termitidis strain CT1112 was comparable to that of the cellulolytic bacterium *C. cellulolyticum*. *C. termitidis* was observed to synthesize greater amounts of H_2 than ethanol when cultured with either cellobiose or α-cellulose, even though the rates of growth of *C. termitidis* with these substrates were vastly different. *C. termitidis* grows faster using cellobiose (doubling-time of 6.5 h) than α-cellulose (doubling-time of 18.9 h). Moreover, a metabolic shift from ethanol to acetate and H_2 synthesis and a trend toward lower H_2:CO_2 ratios were observed when the pH dropped below 6.2 during fermentation.

3.3 Xylose Utilization

Many bacteria in the Phylum *Firmicutes*, including *Clostridium* species, are able to utilize xylose as a carbon source. Depolymerization of xylan and xyloglucan, which are major constituents of hemicellulose in plant cell walls, produces α- and β-xylosides, respectively. These compounds are transported into the cell and converted into D-xylose and then transformed to xylulose 5-phosphate. It has already been reported that *C. termitidis* can utilize different types of simple and complex carbohydrates such as cellulose, cellobiose, xylose, glucose, and mannose and produces acetate, formate, ethanol, H_2, and CO_2 during fermentation [21–23, 72]. Recently, Munir et al. [42] reported that *C. termitidis* is also able to grow well on xylan polymers. A computational search, based on gene homology, was used to determine the complete xylose degradation and pentose phosphate pathway in *C. termitidis* (Fig. 3). This analysis showed that *C. termitidis* contains most D-xylose pathway genes.

In the xylose utilization pathway, the first reaction is the conversion of D-xylose into D-xylulose by xylose isomerase (EC 5.3.1.5) (CTER_4329). In the second reaction, phosphorylation of D-xylulose to D-xylulose 5-phosphate via xylulokinase (EC 2.7.1.17) (CTER_4331) was observed as a key intermediate in the pentose phosphate pathway [84]. D-Xylulose 5-phosphate is finally converted into D-ribose, 5-phospho-α-D-ribose 1-diphosphate, L-ribulose 5-phosphate, and α-D-ribose 1-phosphate via several steps. All locus tags belonging to these enzymes involved in the xylose pathway are present in *C. termitidis* (Fig. 5).

Enzymes involved in glutamine synthesis were also screened and hits for almost all the enzymes were found in *C. termitidis* (Fig. 6). Glutamine synthetase (GS) (EC 6.3.1.2) is an enzyme that plays an important role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine. Glutamine synthetase uses ammonia produced by nitrate reduction, amino acid degradation, and photorespiration [85] The amide group of glutamate is a nitrogen source for the synthesis of glutamine pathway metabolites [86] (Fig. 6).

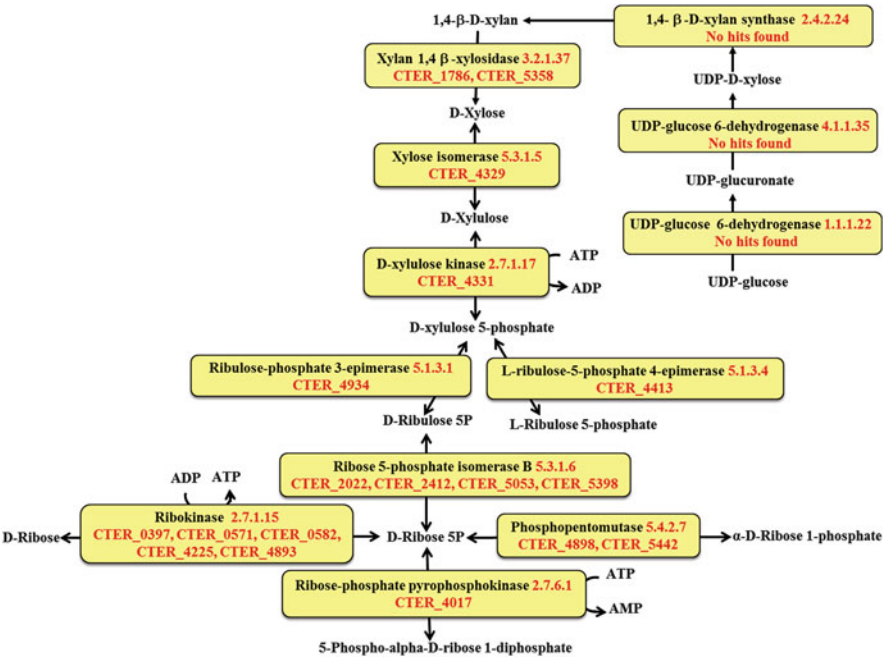


Fig. 5 Detailed schematic representation of xylose catabolism in *C. termitidis*. The KEGG database was used as the reference for construction of pathways. EC numbers and locus tags for each enzyme are written in *red*. "No hits found" means genes encoding these enzymes were not observed in *C. termitidis* genome

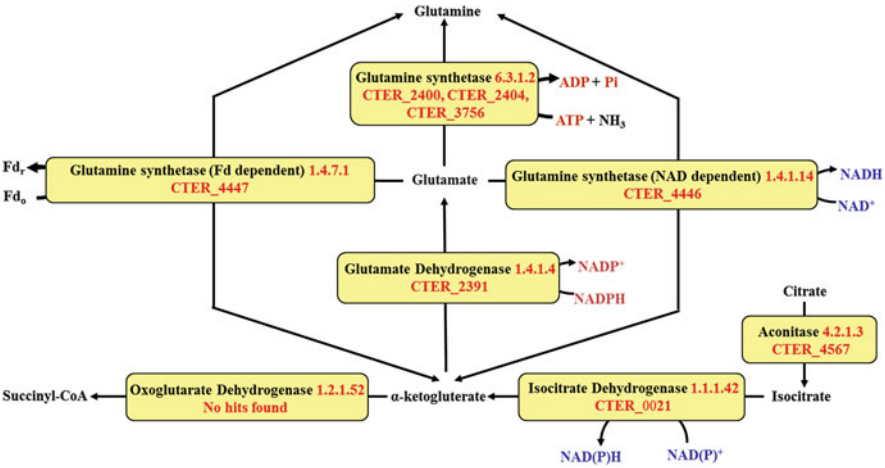


Fig. 6 Detailed schematic representation of glutamine synthesis in *C. termitidis*. The KEGG database was used as the reference for construction of pathways. EC numbers and locus tags for each enzyme are written in *red*. "No hits found" means genes encoding these enzymes were not observed in *C. termitidis* genome

4 Comparative Analysis of Whole Genome of *Clostridium* Species

4.1 Comparative Synteny Dot Plot Analyses of Clostridial Genomes

Comparative synteny dot plot analyses based on protein sequences were carried out using *C. termitidis* and 8 cellulolytic *Clostridium* species (Fig. 7). In this analysis, pairwise comparisons of the *C. termitidis* genome and each of the other genomes

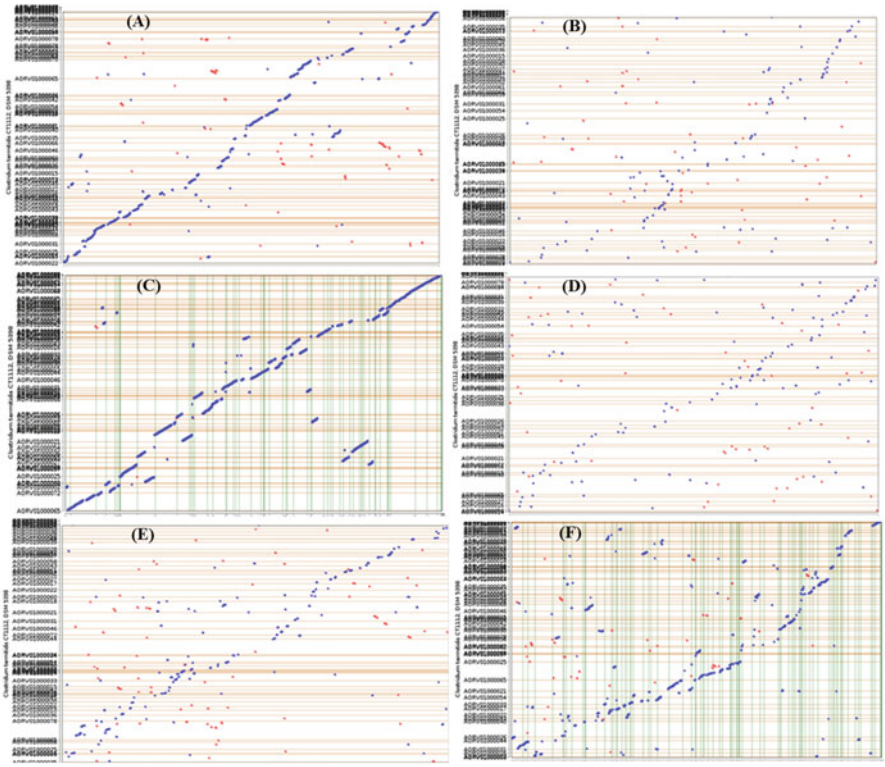


Fig. 7 Comparative synteny *dot* plots between the *C. termitidis* genome and the genomes of other *Clostridiu*m species. The synteny plots reveal orthologous relationships between *C. termitidis* and six *Clostridium* species. (**a**) *C. termitidis* CT1112, DSM 5398 vs *C. cellulolyticum* H10. (**b**) *C. termitidis* CT1112 vs *C. cellulovorans* 743B, ATCC 35296. (**c**) *C. termitidis* CT1112 vs *C. cellobioparum* ATCC 15832. (**d**) *C. termitidis* CT1112 vs *C. phytofermentans* ISDg. (**e**) *C. termitidis* CT1112 vs *C. papyrosolvens* C7. (**f**) *C. termitidis* CT1112 vs *C. thermocellum* ATCC 27405. Comparison was based on amino acid sequences. The *blue points* in the *dot plots* represent the regions of similarity found on parallel strands (fplot) and the *red points* represent the regions of similarity found on antiparallel strands (rplot). The tooltip for each point shows which plot it is and the coordinates and scaffolds of the alignment

was accomplished using Mummer to generate dot plot diagrams [87]. The diagonal line in Fig. 7 show the co-linearity of DNA strands. The blue points in the dot plots represent the regions of similarity found on parallel strands and the red points represent the regions of similarity found on antiparallel strands. On the basis of genome arrangement, *C. termitidis* showed high synteny (co-linearity) with *C. cellobioparum* and *C. cellulolyticum*, but displayed markedly different genome organization from *C. cellulovorans*, *C. papyrosolvens*, *C. phytofermentans*, and *C. thermocellum*.

4.2 *Whole Genome Comparisons to Identify Orthologous Genes*

Genomes can be compared in terms of gene content using the "Phylogenetic Profiler" which allows one to identify genes in a query genome in terms of presence or absence of homologs in other genomes. Whole genome comparisons were conducted to find orthologous genes in 24 genomes of cellulolytic and non-cellulolytic *Clostridium* species using the *C. termitidis* genome as the reference organism. The analysis was based on the selection of "best gene homologs" between the *C. termitidis* genome and other *Clostridium* species genomes at a 60–90% amino acid sequence identity level. Comparative analysis revealed the highest number of orthologs was observed between *C. termitidis* and *C. cellobioparum*. This observation was supported by phylogenetic analyses based on 16S *rRNA*, *cpn60*, and COG functional profiles. No homologs (0) were found with *C. acetobutylicum* ATCC 824 and *C.* cf. *saccharolyticum* K10 at 90% identity level (Table 4).

4.3 *Comparative Analysis of COGs in Eight* Clostridium *Species*

An analysis of Clusters of Orthologous Groups (COGs) was conducted using the "Abundance Profiles" tools in IMG, which provide a comprehensive examination of the functional components of genomes between strains. In other words, "Abundance Profiles" are based on annotation profiles (e.g., COGs, Pfam, Enzyme EC Numbers, and TIGRfams) to compare and contrast the genome content. In this analysis, eight *Clostridium* species – *C. termitidis* CT1112, DSM 5398, *C. cellulolyticum* H10, *C. thermocellum* ATCC 27405, *C. thermocellum* LQ8, *C. acetobutylicum* ATCC 824, *C. cellulovorans* 743B, ATCC 35296, *C. papyrosolvens* DSM 2782, and *C. phytofermentans* ISDg – were used for comparative COG matrix analysis. On the basis of this analysis, 2,362 common COG families were identified in the genomes of the 8 *Clostridium* species. Some

Table 4 Pairwise whole genome comparison for "best" homologs in cellulolytic and non-cellulolytic *Clostridium* species

Cellulolytic and non-cellulolytic *Clostridium* species	Reference genome (Cter[a]) vs query genome	Genome identity (%)			
		60+	70+	80+	90+
		Number of homologs			
Clostridium cellobioparum ATCC 15832	Cter-Ccellobio	4,317	4,268	4,228	4,138
C. papyrosolvens DSM 2782	Cter-Cpap	1,947	1,449	791	190
Clostridium species BNL1100	Cter-Clo1100	1,944	1,474	812	218
C. josui JCM 17888	Cter-Cjos	1,830	1,400	781	214
C. cellulolyticum H10	Cter-Ccel	1,775	1,342	739	165
C. cellulosi CS-4-4	Cter-CcelCS-4-4	849	306	48	1
C. clariflavum EBR 45, DSM 19732	Cter-Cclari	824	441	128	8
C. thermocellum ATCC 27405	Cter-Cthe	775	417	137	6
C. thermocellum LQ8, DSM 1313	Cter-Cthe1313	773	415	137	6
C. alkalicellulosi Z-7026, DSM 17461	Cter-Calkali	747	360	111	11
C. stercorarium stercorarium DSM 8532	Cter-Cster	512	207	36	2
C. cellulovorans 743B, ATCC 35296	Cter-Ccel743B	364	113	19	1
C. phytofermentans ISDg	Cter-Cphy	325	107	26	1
C. saccharolyticum WM1, DSM 2544	Cter-CsacWM1	300	79	15	1
C. saccharoperbutylacetonicum N1-4(HMT)	Cter-CsacN1	448	136	21	2
C. pasteurianum BC1	Cter-Cpast	386	132	19	1
C. intestinale URNW	Cter-Cinte	371	139	21	2
C. kluyveri DSM 555	Cter-Cklu	352	109	17	1
C. carboxidivorans P7, DSM 15243	Cter-Ccarboxi	341	106	18	2
C. ljungdahlii PETC, DSM 13528	Cter-Cljung	320	97	19	2
C. saccharobutylicum DSM 13864	Cter-Csac13864	318	100	16	2
C. acetobutylicum ATCC 824	Cter-Cace	315	95	16	0
C. autoethanogenum DSM 10061	Cter-Cauto	312	98	18	2
C. cf. *saccharolyticum* K10	Cter-CsacK10	179	46	9	0

[a]Cter, *C. termitidis*

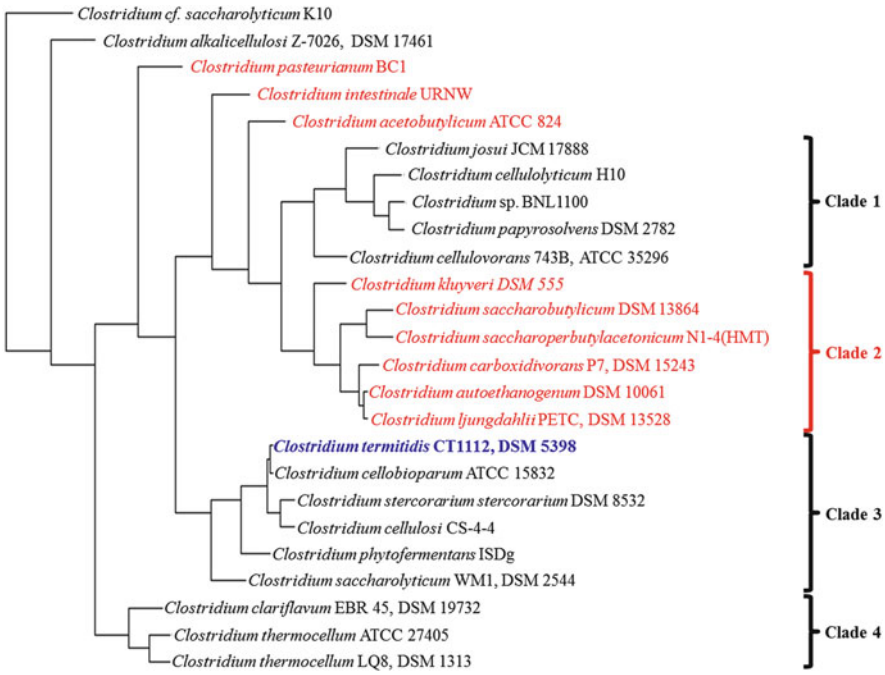


Fig. 8 Phylogenetic tree based on annotated COG functional profiles for 25 sequenced cellulolytic and non-cellulolytic *Clostridium* species genomes. Clustering is based on Cluster 3.0 analysis software [70], and was performed within the IMG-ER platform using the COG profile for each of the genome. Branch lengths correspond to calculated distances between functional profiles. The tree was generated using the Archaeopteryx applet on IMG platform

COGs were present in all species, some were present in more than one copy in some genomes, and some were absent in one or more genomes. Out of 2,362 COG families, 694 COG families were absent in the *C. termitidis* genome. *C. termitidis* genome showed 3,392 protein coding genes with COG families, which was greater than all other *Clostridium* species (Ccel-2036; Cthe27405-1892; Cthe1313-1814; Clocel-2514; Cphy-2497, and Cac-2648).

An analysis of hierarchical clustering based upon COGs was conducted with the genomes of 25 cellulolytic and non-cellulolytic *Clostridium* species to determine whether similar functional profiles among the species could be identified. This analysis revealed four distinct Clades within the genus. Clades 1, 3, and 4 contained the cellulolytic *Clostridium* species, whereas Clade 2 contained non-cellulolytic solventogenic *Clostridium* species (Fig. 8).

5 Conclusions

Although the genus *Clostridium* contains a large number of species with seeming great genetic variation and metabolic capabilities, comparative genomic analyses enabled rapid visualization of the evolutionary relationships among cellulolytic and non-cellulolytic species. Phylogenetic analyses of 16S *rRNA* and *cpn60* genes have suggested a close evolutionary relationship between *C. termitidis* and *C. cellobioparum*. This observation was supported by whole genome comparisons. Genome analyses also revealed that *C. termitidis* has the largest genome (6.42 Mb) [23] of all the cellulolytic *Clostridium* species studied and its genome encodes all enzymes required for pyruvate metabolism and catabolism, xylose utilization, ethanol synthesis, and hydrogen synthesis. Our comparative genomic analyses identified homologs of [NiFe] and [FeFe] hydrogenases in seven cellulolytic *Clostridium* species.

Genomic analyses further determined that the *C. termitidis* genome encodes the greatest number of carbohydrate active enzymes (CAZymes) in comparison to other cellulolytic *Clostridium* species. These CAZymes have the potential ability to degrade a wide variety of complex and simple carbohydrates, such as cellulose, hemicellulose, starch, chitin, fructans, pectin, glucose, cellobiose, and xylose, thus making *C. termitidis* an attractive microorganism for biofuel production through CBP [42]. Although many of these CAZymes have homologs in other bacteria, a multidomain GH5 protein, Cter_2817, seems to be unique to *C. termitidis* because BLAST searches did not give any hits in other *Clostridium* species with this protein. This protein has a modular structure CBM66-CBM66-CBM66-GH5_distGH43-CBM35-CBM66-GH43-SLH-SLH-SLH and would be putatively bound to the cell via the SLH domains. In addition to the non-cellulosomal enzymes, *C. termitidis* has been shown to harbor the genes and express the products of cellulosomal components and CAZymes, suggesting cellulosome assembly [42, 88]. In conclusion, computational approaches and comparative genomic analyses can facilitate deep insight into the genetic basis of metabolic pathways involved in synthesis of various useful products by cellulolytic and non-cellulolytic biofuel and solvent producing *Clostridium* species. Searching pathways using in silico approaches generates valuable information concerning the presence or absence of the genes involved in particular pathways in a much shorter time. On the basis of this information, we can divert or delete the particular pathway to manipulate or engineer any organisms to enhance the production of various bioproducts.

Acknowledgements This work was supported by funds from Genome Canada through the Applied Genomics Research in Bioproducts or Crops (ABC) program for the grant titled "Microbial genomics for biofuels and coproducts from biorefining processes," and the Province of Manitoba from the Manitoba Research Innovation Fund (MRIF).

References

1. Levin DB, Zhu H, Beland M, Cicek N, Holbein BE (2007) Potential for hydrogen and methane production from biomass residues in Canada. Bioresour Technol 98:654–660
2. Zhou C, Ma Q, Mao Z, Yanbin Y, Ying X (2014) New insights into *Clostridia* through comparative analyses of their 40 genomes. BioEnergy Res 7:1481–1492
3. Shoseyov O, Takagi M, Goldstein M, Doi RH (1992) Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein a (CbpA). Proc Natl Acad Sci U S A 89:3483–3487
4. Tamaru Y, Karita S, Ibrahim A, Chan H, Doi RH (2000) A large gene cluster for the *Clostridium cellulovorans* cellulosome. J Bacteriol 182:5906–5910
5. Tamaru Y, Miyake H, Kuroda K, Nakanishi A, Kawade Y, Yamamoto K, Uemura M, Fujita Y, Doi RH, Ueda M (2010) Genome sequence of the cellulosome-producing mesophilic organism *Clostridium cellulovorans* 743B. J Bacteriol 192:901–902
6. Pagès S, Gal L, Bélaich A, Gaudin C, Tardif C, Bélaich JP (1997) Role of scaffolding protein CipC of *Clostridium cellulolyticum* in cellulose degradation. J Bacteriol 179:2810–2816
7. Petitdemange E, Caillet F, Giallo J, Gaudin C (1984) *Clostridium cellulolyticum* species nov., a cellulolytic, mesophilic species from decayed grass. Int J Syst Bacteriol 34:155–159
8. Murray WD, Khan AW, van den Berg L (1982) *Clostridium saccharolyticum* species nov., a saccharolytic species from sewage sludge. Int J Syst Bacteriol 32:132–135
9. Warnick TA, Methé A, Leschine SB (2002) *Clostridium phytofermentans* species nov., a cellulolytic mesophile from forest soil. Int J Syst Evol Microbiol 52:1155–1160
10. Lamed R, Kenig R, Morgenstern E, Calzada JF, Micheo FD, Bayer EA (1991) Efficient cellulose solubilization by a combined cellulosome β-glucosidase system. Appl Biochem Biotechnol 27:173–183
11. Stevenson DM, Weimer PJ (2005) Expression of 17 genes in *Clostridium thermocellum* ATCC 27405 during fermentation of cellulose or cellobiose in continuous culture. Appl Environ Microbiol 71:4672–4678
12. Feinberg L, Foden J, Barrett T, Davenport KW, Bruce D, Detter C, Tapia R, Han C, Lapidus A, Lucas S, Cheng JF, Pitluck S, Woyke T, Ivanova N, Mikhailova N, Land M, Hauser L, Argyros DA, Goodwin L, Hogsett D, Caiazza N (2011) Complete genome sequence of the cellulolytic thermophile *Clostridium thermocellum* DSM1313. J Bacteriol 193:2906–2907
13. Shiratori H, Sasaya K, Ohiwa H, Ikeno H, Ayame S, Kataoka N, Miya A, Beppu T, Ueda K (2009) *Clostridium clariflavum* species nov. and *Clostridium caenicola* species nov., moderately thermophilic, cellulose-/cellobiose-digesting bacteria isolated from methanogenic sludge. Int J Syst Evol Microbiol 59:1764–1770
14. Izquierdo JA, Goodwin L, Davenport KW, Teshima H, Bruce D, Detter C, Tapia R, Han S, Land M, Hauser L, Jeffries CD, Han J, Pitluck S, Nolan M, Chen A, Huntemann M, Mavromatis K, Mikhailova N, Liolios K, Woyke T, Lynd LR (2012) Complete genome sequence of *Clostridium clariflavum* DSM 19732. Stand Genomic Sci 6:104–115
15. Schwarz W, Bronnenmeier K, Landmann B, Wanner G, Staudenbauer W, Kurose N, Takayama T (1995) Molecular characterization of four strains of the cellulolytic thermophile *Clostridium stercorarium*. Biosci Biotechnol Biochem 59:1661–1665
16. Poehlein A, Hartwich K, Krabben P, Ehrenreich A, Liebl W, Dürre P, Gottschalk G, Daniel R (2013) Complete genome sequence of the solvent producer *Clostridium saccharobutylicum* NCP262 (DSM 13864). Genome Announc 1(6pii):e00997–e001013
17. Schellenberg JJ, Verbeke TJ, McQueen P, Krokhin OV, Zhang X, Alvare GM, Fristensky B, Thallinger GG, Henrissat B, Wilkins JA, Levin DB, Sparling R (2014) Enhanced whole genome sequence and annotation of *Clostridium stercorarium* DSM8532T using RNA-seq transcriptomics and high-throughput. BMC Genomics 15(1):567
18. Li LL, Taghavi S, Izquierdo JA, van der Lelie D (2012) Complete genome sequence of *Clostridium* species strain BNL1100, a cellulolytic mesophile isolated from corn stover. J Bacteriol 194:6982–6983

19. Madden RH, Bryder MJ, Poole NJ (1982) Isolation and characterization of an anaerobic, cellulolytic bacterium *Clostridium papyrosolvens* sp nov. Int J Syst Bacteriol 32:87–91
20. Leschine SB, Canale-Parola E (1983) Mesophilic cellulolytic clostridia from freshwater environments. Appl Environ Microbiol 46:728–737
21. Hethener P, Brauman A, Garcia JL (1992) *Clostridium termitidis* species nov., a cellulolytic bacterium from the gut of the woodfeeding termite, *Nasutitermes lujae*. Syst Appl Microbiol 15:52–58
22. Ramachandran U, Wrana N, Cicek N, Sparling R, Levin DB (2008) Hydrogen production and end-product synthesis patterns by *Clostridium termitidis* strain CT1112 in batch fermentation cultures with cellobiose or α-cellulose. Int J Hydrogen Energy 33:7006–7012
23. Lal S, Ramachandran U, Zhang X, Munir R, Sparling R, Levin DB (2013) Draft genome sequence of the cellulolytic, mesophilic, anaerobic bacterium *Clostridium termitidis* strain CT1112 (DSM 5398). Genome Announc 1(3):e00281–13
24. Yanling H, Youfang D, Yanquan L (1991) Two cellulolytic *Clostridium* species: *Clostridium cellulosi* species nov. and *Clostridium cellulofermentans* species nov. Int J Syst Bacteriol 41:306–309
25. Lamed R, Naimark J, Morgenstern E, Bayer EA (1987) Specialized cell surface structures in cellulolytic bacteria. J Bacteriol 169:3792–3800
26. Zhilina TN, Kevbrin VV, Turova TP, Lysenko AM, Kostrikina NA, Zavarzin GA (2005) *Clostridium alkalicellum* species nov., an obligately alkaliphilic cellulolytic bacterium from a soda lake in the Baikal region. Mikrobiologiia 74:642–653
27. Sukhumavasi J, Ohmiya K, Shimizu S, Ueno K (1988) *Clostridium josui* species nov., a cellulolytic, moderate thermophilic species from Thai compost. Int J Syst Bacteriol 38:179–182
28. Kelly WJ, Asmundson RV, Hopcroft DH (1987) Isolation and characterization of a strictly anaerobic, cellulolytic spore former: *Clostridium chartatabidum* species nov. Arch Microbiol 147:169–173
29. Yang JC, Chynoweth DP, Williams DS, Li A (1990) *Clostridium aldrichii* species nov., a cellulolytic mesophile inhabiting a wood-fermenting anaerobic digester. Int J Syst Bacteriol 40:268–272
30. Palop ML, Valles S, Pinaga F, Flors A (1989) Isolation and characterization of anaerobic, cellulolytic bacterium *Clostridium celerecrescens* species nov. Int J Syst Bacteriol 39:68–71
31. Keis S, Shaheen R, Jones DT (2001) Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions of *Clostridium saccharoperbutylacetonicum* species nov. and *Clostridium saccharobutylicum* species nov. Int J Syst Evol Microbiol 51:2095–2103
32. Poehlein A, Krabben P, Dürre P, Daniel R (2014) Complete genome sequence of the solvent producer *Clostridium saccharoperbutylacetonicum* strain DSM 14923. Genome Announc 2 (5pii):e01056–14
33. Seedorf H, Fricke WF, Veith B, Brüggemann H, Liesegang H, Strittmatter A, Buckel W, Hinderberger J, Hagemeier C, Thauer RK, Gottschalk G (2008) The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. Proc Natl Acad Sci 105:2128–2133
34. Pyne ME, Utturkar S, Brown SD, Moo-Young M, Chung DA, Chou CP (2014) Improved draft genome sequence of *Clostridium pasteurianum* strain ATCC 6013 (DSM 525) using a hybrid next-generation sequencing approach. Genome Announc 2(4pii):e00790–14
35. Köpke M, Held C, Hujer S, Liesegang H, Wiezer A, Wollherr A, Ehrenreich A, Liebl W, Gottschalk G, Dürre P (2010) *Clostridium ljungdahlii* represents a microbial production platform based on syngas. Proc Natl Acad Sci U S A 107:13087–13092
36. Bruno-Barcena JM, Chinn MS, Grunden AM (2013) Genome sequence of the autotrophic acetogen *Clostridium autoethanogenum* JA1-1 strain DSM 10061, a producer of ethanol from carbon monoxide. Genome Announc 1(4pii):e00628–13
37. Liou JSC, Balkwill DL, Drake GR, Tanner RS (2005) *Clostridium carboxidivorans* species nov., a solvent-producing *Clostridium* isolated from an agricultural settling lagoon,

and reclassification of the acetogen *Clostridium scatologenes* strain SL1 as *Clostridium drakei* species nov. Int J Syst Evol Microbiol 55:2085–2091
38. Bruant G, Lévesque MJ, Peter C, Guiot SR, Masson L (2010) Genomic analysis of carbon monoxide utilization and butanol production by *Clostridium carboxidivorans* strain P7. PLoS One 5(9), e13033
39. Lal S, Ramachandran U, Zhang X, Sparling R, Levin DB (2013) Draft genome sequence of the hydrogen- and ethanol-producing bacterium *Clostridium intestinale* strain URNW. Genome Announc 1(5pii):e00871–13
40. Markowitz VM, Mavromatis K, Ivanova NN, Chen IM, Chu K, Kyrpides NC (2009) IMG ER: a system for microbial genome annotation expert review and curation. Bioinformatics 25:2271–2278
41. Hemme CL, Mouttaki H, Lee YJ, Zhang G, Goodwin L, Lucas S, Copeland A, Lapidus A, Glavina del Rio T, Tice H, Saunders E, Brettin T, Detter JC, Han CS, Pitluck S, Land ML, Hauser LJ, Kyrpides N, Mikhailova N, He Z, Wu L, Van Nostrand JD, Henrissat B, He Q, Lawson PA, Tanner RS, Lynd LR, Wiegel J, Fields MW, Arkin AP, Schadt CW, Stevenson BS, McInerney MJ, Yang Y, Dong H, Xing D, Ren N, Wang A, Huhnke RL, Mielenz JR, Ding SY, Himmel ME, Taghavi S, van der Lelie D, Rubin EM, Zhou J (2010) Sequencing of multiple clostridial genomes related to biomass conversion and biofuel production. J Bacteriol 192:6494–6496
42. Munir RI, Schellenberg J, Henrissat B, Verbeke TJ, Sparling R, Levin DB (2014) Comparative analysis of carbohydrate active enzymes in *Clostridium termitidis* CT1112 reveals complex carbohydrate degradation ability. PLoS One 9(8), e104260
43. Overbeek R, Fonstein M, D'Souza M, Pusch GD, Maltsev N (1999) The use of gene clusters to infer functional coupling. Proc Natl Acad Sci 96:2896–2901
44. Pati A, Ivanova NN, Mikhailova N, Ovchinnikova G, Hooper SD, Lykidis A, Kyrpides NC (2010) GenePRIMP: a gene prediction improvement pipeline for prokaryotic genomes. Nat Methods 7:455–457
45. Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Grechkin Y, Ratner A, Jacob B, Huang J, Williams P, Huntemann M, Anderson I, Mavromatis K, Ivanova NN, Kyrpides NC (2012) IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Res 40:D115–D122
46. Carver T, Harris SR, Berriman M, Parkhill J, McQuillan JA (2012) Artemis: an integrated platform for visualization and analysis of high throughput sequence-based experimental data. Bioinformatics 28:464–469
47. Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR, Stahl DA (1986) Microbial ecology and evolution: a ribosomal RNA approach. Annu Rev Microbiol 40:337–365
48. Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains archaea, bacteria, and eucarya. Proc Natl Acad Sci U S A 87:4576–4579
49. Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31:442–443
50. Hill JE, Penny SL, Crowell KG, Goh SH, Hemmingsen SM (2004) CpnDB: a chaperonin sequence database. Genome Res 14:1669–1675
51. Boucher Y, Douady CJ, Papke RT, Walsh DA, Boudreau ME, Nesbo CL, Case RJ, Doolittle WF (2003) Lateral gene transfer and the origins of prokaryotic groups. Annu Rev Genet 37:283–328
52. Jian W, Zhu L, Dong X (2001) New approach to phylogenetic analysis of the genus Bifidobacterium based on partial HSP60 gene sequences. Int J Syst Evol Microbiol 51:1633–1638
53. Hill JE, Seipp RP, Betts M, Hawkins L, Van Kessel AG, Crosby WL, Hemmingsen SM (2002) Extensive profiling of a complex microbial community by high-throughput sequencing. Appl Environ Microbiol 68:3055–3066

54. Verbeke TJ, Sparling R, Hill JE, Links MG, Levin D, Dumonceaux TJ (2011) Predicting relatedness of bacterial genomes using the chaperonin-60 universal target (cpn60 UT): application to *Thermoanaerobacter* species. Syst Appl Microbiol 34:171–179
55. Verbeke TJ, Dumonceaux TJ, Wushke S, Cicek N, Levin DB, Sparling R (2011) Isolates of *Thermoanaerobacter thermohydrosulfuricus* from decaying wood compost display genetic and phenotypic microdiversity. FEMS Microbiol Ecol 78:473–487
56. Brousseau R, Hill JE, Préfontaine G, Goh SH, Harel J, Hemmingsen SM (2001) *Streptococcus suis* serotypes characterized by analysis of chaperonin 60 gene sequences. Appl Environ Microbiol 67:4828–4833
57. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acids Res 42(Database issue):D633–D642
58. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
59. Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Pillay M, Ratner A, Huang J, Woyke T, Huntemann M, Anderson I, Billis K, Varghese N, Mavromatis K, Pati A, Ivanova NN, Kyrpides NC (2014) IMG 4 version of the integrated microbial genomes comparative analysis system. Nucleic Acids Res 42:D560–D567
60. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98
61. Felsenstein J (1993) Phylip (Phylogeny Inference Package) version 3.57c. Department of Genetics, University of Washington, Seattle. http://evolution.genetics.washington.edu/phylip.html
62. Saitou N, Nei M (1987) The neighbor-joining method – a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
63. Page RDM (1996) TreeView: an application to display phylogenetic trees on personal computer. Comput Appl Biosci 12:357–358
64. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Bryant SH (2015) CDD: NCBI's conserved domain database. Nucleic Acids Res 43(D):222–226
65. Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, Gavin OL, Gunasekaran P, Ceric G, Forslund K et al (2010) The Pfam protein families database. Nucleic Acids Res 38: D211–D222
66. Tatusov RL, Galperin MY, Natale DA, Koonin EV (2000) The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 28:33–36
67. Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, Yamanishi Y (2008) KEGG for linking genomes to life and the environment. Nucleic Acids Res 36:D480–D484
68. Haft DH, Selengut JD, White O (2003) The TIGRFAMs database of protein families. Nucleic Acids Res 31:371–373
69. de Hoon MJL, Imoto S, Nolan J, Miyano S (2004) Open source clustering software. Bioinformatics 20:1453–1454
70. Kanehisa M, Goto S, Kawashima S, Nakaya A (2002) The KEGG databases at GenomeNet. Nucleic Acids Res 30:42–46
71. Sauer U, Eikmanns BJ (2005) The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. FEMS Microbiol Rev 29:765–794
72. Carere CR, Rydzak T, Verbeke TJ, Cicek N, Levin DB, Sparling R (2012) Linking genome content to biofuel production yields: a meta-analysis of major catabolic pathways among select H_2 and ethanol-producing bacteria. BMC Microbiol 12:295
73. Li Y, Tschaplinski TJ, Engle NL, Hamilton CY, Rodriguez M Jr, Liao JC, Schadt CW, Guss AM, Yang Y, Graham DE (2012) Combined inactivation of the *Clostridium cellulolyticum*

lactate and malate dehydrogenase genes substantially increases ethanol yield from cellulose and switchgrass fermentations. Biotechnol Biofuels 5(1):2
74. Levin DB, Pitt L, Love M (2004) Biohydrogen production prospects and limitations to practical application. Int J Hydrogen Energy 29:173–185
75. Levin DB, Islam R, Cicek N, Sparling R (2006) Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. Int J Hydrogen Energy 31:1496–1503
76. Kalia VC, Lal S, Ghai R, Mandal M, Chauhan A (2003) Mining genomic databases to identify novel hydrogen producers. Trends Biotechnol 21:152–156
77. Kim DH, Kim MS (2001) Hydrogenases for biological hydrogen production. Bioresour Technol 102:8423–8431
78. Masukawa H, Mochimaru M, Sakurai H (2002) Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* species PCC 7120. Appl Microbiol Biotechnol 58:618–624
79. Hallenbeck PC, Benemann JR (2002) Biological hydrogen production; fundamentals and limiting processes. Int J Hydrogen Energy 27:1185–1193
80. Calusinska M, Happe T, Joris B, Wilmotte A (2010) The surprising diversity of clostridial hydrogenases: a comparative genomic perspective. Microbiology 156:1575–1588
81. Vignais PM, Magnin JP, Willison JC (2006) Increasing biohydrogen production by metabolic engineering. Int J Hydrogen Energy 31:1478–1483
82. Pilak O, Mamat B, Vogt S, Hagemeier CH, Thauer RK, Shima S, Vonrhein C, Warkentin E, Ermler U (2006) The crystal structure of the apoenzyme of the iron-sulfur cluster-free hydrogenase. J Mol Biol 358:798–809
83. Gardy JL, Laird MR, Chen F, Rey S, Walsh CJ, Ester M, Brinkman FSL (2005) PSORTb v. 2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. Bioinformatics 21:617–623
84. Gu Y, Ding Y, Ren C, Sun Z, Rodionov DA, Zhang W, Yang S, Yang C, Jiang W (2010) Reconstruction of xylose utilization pathway and regulons in *Firmicutes*. BMC Genomics 11:255
85. Liaw SH, Kuo I, Eisenberg D (1995) Discovery of the ammonium substrate site on glutamine synthetase, a third cation binding site. Protein Sci 4:2358–2365
86. Liaw SH, Pan C, Eisenberg D (1993) Feedback inhibition of fully unadenylylated glutamine synthetase from *Salmonella typhimurium* by glycine, alanine, and serine. Proc Natl Acad Sci U S A 90:4996–5000
87. Huang Y, Zhang L (2004) Rapid and sensitive dot matrix methods for genome analysis. Bioinformatics 20:460–466
88. Munir RI, Spicer V, Shamshurin D, Krokhin OV, Wilkins J, Ramachandran U, Sparling R, Levin DB (2015) Quantitative proteomic analysis of the cellulolytic system of *Clostridium termitidis* CT1112 reveals distinct protein expression profiles upon growth on α-cellulose and cellobiose. J Proteomics 125:41–53

Adv Biochem Eng Biotechnol (2016) 156: 113–138
DOI: 10.1007/10_2015_5002

Published online: 24 February 2016

Enzyme Systems of Anaerobes for Biomass Conversion

Riffat Munir and David B. Levin

Abstract Biofuels from abundantly available cellulosic biomass are an attractive alternative to current petroleum-based fuels (fossil fuels). Although several strategies exist for commercial production of biofuels, conversion of biomass to biofuels via consolidated bioprocessing offers the potential to reduce production costs and increase processing efficiencies. In consolidated bioprocessing (CBP), enzyme production, cellulose hydrolysis, and fermentation are all carried out in a single-step by microorganisms that efficiently employ a multitude of intricate enzymes which act synergistically to breakdown cellulose and its associated cell wall components. Various strategies employed by anaerobic cellulolytic bacteria for biomass hydrolysis are described in this chapter. In addition, the regulation of CAZymes, the role of "omics" technologies in assessing lignocellulolytic ability, and current strategies for improving biomass hydrolysis for optimum biofuel production are highlighted.

Keywords Biomass, CAZymes, Cellulolytic bacteria, Cellulosomes, Genetic manipulation

Contents

R. Munir and D.B. Levin (✉)
Department of Biosystems Engineering, University of Manitoba, Winnipeg, MB, Canada R3T 5V6
e-mail: david.levin@umanitoba.ca

Abbreviations

AA	Auxiliary activities
CAZy	Carbohydrate-active enzyme (database)
CAZymes	Carbohydrate-active enzymes
CBM	Carbohydrate binding module
CBP	Consolidated bioprocessing
CCR	Carbon catabolite repression
CE	Carbohydrate esterase
Doc	Dockerin domain
Fn3	Fibronectin type III domain
GH(s)	Glycoside hydrolase(s)
GT(s)	Glycosyl transferase(s)
Ig	Immunoglobulin (Ig)-like domain
kDa	Kilodalton
PA14	Conserved domain proposed to have a sugar-binding function
PL(s)	Polysaccharide lyase(s)
Sca	Scaffoldin
SLH	Surface-layer homology
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
TCS	Two component regulatory systems

1 Importance of Alternative Energy Sources

Increasing concerns about climate change coupled with high demands for fossil-based energy carriers have driven the search for alternative, abundantly available, sustainable, and biodegradable energy sources to meet the future needs of the transportation sector. Biofuels, which include bioalcohols (ethanol, butanol),

biogases (hydrogen, methane, syngas), and bio-oils (biodiesel, vegetable oils), are an attractive alternative to fossil fuels. In addition to being sustainable and environment friendly, they can be used as transport fuels with little or no alterations to present technologies [1]. Fossil-based energy resources, such as petroleum, coal, and natural gas, are responsible for about three quarters of the worlds' primary energy consumption, each corresponding to a world consumption of 33, 24, and 19%, respectively. Alternatives to fossil-based energy resources are nuclear power (5%), hydropower (6%), and biomass (13%), representing about one quarter of the world's primary energy consumption [2, 3]. The combustion of fossil fuels is by far the largest contributor to the increased levels of carbon dioxide observed in the atmosphere. Comparatively, conversion of biomass to bioenergy emits smaller amounts of greenhouse gases (carbon dioxide, methane, nitrous oxide. and various engineered chemicals including chloroflurocarbons), and the carbon dioxide generated is consumed during subsequent biomass re-growth [2].

Biomass, which includes microbes, plants, animals, and their organic waste products, is an abundantly available energy resource for the production of biofuels. It contains large amounts of the sugar polymers, cellulose and hemicelluloses, which can be broken down by a mixture of enzymes usually produced by microorganisms into simple sugars that are fermented to produce value-added products such as ethanol [3]. Perlack et al. [4] reported that, by 2030, over 1.3 billion dry tons of biomass could be available for large-scale bioenergy and biorefinery industries, enough to displace 30% or more of the current consumption of petroleum in the United States. Canada has vast amounts of biomass too, much of which remains unused. It is estimated that there may be enough unused biomass (agricultural wastes, mill wastes, unused tree branches) from Canada's forestry and farming operations to provide about 27% of Canada's energy needs. However, biomass remains an important contributor and currently provides about 4.7% of our primary energy needs, the second largest source of renewable energy after hydroelectricity [5].

2 Structure and Composition of Lignocellulosic Biomass

Plant-based lignocellulosic biomass is the most abundant renewable natural resource available for conversion to biofuels [1]. Terrestrial plants produce 1.3×10^{10} metric tonnes of wood per year on a worldwide basis. This is equivalent to providing approximately two-thirds of the world's energy requirements [6]. Lignocellulosic biomass is mainly composed of cellulose, hemicelluloses, and lignin. The long cellulose chains are held together by hydrogen bonds and tangled with hemicellulose molecules to form a highly crystalline structure, which is surrounded by lignin [7]. The relative composition of these polymers varies depending on the type, species, and source of the biomass, although cellulose is the predominant polysaccharide.

2.1 Cellulose

Cellulose, comprising on average about 41% of biomass, is the most abundant biopolymer found in nature. It is almost exclusively found in plant cell walls. Some animals, microalgae, and a few bacterial species, however, can also synthesize cellulose [8]. Cellulose is a highly recalcitrant substrate for enzymatic hydrolysis. It is a linear polymer consisting of up to 10,000 or more D-glucose molecules linked by β-1,4-glycosidic bonds. Because each glucose molecule is tilted by 180° towards its neighbour, the repeating unit of cellulose is cellobiose, a simple disaccharide. As a result, cellulose exhibits a high degree of polymerization [9]. Approximately 30 individual cellulose molecules are assembled into larger units known as fibrils. Cellulose molecules in fibrils are oriented in parallel and maintained together by inter-chain hydrogen bonds and van der Waals interactions between pyranose rings. Although individually the hydrogen bonds are relatively weak, collectively they become a strong associative force as the degree of polymerization increases [10]. The fibrils are in a *para*-crystalline state, which adds to the structural rigidity of cellulose and amorphous regions, which contain large voids for easy hydrolysis [11]. The presence of hemicellulose and lignin, however, restricts their accessibility to hydrolytic enzymes.

2.2 Hemicellulose

Hemicelluloses are an amorphous and heterogeneous group of branched polysaccharides composed of both hexoses and pentoses. D-Xylose and L-arabinose are the major constituents of pentosans (xylans), and D-glucose, D-galactose, and D-mannose are the constituents of hexosans (mannans). Hemicelluloses are composed both of neutral sugars and uronic acids. The sugars are all present as their respective polysaccharides, i.e. xylan, araban, glucan, galactan, and mannan (substituted with acetyl groups). Hemicelluloses constitute on average about 26% of hardwood, 22% of softwood, and 30% of various agricultural residues. Most hemicelluloses are built up by β-l,4-linkages between their backbone sugars. Although hemicelluloses are not digestible by animals, they can be fermented by yeasts and bacteria. Hemicellulose generally surrounds the cellulose fibers and forms a linkage between cellulose and lignin [12, 13].

2.3 Lignin

Lignin is a highly complex three-dimensional polymer of different aromatic phenylpropanoid units bound together by ether and carbon–carbon bonds. Lignin is concentrated between the outer layers of the fibers, leading to structural rigidity

and holding the fibers of polysaccharides together. Lignin content can range from 15% to 25% for most grasses and hardwoods, up to 40% for softwoods [14]. Lignin is much more resistant to microbial degradation than polysaccharides and other natural polymers [12, 13, 15] and is devoid of any sugars. Some fungal species have, however, been shown to decompose lignin [16].

3 Lignocellulosic Biofuel Production

The production of biofuel from cellulosic biomass (lignocellulosic biomass) involves the collection of biomass, deconstruction of cell wall polymers to release long-chain polysaccharides, specifically cellulose and hemicellulose, subsequent hydrolysis of these polysaccharides into their component 5- and 6-carbon sugars (pre-treatment and saccharification), and fermentative conversion of the sugars to biofuels [17]. The major obstacle in lignocellulose conversion and utilization is its crystallinity and resistance to hydrolysis. A variety of pre-treatment procedures have been evaluated for their effectiveness towards cellulose biodegradation. Chemical pre-treatments generally practiced involve sodium hydroxide, perchloric acid, peracetic acid, sulfuric and formic acids, ammonia freeze explosion, and the use of organic solvents. Steam pre-treatment strategies have also been effectively used to loosen the cellulose, hemicellulose, and lignin complex [18–20]. These treatment methods, however, tend to be expensive, slow, and relatively inefficient, and produce enzyme inhibitors such as furfural and 5-hydroxymethylfurfural which decrease the overall efficacy of the fermentation process [21].

Biological strategies for degrading lignocellulose include the use of bacteria such as *Bacillus* and *Clostridium* and a variety of fungi. These microorganisms have the ability to attack the non-cellulosic substances and extract the cellulose fibers [22]. Lignocellulose degradation by a combination of enzymes such as pectinases, hemicellulases, and cellulases are particularly attractive and are generally used with a pre-chemical treatment. Biomass treatment using enzymes have potential advantages such as low cost, low-energy input and high yields without generating polluting by-products. The long treatment time and degradation of the resulting carbohydrates are, however, drawbacks of such processes.

The strategies currently employed to produce biofuel from cellulosic biomass utilize simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and co-fermentation (SSCF) techniques [23]. SSF consolidates hydrolysis and fermentation of cellulose hydrolysis products into one process step, with cellulase production and fermentation of hemicellulose hydrolysis products occurring in two additional discrete process steps. SSCF, on the other hand, involves two process steps: enzyme production and a second step in which hydrolysis and fermentation of both cellulose and hemicellulose hydrolysis products occurs. Both SSF and SSCF require extensive cellulosic feedstock pre-treatment strategies, and the addition of exogenous cellulolytic enzymes to hydrolyse cellulose into simple sugars for fermentation. This accounts for a large portion of the cost

involved in biofuel production [24]. Potential single-step conversion of biomass to biofuels afforded by consolidated bioprocessing (CBP) (third generation biofuels) would provide an alternative cost effective cellulose processing strategy, in which enzyme production, substrate hydrolysis, and fermentation are all carried out in a single-step process by microorganisms that express both cellulolytic and hemicellulolytic enzymes [1, 6]. CBP is considered a potential breakthrough for saving the cost of investing in a multistep process and in expensive pre-treatment steps. In addition, the use of costly enzymatic cocktails for saccharification of the biomass can potentially be minimized or eliminated [25, 26]. It is estimated that CBP can reduce the cost of biomass processing to biofuel by 41% [25]. Successful implementation of CBP, however, requires bioprospecting for microorganisms capable of efficient biomass hydrolysis and biofuel production, an understanding of the metabolism of cellulolytic bacteria, and the development of novel microorganisms into industry-relevant microbes as monocultures or as co-cultures.

4 Microorganisms Involved in the Degradation of Cellulosic Biomass

In nature, cellulosic biomass is degraded by a variety of fungi, archaea, and bacteria that utilize it as a carbon source. These microorganisms produce a complex combination of hydrolytic enzymes (cellulases, hemicellulases, and pectinases) which act synergistically to break down cellulose and its associated cell wall components [27]. Cellulolytic microorganisms can be found in all biota where cellulosic waste accumulates (soil, sewage, compost, wood, mud, termite gut, rumen, etc.). They usually occur in mixed populations comprising cellulolytic and non-cellulolytic species which often interact synergistically. These interactions lead to the complete degradation of cellulose. Cellulolytic organisms mainly exist within the phyla Actinobacteria, Bacteroides, Fibrobacteres, Firmicutes, Proteobacteria, Spirochaetes, and Thermotogae. The vast majority (80%) of these species, however, are found within the Phyla Actinobacteria and Firmicutes. Of these, the Gram-positive cellulolytic bacteria are found within the Firmicutes and belong to the Class Clostridia and Genus *Clostridium* [28].

Among aerobic cellulolytic soil bacteria, several species belonging to the genera *Cellulomonas, Pseudomonas, Thermomonospora,* and *Microbispora* have been studied in detail [11]. Generally, in aerobic conditions, cellulose is converted into water and carbon dioxide. In anaerobic cellulose degradation, ethanol and hydrogen are also produced. *Clostridium thermocellum*, an anaerobic, thermophilic, cellulolytic bacterium, has been extensively considered for potential industrial applications in biofuel production through CBP [1, 26]. *Fibrobacter succinogens, Ruminococcus albus,* and *R. flavifaciens* are considered as the primary organisms responsible for degradation of plant cell walls in the rumen. These bacteria have been shown to harbor a complete set of polysaccharide-degrading enzymes [29].

5 Enzyme Systems Involved in the Degradation of Plant Cell Walls

Converting cellulosic biomass in plant cell wall substrates is of crucial importance for the carbon cycle and for economic success. Bacteria and fungi that decompose plant cell wall polysaccharides efficiently employ a multitude of intricate enzymes, otherwise known as carbohydrate-active enzymes (CAZymes) [30]. These highly specialized enzyme systems include the cellulases, hemicellulases, and other related glycoside hydrolases (GHs), as well as the polysaccharide lyases (PL), glycosyl transferases (GT), carbohydrate esterases (CE), enzymes with auxiliary activities (AA), and carbohydrate binding modules (CBM). The various CAZymes are classified into families based on the amino acid sequence and consequent fold of the protein. A complete list of all the CAZymes so far identified, and their respective function, is available in the CAZy database [31]. Currently, the GHs form 133 different families and membership of a given enzyme in a GH family provides insight into its structural features, its evolutionary relationship with enzymes in a family, and its mechanism of action. In addition, the GTs are divided into 97 families, the PLs into 23, the CEs into 16, the AAs into 13, and the CBM families currently number 71. These enzymes act synergistically to break down cellulose and its associated cell wall components. Although CAZymes represent a significant proportion of protein-encoding genes in any given genome [31], the number and types of extracellular GHs produced by biofuels producing Firmicutes indicate the extent of their lignocellulose hydrolysis capabilities.

5.1 Glycoside Hydrolases (GHs)

The glycoside hydrolases are a large group of enzymes which hydrolyse the glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. GHs are classified into families based on amino acid sequence similarities and the consequent fold of protein [30]. Figure 1 shows a schematic structure of a hypothetical Clostridial glycoside hydrolase. Many extracellular GHs of the anaerobic bacteria have a modular structure: that is, they consist not only of a catalytic module but also of a complex arrangement of different modules. The enzymes may have one or even more than one catalytic module (s) and can be accompanied by one, several, or all of the following modules: carbohydrate binding (CBM), immunoglobulin (Ig)-like, dockerin (Doc), fibronectin type III (Fn3), and surface-layer homology (SLH). These modules have independent folding units often covalently linked by flexible links of amino acids. Because of the presence of several modules, these enzymes are often quite large, and may consist of more than 1,000 amino acids with a molecular mass of more than 100 kDa.

Fig. 1 Schematic representation of the modular structure of a hypothetical Clostridial glycoside hydrolase. Many extracellular GHs of the anaerobic bacteria consist of one or more catalytic modules accompanied by one, several, or all of the following modules: carbohydrate binding (CBM), immunoglobulin (Ig)-like module, dockerin (Doc), fibronectin type III (Fn3), and Surface-layer homology (SLH)

In general, the non-catalytic modules may support, or even modulate, the activity of the catalytic modules, which are responsible for hydrolysis of the glycosidic bonds. Functionally, the most important and best-characterized non-catalytic module in the extracellular enzymes of the Clostridia is the CBM. The function of the Doc module is discussed in the cellulosome section below. The function of Ig has not yet been successfully addressed and very little is known about the function of the Fn3 module. Earlier it was reported that the Fn3 modules serve as spacers or linkers, allowing optimal interaction between the catalytic modules and the CBM [32, 33]. However, Kataeva et al. [34] used scanning electron microscopy and X-ray diffraction studies, to show that the two Fn3 modules of the multi-modular cellobiohydrolase CbhA of *C. thermocellum* are able to modify the surface of cellulose that had been loosened up and crenulated, and thus promote hydrolysis by the catalytic domain.

5.2 Carbohydrate Binding Modules (CBMs)

The CBMs are proteins of about 35–180 amino acid residues that are generally appended to glycoside hydrolases that degrade insoluble polysaccharides. CBMs guide the enzymes to suitable areas on the surface of the substrate where they are most active. This results in increased concentrations of the enzyme on the substrate surface and improves substrate interaction [35]. As with GHs, the CBMs are divided into families based on their amino acid sequence similarity [35]. Not all CBMs bind cellulose, as many families contain starch-, chitin-, xylan-, or mannose-binding domains. Aerobic fungi bear CBMs from family 1, which are very small (about 35 amino acid residues). The important CBMs of bacterial cellulases are often from family 2 or 3, which are much larger, comprising approximately 100–150 amino acid residues, respectively. All cellulose binding CBMs, despite their size differences, exhibit a strip of conserved aromatic amino acid residues located on a relatively flat surface [36]. These align with the hydrophobic side of the glucose rings along the length of a single cellodextrin on the cellulose surface, thus allowing binding between substrate and the CBM appended parent enzyme, the cellulosome, or the entire microbial cell (in cases where the

CBMs have lectin-like specificity and bind to the cell surface via cell surface glycans) [35].

5.3 Surface Layer Homology Domain (SLH)

SLH modules are found in proteins from phylogenetically unrelated bacteria, e.g., Gram-positive and Gram-negative bacteria. These modules are present in three types of proteins: surface-layer (S-layer) proteins, extracellular enzymes/proteins, and outer membrane proteins [37]. The SLH module in most cases is present in three copies of about 50–60 residues each. Even though the overall similarity of SLH modules in proteins from different organisms is low, they contain at least two highly conserved motifs, an FxDV motif at the N-terminus and a TRAE motif at the C-terminus [38, 39].

In S-layer and outer membrane proteins, the SLH modules are generally located at the N-terminus and in enzymes at the C-terminus. Their role in serving as an anchor to the cell wall for the different protein types has been widely explored [40, 41]. Although it was originally proposed that SLH modules bind to peptidoglycan, it is now evident that the secondary cell wall polymer, teichuronic acid, serves as the anchoring structures for SLH modules in the Gram-positive cell wall [42]. The SLH-mediated anchoring mechanism is one of several, but highly conserved strategies bacteria have developed to display proteins on their surface. In Clostridia species, SLH modules have been found in several glycoside hydrolases (e.g., cellulases, xylanases, amylase-pullulanases) [43, 44]. Generally, enzymes can be attached to the cell wall via SLH modules either directly, mediated by a linker protein, or as part of a multienzyme complex [38].

6 Mechanisms of Cellulose/Hemicellulose Hydrolysis

To thrive in their environment, cellulolytic microorganisms have developed several strategies for biomass hydrolysis. These organisms secrete non-complexed (hemi) cellulolytic glycoside hydrolases as single enzymes or enzymes with multiple catalytic sites (free enzymes) and as enzymes in extracellular multi-enzyme complexes (complexed enzymes). Enzymes with multiple catalytic sites have multifunctional properties, distinct CBMs, and exist in both free and cellulosomal enzyme systems. The presence of two different enzymes on the same polypeptide chain may suggest concerted action on a given portion of lignocellulolytic substrate. Based on their primary catalytic domains, four classes have been identified and are reviewed in detail in Himmel et al. [45]. These include: (1) cellulase-cellulase systems which have been identified in *Anaerocellum thermophilum* (renamed *Caldicellulosiruptor bescii*) [46], and may include two or more cellulases such as the catalytic GH5, GH6, GH9, GH48, and other ancillary modules;

(2) hemicellulase-hemicellulase systems which comprise two or more modules of hemicellulases, GH10, GH26, GH43, and GH54, and CBMs 6, 22, and 30, which are related to the binding of various hemicelluloses – this type of enzyme system has been identified in *Caldicellulosiruptor* spp.; (3) cellulase-hemicellulase systems which constitute, in addition to CBMs 3 and 30 capable of binding both to cellulose and hemicellulose, a mixture of cellulase catalytic modules (GH9 and GH48) and hemicellulase catalytic modules (GH10 and GH44) – this type of multicomponent enzyme system has been described in a number of cellulolytic bacteria including *C. thermocellum* [47]; and (4) hemicellulase-carbohydrate esterase systems, which consist of hemicellulase catalytic modules (GH5, GH10, GH11, GH43, and GH53) and carbohydrate esterase modules (CE1, 2, 3, and 4), as well as CBMs 3, 6, and 22. One of the *C. thermocellum* cellulosomal enzymes has a combination of xylanase and CE1 feruloyl esterase on the same polypeptide chain [47].

6.1 *Free Enzyme Systems: Non-Complexed Glycoside Hydrolases*

In cellulolytic bacteria, all cellulose hydrolysing enzymes (the cellulases) hydrolyse the same type of bond of the cellulose chain, i.e., the β-1,4 glycosidic bond, but they use different modes of action. Based on their mode of catalytic action, these enzymes have been classified into three distinct classes: (1) endo-β-(1,4)-glucanases (endoglucanases) – EC 3.2.1.4 – these randomly hydrolyse the amorphous region of the fibrils generating oligosaccharides of various lengths and creating reducing and non-reducing ends; (2) exo-β-(1,4)-glucanases (cellobiohydrolases) – these act on reducing and non-reducing ends of cellulose, liberating glucose, cellobiose or other cellodextrinases – this group includes both cellobiohydrolases (EC 3.2.1.91) which liberates cellobiose in a processive manner from β-1,4-glucans, and cellodextrinases (EC 3.2.1.74), which liberate D-glucose from β-1,4-glucans; and (3) β-glucosidases (EC 3.2.1.21) which act to release D-glucose units from cellobiose and soluble cellodextrins, as well as other glycosides.

Structurally, all endoglucanases have open active sites which are cleft-like in topology. This is probably why a cellulose chain can be accessed in a random fashion by endoglucanases, and bond cleavage can occur anywhere along the cellulose chain [48]. In contrast, exoglucanases have a tunnel shaped active site, formed by long loops of protein molecule that fold over the active residues [49]. As with CBM domains, the active sites contain aromatic residues, usually tryptophans, which stack against the glucose residue. Consequently, once a cellulose chain is bound, it is fed into one end of the tunnel through the active site, and subsequently cleaved inside the tunnel to release cellobiose product from the other end [50]. Successive cleavage events continue processively in a unidirectional manner until the entire strand is hydrolysed [51].

It has been shown by Li et al. [52] that some endoglucanases, such as some members of the GH9 family, can exhibit a processive action on the substrate. These enzymes contain a subfamily of CBM 3, termed CBM3c, which is fused tightly to the catalytic module via a characteristic linker segment. The GH9-CBM3c complex works in coordination, where the CBM3c feeds a single cellulose chain into the active site cleft of the endoglucanase, thereby modifying its character from a simple endoglucanase to an endoglucanase which acts successively to hydrolyse the entire chain.

Unlike microbial degradation of cellulose, bacteria and fungi produce many different types of enzymes (hemicellulases) which act efficiently to break down various types of hemicellulose. Hemicellulases can be grouped into three types: (1) endo-acting enzymes, which attack polysaccharide chains internally – these enzymes exhibit very little activity on short oligomers; (2) exo-acting enzymes which, as in exoglucanases, tend to act processively from either the reducing or non-reducing ends and have a preference for shorter chains; and (3) accessory enzymes, which include a variety of acetyl esterases, lyases, and esterases such as coumaric acid esterase and ferulic acid esterase, which hydrolyse lignin-linked glycoside bonds [53].

The complex nature of hemicellulose structures requires a high degree of coordination between the enzymes involved in hemicellulose degradation. In the case of xylan hydrolysis, for example, microbial enzymes act cooperatively to convert it into simple sugars. These enzymes include β-1,4-endoxylanase which cleaves internal glycosidic bonds within the xylan backbone, α-L-arabinofuranosidase which hydrolyses arabinose side chains, α-glucuronidase which removes glucoronic acid side chains from the xylose units, acetyl-xylan esterase which releases acetate groups, and β-xylosidase which hydrolyses xylobiose to xylose. Ferulic and *p*-coumaric acids are removed by phenolic acid esterases [54]. Even though the structure of xylan is more complex than cellulose and a large number of different enzymes are required for efficient hydrolysis, the polysaccharide does not form tightly packed structures and is thus more accessible to hydrolytic enzymes. Consequently, the specific activity of xylanases is considered to be 2–3 orders of magnitude greater than for cellulase hydrolysis of cellulose [55].

Multifunctional xylanolytic enzyme systems are quite widespread among fungi [56, 57], actinomycetes [58], and bacteria [59]. Various anaerobic bacteria belonging to the Firmicutes have been studied for their ability to produce a number of hemicellulases on a variety of substrates. Some examples include *Clostridium cellulolyticum* [60, 61], *Clostridium cellulovorans* [62, 63], *Cadicellulosiruptor* species [64], *C. thermocellum* [47, 65], *Clostridium termitidis* [66], *Clostridium papyrosolvens* [67], and *Butyvibrio fibrisolvens* [68].

6.2 Cellulosomes: Complexed Enzyme Systems

Early studies on the cellulolytic system of the anaerobic thermophilic bacterium *C. thermocellum* revealed that true cellulase activity was part of a large multienzyme complex termed the cellulosome [69]. More recently, a range of anaerobic bacteria and fungi were shown to produce cellulosome systems similar to those of *C. thermocellum*, particularly the bacteria *C. cellulovorans, C. cellulolyticum*, *Clostridium acetobutylicum*, *Clostridium josui*, *C. papyrosolvens, Acetivibrio cellulolyticus, Bacteroides cellulosolvens, R. albus, Ruminococcus flavefaciens*, *C. termitidis* [67, 70–79], and the anaerobic fungi of the genera *Orpinomyces, Piromyces,* and *Neocalimastix* [80–82]. The genome sequences of some of these are already known, and others will follow soon. Sequence analysis provides a better view of the molecular components of the cellulosome of each organism.

Cellulosomes harbor a large variety of lignocellulolytic enzymes such as cellulases, hemicellulases, carbohydrate esterases, polysaccharide lyases, lichenases, pectinases, chitinases, etc., which have activity against various components of lignocellulosic biomass [47, 65, 83, 84]. Members of the same CAZy families of cellulases and hemicellulases involved in the free enzyme systems of bacteria also serve as cellulosomal enzymes, except GH6 enzymes, which occur both in fungi and bacteria but have not been found in native cellulosome systems [47]. Complexed enzyme systems are ecologically advantageous for cellulolytic bacteria because hydrolysis by cellulosomes requires direct attachment of the cell-bound cellulosome to cellulosic substrate, thereby minimizing diffusion of soluble cello-oligosaccharides into the environment and ensuring efficient uptake of hydrolysis products into the cell. Furthermore, cellulosomes promote synergism of enzymatic components and increase competitiveness of substrate utilization in the natural environment [26].

The cellulosome of *C. thermocellum* (Fig. 2) is perhaps the most extensively studied and can be seen microscopically as protuberances of the outermost layer of the cell envelope. The protuberances elongate and form filamentous protractions tethering the cells to the substrate. Cellulosomes may act as contact corridors enabling diffusion of soluble degradation products from the cellulose substrate into the cell. Cellulosomes can, however, detach from the cells in the latter part of the growth phase and hydrolyse the substrate independently [85]. In general, the multienzyme cellulosome complex is composed of two major subunits: the non-catalytic scaffoldin (cipA) and the catalytic enzymes. The scaffoldin contains functional modules that carry out various activities. These modules include a single CBM and varying numbers (1 to 11, but usually higher than 4) of type 1 cohesion domains which interact with multiple cellulosomal enzymes [85].

In many ways, cellulosomal enzymes are very similar to their free counterparts except that they possess an additional domain called dockerin type 1 which binds tightly with the type 1 cohesion of the scaffoldin, thereby governing the assembly of the complex [86]. Dockerin modules are usually present in a single copy at the C-terminus of cellulosomal enzymes. Significantly, cohesin domains in any given

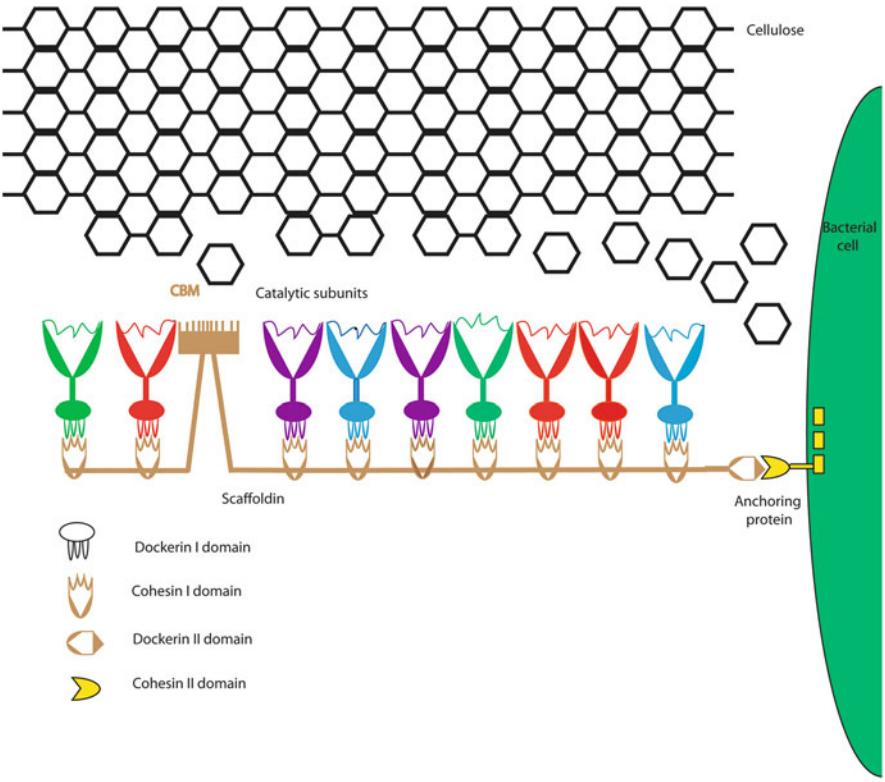


Fig. 2 Diagrammatic representation of the cellulosome components of *C. thermocellum* [79]. Enzymatic components (colored differently to indicate enzyme variety) produced by anaerobic bacteria contain a dockerin domain. Dockerin domains bind to the cohesin domains of a non-catalytic scaffoldin, providing a mechanism for cellulosome assembly. Scaffoldins also contain a cellulose-specific family 3 CBM (cellulose binding module) and a C-terminal dockerin domain II that targets the cellulosome to cellulose and the bacterial cell envelope, respectively

scaffoldin in a particular *Clostridium* species are unable to discriminate between the dockerins present in the various cellulosomal enzymes [87], suggesting that cellulosome complexes may comprise a different ensemble of catalytic subunits influenced by the induction of specific genes by the type of substrate present. This is, however, not true between species, where the dockerin domains of one organism do not cross-react with the cohesion domains of another, suggesting organism-dependent dockerin cohesion specificity/interactions [88].

The interaction of the cellulosome complex with cellulose is mediated by the scaffoldin-borne CBM, which serves to deliver the entire complement of cellulosome enzymes collectively to the lignocellulosic substrate. The attachment of the cellulosomes to the cell surface is, in most cellulosome producers, mediated by type II dockerin domains located on the scaffoldin. Type II dockerin domains do not bind to type 1 cohesions, but instead interact with the complementary type II

cohesions of cell surface anchoring proteins [10]. The majority of the anchoring proteins (encoded by Orf2, SdbA, OlpB, and OlpA in *C. thermocellum*) are non-covalently bound to the peptidoglycan cell wall via repeats of SLH modules, thereby incorporating the cellulosomes into the bacterial cell surface [1]. Anchoring proteins OlpA and OlpC do not have a type II cohesion domain, and may allow direct adherence of dockerin-containing cellulosomal enzymes via the single C-terminal cohesion I domain [47]. Anchoring scaffoldin Cthe_0736 harbors more than one type II cohesion domain and appears to contribute to the assembly of polycellulosomes which may contain up to 63 catalytic subunits. Lack of SLH domain in Cthe_0736 suggests the formation of extracellular cell-free complexes [89]. In the case of *C. cellulovorans*, there is no evidence for the presence of type II dockerin domains. However, a cellulosomal cellulase EngE was found to contain both a dockerin domain that binds the scaffoldin, and three repeated SLH domains, which are thought to contribute to the anchoring of the complete cellulosome on to the bacterial cell surface [83]. Similarly, analysis of the *C. cellulolyticum* genome has so far failed to identify genes that may encode any cellulosome anchoring proteins [90, 91].

Genome sequencing and biochemical analyses have provided novel information on the architecture and components of cellulosome systems from different cellulolytic bacteria. This has enabled bacterial cellulosomes to be classified into two types: (1) those that present multiple types of scaffoldin, such as those of *C. thermocellum* and (2) those that contain a single scaffoldin, which is characteristic of most mesophilic Clostridia. Furthermore, the cellulosomes produced by *Acetovibrio cellulolyticus, Bacteroides cellulosolvens,* and *R. flavefaciens* are of a more complex nature and differ in organization from the commonly known cellulosomes of *C. thermocellum and C. cellulolyticum*.

Even though the cellulosomes of *B. cellulosolvens* and *C. thermocellum* are similar in architecture and are able to form polycellulosomes, the primary scaffoldin in *B. cellulosolvens* contains type II cohesions and a type I dockerin, whereas the anchoring scaffoldin contains type I cohesins. It is, however, unclear whether the reverse disposition of type I and type II cohesins has any biological significance [73]. In the case of *A. cellulolyticus,* two distinct cellulosome systems with three different cohesin-dockerin specificities have been identified. The two systems are able to bind up to 113 enzymes at any given time [74]. The cellulosome system of *R. flavefaciens* strain FD-1 is probably the most intricate, and potentially versatile cellulosomal complex described to date. Of the five structural cellulosome components, three – ScaA, ScaB, and ScaE – have cohesion modules that are phylogenetically distinct from the previously described type I and type II cohesins and are classified into type III cohesins [92]. The functional significance for the large array of scaffoldins in different cellulolytic bacteria, which leads to a highly complex cellulosome structures, is currently unclear. Significantly, cellulosome structural organization varies between strains, and this cellulosome heterogeneity may reflect the complexity and diversity of the lignocellulosic substrates found in different ecological environments.

6.3 *Attachment of GHs and Cellulosomes to the Bacterial Cell Surface*

In addition to the cohesion dockerin interactions of the cellulosome described above, some individual enzymes can interact non-covalently either with the peptidoglycan layer or with secondary cell wall polymers of the bacterial cell surface via a C-terminal SLH module [93]. This phenomenon has been observed in at least two *C. thermocellum* enzymes, a xylanase and a lichenase [43, 94]. Single and multidomain GHs from several other plant cell wall-degrading bacteria such as *C. stercorarium, C. termitidis,* and *C. josui* have also been known to carry SLH domains for attachment to the cell [79, 95, 96].

In *R. flavefaciens*, the cellulosome is attached covalently to the cell surface via the scaffoldin ScaE, which contains a sortase signal motif at its C-terminus. Similar sortase signal motifs have been identified in other cell surface proteins and in at least one GH (GH10 xylanase) from this cellulose degrader. This may represent a more common mode of enzyme attachment to the bacterial cell surface [92].

CBMs, classified as family 37, have recently been discovered and implicated in the non-covalent attachment of cell wall-degrading enzymes to the bacterial cell surface in the rumen bacterium *R. albus* strain 8 [97]. Similarly, in the soil bacterium *Amycolatopsis orientalis,* the CBM35 component of *exo*-D-glucosaminidase, CsxA, has been shown to anchor the enzyme to the bacterial cell wall via its capacity to bind uronic acid sugars [98]. In the case of *R. albus*, the draft genome sequence shows that at least 40 proteins contain CBM37, with half of them classified as putative carbohydrate active enzymes [97]. Even though no cohesion domains and scaffoldin have been identified in this bacterium, many of the GHs do contain dockerin domains. If *R. albus* populates the rumen in multiple strains, as is the case with *R. flavefaciens,* then it is quite possible that this strain may produce and secrete various dockerin-bearing proteins while other strains produce complimentary cohesion-containing scaffoldin, thus providing a synergistic action in degrading plant cell walls [45].

7 Role of Proteomic and Transcriptomic Studies in Assessing Lignocellulolytic Ability

While genome sequencing studies have identified genes encoding a diversity of CAZymes in a variety of organisms, the presence of a gene does not warrant actual production of protein, nor does it adequately indicate the expression profile of the genes under a given condition. In the last decade, "omics"-based technologies such as proteomics and transcriptomics have progressed rapidly and have been applied to elucidate mechanisms of biomass destruction by examining the expression and dynamics of complexed and non-complexed CAZymes in different microbes under different substrate conditions [47, 60, 99, 100]. This is important because, in

addition to bioprospecting and identifying strains with maximum lignocellulose hydrolytic capability, which are potentially useful in CBP, understanding the expression of various glycoside hydrolases under various conditions may help to identify specific limitations that can be resolved through targeted strategies.

Proteomic analysis was conducted to assess quantitative alterations in the expression patterns of catalytic subunits within cellulosomes of *C. thermocellum* grown on either α-cellulose or cellobiose [101]. Of the 41 cellulosomal proteins detected, 16 new subunits were identified. Varying differences were observed in protein expression from cells grown on the two substrates. However, the glycoside hydrolase (GH) family 9 was the most abundant group of enzymes when cells were grown on cellulose, while hemicellulases were the most abundant group on cellobiose. Proteomic studies were similarly employed to examine the cellulosome composition of *C. thermocellum* ATCC 27405 grown on a variety of carbon sources, dilute-acid pretreated switch grass, cellobiose, amorphous cellulose, crystalline cellulose (Avicel), and combinations of crystalline cellulose with pectin or xylan (or both) [47].

These quantitative results suggest a coordinated substrate-specific regulation of cellulosomal subunit composition in *C. thermocellum* to suit better the organism's needs for growth under specific carbon source conditions. Interestingly, xylanases were expressed highly on cellobiose and showed decreased expression during growth on pretreated switchgrass, which contains xylan relative to growth on cellulose. While this suggests xylan-independent expression of xylanases, the increased expression of cellulases observed on polymeric cellulose compared to cellobiose suggests cellulase production is connected to the presence of cellulose and not its hydrolysis products. In contrast to expression in *C. thermocellum*, endo- and exo-glucanases in *C. obsidiansis* secretomes showed increased expression on cellobiose relative to cellulose grown cells, suggesting that cellulose hydrolysis products induce rather than repress cellulases [102]. This indicates that significant differences exist in hydrolytic mechanisms of different Firmicutes, which need to be thoroughly understood before industrial processes should be developed.

More recently, the transcriptional profiles of *C. thermocellum* grown on cellobiose and pretreated yellow poplar indicated increased expression of 47 cellulosomal protein encoding genes which included, among others, both cellulases and hemicellulases on pretreated yellow popular compared to cellobiose. In addition to other genes, genes for glycosidase regulation were also identified and up-regulated, which could be important for studying regulatory mechanisms in this organism [100]. Furthermore, the secretomes of various bacteria and fungi have been analysed to examine secreted lignocellulolytic enzymes [64, 103, 104]. Secretome analysis of *C. bescii* and *Caldicellulosiruptor obsidiansis* during growth on crystalline cellulose identified more than 400 proteins, of which the most abundant were multi-domain glycosidases belonging to the GH family 5, 9, 10, 44, or 48. In addition to their orthologous cellulases, the organisms expressed unique glycosidases with different domain organizations: *C. obsidiansis* expressed the COB47_1671 protein with GH10/5 domains whereas *C. bescii* expressed the Athe_1857 (GH10/48) and Athe_1859 (GH5/44) proteins [64].

"Omics" technologies have similarly been used to examine expression and regulation of GH production in fungal species [105]. The transcriptomes of the softwood-degrading white-rot fungus *Phanerochaete carnosa* were evaluated to identify enzymes capable of reducing recalcitrance of softwood resources. Of the 30 transcripts that were on average over 100 times more abundant during growth on wood than on nutrient medium, 5 were cellulases and 2 were hemicellulases. Overall, transcripts predicted to encode lignin-degrading activities were more abundant than those predicted to encode carbohydrate-active enzymes [106]. Coupling enzymes with different functions and specificities from divergent organisms through genetic engineering or through co-culturing is a promising potential strategy to improve lignocellulosic biomass hydrolysis.

8 Regulation of Carbohydrate-Active Enzyme Synthesis

The production of extracellular CAZymes is an energy-consuming process. These enzymes are therefore only produced when the bacteria needs to use cellulosic substrates as an energy and carbon source – so called induced expression. Studies in *C. thermocellum* have shown that cellulase synthesis and the assembly of cellulosomes, is inhibited in the presence of soluble sugars such as cellobiose compared to growth on crystalline cellulose. This suggests that the expression of cellulolytic enzymes is controlled by a carbon catabolite repression (CCR) mechanism [1, 107, 108] where CCR-related regulators sense intracellular glucose or cellodextrin levels and control CAZyme expression. A similar phenomenon was observed in *C. acetobutylicum, C. cellulolyticum,* and *C. cellulovorans*, where cellulases were repressed under glucose and de-repressed upon glucose exhaustion [63, 109, 110].

Additionally, a number of membrane-associated anti-sigma factors, and two-component regulatory systems (TCSs) have been implicated in extracellular carbohydrate sensing and CAZyme gene regulation in cellulolytic Clostridia. Kahel-Raifer et al. [111] identified several putative bicistronic operons in the genome of *C. thermocellum*, with each encoding an anti-sigma factor, which senses the presence of various polysaccharides in the extracellular environment via its CBM, GH10, GH5, and PA14 domains [112], and an alternative sigma factor which mediates the intracellular activation of appropriate CAZyme genes. Furthermore, TCSs have been implicated in inducing the regulation of both cellulosomal (*xyl-doc* cluster) and non-cellulosomal CAZymes and associated transporters in *C. cellulolyticum* [60]. From an ecological point of view, environment sensing coupled with CAZyme expression may be crucial for cellulose utilization in order to survive in the natural competitive environment.

9 Improving Lignocellulose Hydrolysis and Biofuel Production by Genetic Manipulation

Complete understanding of the genomic sequences of cellulolytic microorganisms coupled with proteomics, transcriptomics, and metabolomics is important to improve/increase the production of lignocellulolytic enzymes and desired end products such as ethanol through metabolic engineering. Blocking undesirable pathways, gene knockout experiments, and over-expression of genes associated with desirable pathways have been applied to both cellulolytic and non-cellulolytic organisms for enhanced substrate hydrolysis and production of desired products. Some examples include the expression of cellulases in *Saccharomyces cerevisiae* [113], construction of ethanologenic *Escherichia coli* KO11 and *Klebsiella oxytoca* M5A1 by the integration of ethanol producing *Zymomonas mobilis* genes [114], improved cellulase production and xylanase expression by the deletion of *Cre1* and *ACE1* genes from *Trichoderma reesi* [115, 116], expressing bacterial cellulase genes efficiently in other microbial systems such as *Penicillium crysogenum, T. reesei, Pseudomonas fluorescens,* and yeast [18], metabolic engineering of *Corynebacterium glutamicum* to broaden its lignocellulosic substrate utilization for the production of fermentable sugars, and construction of recombinant *C. glutamicum* strains by cloning the *xylA* and *xylB* genes encoding xylose isomerase from *E. coli* to enable the utilization of xylose as a carbon source [117].

S. cerevisiae is industrially used for the production of bioethanol from glucose. Its inability to ferment five carbon sugars led to the importation of genes for xylose metabolism [118]. Ha and colleagues [119] successfully engineered yeasts to co-ferment mixtures of xylose and cellobiose simultaneously. These strains exhibited improved ethanol yield when compared to fermentation with either cellobiose or xylose as sole carbon sources. Similarly, in an effort to improve xylose fermentation capacity of *S. cerevisiae*, several groups investigated the effects of importation of xylose isomerase genes from *E. coli* and *Piromyces* specie E2 [120–123]. While *E. coli* xylose isomerase (encoded by xylA) had little activity in yeast, the two-enzyme pathway (xylose to xylulose) using *Piromyces* xylose isomerase enzyme resulted in yeast strains with higher ethanol yields.

Biobutanol, an attractive alternative biofuel, is produced as part of a natural process which is catalysed by *C. acetobutylicum* [124]. Because it is formed as a mixture with acetone and ethanol, the resulting yield is quite low. Genes involved in an alternative butanol pathway were cloned into *E. coli*, allowing the production of reasonable amounts of butanol by an aerobically grown microorganism possessing the genes of a strict anaerobe, *C. acetobutylicum* [125].

During the past several years, progress has been made towards the development of "designer cellulosomes" which have shown enhanced activity on complex substrates [126–128]. Cellulosome chimeras or "mini cellulosomes" have been produced that contain two or more cohesins of different specificities which anchor different dockerin-containing enzymes in precise locations. Wieczorek and Martin [129] reported the successful display of cellulosome-inspired recombinant

complexes on the surface of *Lactococcus lactis* as a key step in the development of recombinant microorganisms capable of carrying out a variety of metabolic processes, including the direct conversion of cellulosic substrates into fuels and chemicals. The efficiency of these recombinant molecules was, however, still much lower than naturally occurring cellulosomes. The same concept has been applied to generate cellulosomes integrating fungal and bacterial enzymes [130] which display promise to increase hydrolytic activities in biomass hydrolysis.

10 Conclusions

Production of biofuels from abundantly available biodegradable lignocellulosic biomass through consolidated bioprocessing is an attractive alternative to fossil-based energy carriers. Although a number of microorganisms have been discovered that have the inherent ability to degrade the components of biomass, only glimpses of the molecular mechanisms of their enzymes have been accomplished. It is expected that, with the discovery of new plant cell wall-degrading organisms and new enzymes, new paradigms can be found. This could potentially enhance our understanding of biomass conversion and improve/increase biofuel production. Research in both native and recombinant microorganisms is currently underway to find/develop the ideal organism(s) for lignocellulosic biomass hydrolysis and biofuel production, and a synthetic biology route may alleviate the problem of limited enzyme production capacity inherent in the anaerobic setting. This is because cellulosomes have so far been identified in slow growing strict anaerobes, and therefore designer cellulosomal components can be produced independently in a faster growing aerobic host cell system and potentially result in increased enzyme production.

Acknowledgements This work was supported by funds from Genome Canada through the Applied Genomics Research in Bioproducts or Crops (ABC) program for the grant entitled "Microbial genomics for biofuels and coproducts from biorefining processes," and the Province of Manitoba from the Manitoba Research Innovation Fund (MRIF).

References

1. Demain AL, Newcomb M, Wu JHD (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69(1):124–154
2. Stöcker M (2008) Biofuels and biomass-to-liquid fuels in the biorefinery: catalytic conversion of lignocellulosic biomass using porous materials. Angew Chem 47(48):9200–9211
3. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Tschaplinski T (2006) The path forward for biofuels and biomaterials. Science 311:484–489
4. Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes BJ, Erbach DC (2005) Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. DTIC Document, Oak Ridge National Lab, TN, USA

5. Canadian Centre for Energy (2012) http://www.centreforenergy.com/AboutEnergy/Biomass/Overview.asp?page=6
6. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16(5):577–583
7. Badua MA (2011) The challenge for lignocellulosics ethanol., http://alternativerenewableenergyphilippines.blogspot.com/2011/08/challenge-for-lignocellulosic-ethanol.html
8. Romeo T (2008) Bacterial biofilms. Springer, Berlin, pp 295
9. Smith AM (2001) The biosynthesis of starch granules. Biomacromolecules. doi:10.1021/bm000133c
10. Béguin P, Lemaire M (1996) The cellulosome: an exocellular, multiprotein complex specialized in cellulose degradation. Crit Rev Biochem Mol Biol 31:201–236
11. Lamed R, Bayer EA (1988) The cellulosome of *Clostridium thermocellum*. Adv Appl Microbiol 33:1–46
12. Lynd LR, Wyman CE, Gerngross TU (1999) Biocommodity engineering. Biotechnol Prog 15:777–793
13. Marchessault RH, Sundararajan PR (1993) Cellulose. In: Aspinall GO (ed) The polysaccharides, vol 2. Academic, New York, pp 11–95
14. Keshwani KR (2010) Biomass chemistry. In: Cheng JJ (ed) Biomass to renewable energy processes. CRC, Boca Raton, pp 209–270
15. Abbas CA (2003) Lignocellulosics to ethanol: meeting ethanol demand in the future. In: Jacques KA, Kelsall DR, Lyons TP (eds) The alcohol texbook, 4th edn. Nottingham University Press, Nottingham, pp 41–58
16. Vane CH, Abbott GD, Head IM (2001) The effect of fungal decay (*Agaricus bisporus*) on wheat straw lignin using pyrolysis–GC–MS in the presence of tetramethylammonium hydroxide (TMAH). J Anal Appl Pyrolysis 60(1):69–78
17. Rubin EM (2008) Genomics of cellulosic biofuels. Nature 454(7206):841–845
18. Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35(5):377–391
19. Van Wyk JPH (2011) Biowaste as a resource for bio-product development. In: Environmental earth sciences, pp 875–883. doi:10.1007/978-3-540-95991-5-82
20. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pre-treatment of lignocellulosic biomass. Bioresour Technol 96:673–686
21. Mes-Hartree M, Yu EKC, Reid ID, Saddler JN (1987) Suitability of aspenwood biologically delignified with *Pheblia tremellosus* for fermentation to ethanol or butanediol. Appl Microbiol Biotechnol 26(2):120–125
22. Reddy N, Yang Y (2005) Biofibers from agricultural byproducts for industrial applications. Trends Biotechnol 23:22–27
23. Sutton KB (2011) Top process designs for c5/c6 sugar fermentations. Novozyme report. http://bioenergy.novozymes.com/Documents/Ferm_SR_C5-C6.pdf
24. Cardona CA, Sánchez OJ (2007) Fuel ethanol production: process design trends and integration opportunities. Bioresour Technol 98:2415–2457
25. Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Wyman CE (2008) How biotech can transform biofuels. Nat Biotechnol 26(2):169–172
26. Lynd LR, Weimer PJ, Zyl WHV, Isak S, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66(3):506–739
27. Watanabe H, Tokuda G (2001) Animal cellulases. Cellular Mol Life Sci 58:1167–1178
28. Bergquist PL, Gibbs MD, Morris DD, Te'o VSJ, Saul DJ, Morgan HW (1999) Molecular diversity of thermophilic cellulolytic and hemicellulolytic bacteria. FEMS Microbiol Ecol 28:99–110
29. Wang Y, McAllister TA (2002) Rumen microbes, enzymes and feed digestion-a review. Asian-Australas J Anim Sci 15:1659–1676

30. Cantarel BI, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:233–238
31. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B (2014) The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42:490–495. http://www.cazy.org/
32. Little E, Bork P, Doolittle RF (1994) Tracing the spread of fibronectin type III domains in bacterial glycohydrolases. J Mol Evol 39:631–643
33. Watanabe T, Ito Y, Yamada T, Hashimoto M, Sekine S, Tanaka H (1994) The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. J Bacteriol 176(15):4465–4472
34. Kataeva IA, Seidel RD, Shah A, West LT, Li X-L, Ljungdahl LG (2002) The Fibronectin type 3-like repeat from the *Clostridium thermocellum* cellobiohydrolase CbhA promotes hydrolysis of cellulose by modifying its surface. Appl Environ Microbiol 68(9):4292–4300
35. Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382(Pt 3):769–781
36. Guillén D, Sánchez S, Rodríguez-Sanoja R (2010) Carbohydrate-binding domains: multiplicity of biological roles. Appl Microbiol Biotechnol 85(5):1241–1249
37. Engelhardt H, Peters J (1998) Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer-cell wall interactions. J Struct Biol 124:276–302
38. Schwarz WH (2001) The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol 56:634–649
39. Lupas A, Engelhardt H, Peters J, Santarius U, Volker S, Baumeister W (1994) Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. J Bacteriol 176:1224–1233
40. Brechtel E, Matuschek M, Hellberg A, Egelseer EM, Schmid R, Bahl H (1999) Cell wall of *Thermoanaerobacterium thermosulfurigenes* EM1: Isolation of its components and attachment of the xylanase XynA. Arch Microbiol 171:159–165
41. Lemaire M, Ohayon H, Gounon P, Fujino T, Beguin P (1995) OlpB, a new outer layer protein of *Clostridium thermocellum*, and binding of its S-layer-like domains to components of the cell envelope. J Bacteriol 177:2451–2459
42. Sára M (2001) Conserved anchoring mechanisms between crystalline cell surface S-layer proteins and secondary cell wall polymers in Gram-positive bacteria? Trends Microbiol 9 (2):47–50
43. Fuchs KP, Zverlov VV, Velikodvorskaya GA, Lottspeich F, Schwarz WH (2003) Lic16A of *Clostridium thermocellum*, a non-cellulosomal, highly complex endo-1,3-glucanase bound to the outer cell surface. Microbiology 149:1021–1031
44. Matuschek M, Sahm K, Zibat A, Bahl H (1996) Characterization of genes from *Thermoanaerobacterium thermosulfurigenes* EM1 that encode two glycosyl hydrolases with conserved S-layer-like domains. Mol General Genet 252:493–496
45. Himmel ME, Xu Q, Luo Y, Ding S-Y, Lamed R, Bayer EA (2010) Microbial enzyme systems for biomass conversion: emerging paradigms. Biofuels 1:323–341
46. Kataeva IA, Yang SJ, Dam P, Poole FL, Yin Y, Zhou F, Adams MWW (2009) Genome sequence of the anaerobic, thermophilic, and cellulolytic bacterium "*Anaerocellum thermophilum*" DSM 6725. J Bacteriol 191:3760–3761
47. Raman B, Pan C, Hurst GB, Rodriguez M, McKeown CK, Lankford PK, Mielenz JR (2009) Impact of pretreated Switchgrass and biomass carbohydrates on *Clostridium thermocellum* ATCC 27405 cellulosome composition: a quantitative proteomic analysis. PLoS One 4 (4):1–13
48. Wilson DB (2012) Processive and nonprocessive cellulases for biofuel production — lessons from bacterial genomes and structural analysis. Appl Microbiol Biotechnol 93:497–502

49. Davies G, Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. Structure 3 (9):853–859
50. Rouvinen J, Bergfors T, Teeri T, Knowles JK, Jones TA (1990) Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science 249:380–386
51. Teeri TT (1996) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases G3. Trends Biotech 7799:141–148
52. Li Y, Irwin DC, Wilson DB (2007) Processivity, substrate binding, and mechanism of cellulose hydrolysis by *Thermobifida fusca* Cel9A. Appl Environ Microbiol 73 (10):3165–3172
53. Tenkanen M, Poutanen K (1991) Significance of esterases in the degradation of xylans. Prog Biotechnol 7:203–212
54. Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56:326–338
55. Gilbert HJ, Hazlewood GP (1993) Bacterial cellulases and xylanases. J Gen Microbiol 139:187–194
56. Belancic A, Scarpa J, Peirano A, Díaz R, Steiner J, Eyzaguirre J (1995) *Penicillium purpurogenum* produces several xylanases: purification and properties of two of the enzymes. J Biotechnol 41:71–79
57. Biely P, MacKenzie CR, Puls J, Schneider H (1986) Cooperativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. Nat Biotechnol 4(8):731–733
58. Elegir G, Sykes M, Jeffries TW (1995) Differential and synergistic action of *Streptomyces* endoxylanases in prebleaching of kraft pulps. Enzyme Microb Technol 17:954–959
59. Dey D, Hinge J, Shendye A, Rao M (1992) Purification and properties of extracellular endoxylanases from alkalophilic thermophilic *Bacillus* sp. Can J Microbiol 38(5):436–442
60. Xu C, Huang R, Teng L, Wang D, Hemme CL, Borovok I, Xu J (2013) Structure and regulation of the cellulose degradome in *Clostridium cellulolyticum*. Biotechnol Biofuels 6:73
61. Mohand-Oussaid O, Payot S, Guedon E, Gelhaye E, Youyou A, Petitdemange H (1999) The extracellular xylan degradative system in *Clostridium cellulolyticum* cultivated on xylan: evidence for cell-free cellulosome production. J Bacteriol 181(13):4035–4040
62. Doi RH, Tamaru Y (2001) The *Clostridium cellulovorans* cellulosome: an enzyme complex with plant cell wall degrading activity. Chem Record (New York, N.Y.) 1:24–32
63. Han SO, Yukawa H, Inui M, Doi RH (2003) Transcription of *Clostridium cellulovorans* cellulosomal cellulase and hemicellulase genes. J Bacteriol 185:2520–2527
64. Lochner A, Giannone RJ, Rodriguez M, Shah MB, Mielenz JR, Keller M, Hettich RL (2011) Use of label-free quantitative proteomics to distinguish the secreted cellulolytic systems of *Caldicellulosiruptor bescii* and *Caldicellulosiruptor obsidiansis*. Appl Environ Microbiol 77 (12):4042–4054
65. Morag E, Bayer EA, Lamed R (1990) Relationship of cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum* to cellulose-degrading enzymes. J Bacteriol 172:6098–6105
66. Munir RI, Spicer V, Shamshurin D, Krokhin OV, Wilkins J, Ramachandran U, Levin DB (2015) Quantitative proteomic analysis of the cellulolytic system of *Clostridium termitidis* CT1112 reveals distinct protein expression profiles upon growth on α-cellulose and cellobiose. J Proteomics 125:41–53
67. Pohlschroder M, Leschine SB, Canale-Parola E (1994) Multicomplex cellulase-xylanase system of *Clostridium papyrosolvens* C7. J Bacteriol 176(1):70–76
68. Lin LL, Thomson JA (1991) An analysis of the extracellular xylanases and cellulases of *Butyrivibrio fibrisolvens* H17c. FEMS Microbiol Lett 68:197–203
69. Lamed R, Setter E, Bayer E (1983) Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. J Bacteriol 156(2):828–836

70. Tamaru Y, Miyake H, Kuroda K, Nakanishi A, Matsushima C, Doi RH, Ueda M (2011) Comparison of the mesophilic cellulosome-producing *Clostridium cellulovorans* genome with other cellulosome-related Clostridial genomes. J Microbial Biotechnol 4(1):64–73
71. Alber O, Noach I, Rincon MT, Flint HJ, Shimon LJW, Lamed R, Bayer EA (2009) Cohesin diversity revealed by the crystal structure of the anchoring cohesin from *Ruminococcus flavefaciens*. Proteins 77(3):699–709
72. Bayer EA, Lamed R, White BA, Flint HJ (2008) From cellulosomes to cellulosomics. Chem Record (New York, N.Y.), 8(6):364–377
73. Xu Q, Bayer EA, Goldman M, Kenig R, Shoham Y, Lamed R (2004) Architecture of the *Bacteroides cellulosolvens* cellulosome: description of a cell surface-anchoring scaffoldin and a family 48 cellulase. J Bacteriol 186:968–977
74. Xu Q, Morrison M, Nelson KE, Bayer EA, Atamna N, Lamed R (2004) A novel family of carbohydrate-binding modules identified with *Ruminococcus albus* proteins. FEBS Lett 566:11–16
75. Sabathé F, Bélaïch A, Soucaille P (2002) Characterization of the cellulolytic complex (cellulosome) of *Clostridium acetobutylicum*. FEMS Microbiol Lett 217:15–22
76. Morrison M, Miron J (2000) Adhesion to cellulose by *Ruminococcus albus*: a combination of cellulosomes and Pili proteins? 1. FEMS Microbiol Lett 185(2):109–115
77. Fujino T, Béguin P, Aubert JP (1993) Organization of a *Clostridium thermocellum* gene cluster encoding the cellulosomal scaffolding protein CipA and a protein possibly involved in attachment of the cellulosome to the cell surface. J Bacteriol 175(7):1891–1899
78. Bagnara-Tardif C, Gaudin C, Belaich A, Hoest P, Citard T, Belaich JP (1992) Sequence analysis of a gene cluster encoding cellulases from *Clostridium cellulolyticum*. Gene 119:17–28
79. Munir RI, Schellenberg J, Henrissat B, Verbeke TJ, Sparling R, Levin DB (2014) Comparative analysis of carbohydrate active enzymes in *Clostridium termitidis* CT1112 reveals complex carbohydrate degradation ability. PLoS One 9(8), e104260
80. Ljungdahl LG (2008) The cellulase/hemicellulase system of the anaerobic fungus *OrpinomycesPC-2* and aspects of its applied use. Ann N Y Acad Sci 1125(1):308–321
81. Raghothama S, Eberhardt RY, Simpson P, Wigelsworth D, White P, Hazlewood GP, Williamson MP (2001) Characterization of a cellulosome dockerin domain from the anaerobic fungus *Piromyces equi*. Nat Struct Mol Biol 8(9):775–778
82. Wilson C, Wood T (1992) The anaerobic fungus *Neocallimastix frontalis*: isolation and properties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen-bond-ordered cellulose. Appl Microbiol Biotechnol 37(1):125–129
83. Kosugi A, Murashima K, Tamaru Y, Doi RH (2002) Cell-surface-anchoring role of N-terminal surface layer homology domains of *Clostridium cellulovorans* EngE. J Bacteriol 184:884–888
84. Tamaru Y, Doi RH (2001) Pectate lyase A, an enzymatic subunit of the *Clostridium cellulovorans* cellulosome. Proc Natl Acad Sci USA 98:4125–4129
85. Bayer EA, Morag E, Shoham Y, Tormo J, Lamed R (1996) The cellulosome: a cell surface organelle for the adhesion to and degradation of cellulose. In: Bacterial adhesion: molecular and ecological diversity. Wiley, New York, pp 155–182
86. Miras I, Schaeffer F, Béguin P, Alzari PM (2002) Mapping by site-directed mutagenesis of the region responsible for cohesin – Dockerin interaction on the surface of the seventh cohesin domain of *Clostridium thermocellum* CipA. Biochemistry 41:2115–2119
87. Ciruela A, Gilbert HJ, Ali BRS, Hazlewood GP (1998) Synergistic interaction of the cellulosome integrating protein (CipA) from *Clostridium thermocellum* with a cellulosomal endoglucanase. FEBS Lett 422:221–224
88. Mechaly A, Fierobe HP, Belaich A, Belaich JP, Lamed R, Shoham Y, Bayer EA (2001) Cohesin-dockerin interaction in cellulosome assembly: a single hydroxyl group of a dockerin domain distinguishes between nonrecognition and high affinity recognition. J Biol Chem 276:9883–9888

89. Fontes CMG, Gilbert HJ (2010) Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. Annu Rev Biochem 79:655–681
90. Bélaich JP, Tardif C, Bélaich A, Gaudin C (1997) The cellulolytic system of *clostridium cellulolyticum*. J Biotechnol 57:3–14
91. Ferdinand PH, Borne R, Trotter V, Pagès S, Tardif C, Fierobe HP, Perret S (2013) Are cellulosome scaffolding protein CipC and CBM3-containing protein HycP, involved in adherence of *Clostridium cellulolyticum* to cellulose? PLoS One 8:1–11
92. Rincon MT, Čepeljnik T, Martin JC, Lamed R, Barak Y, Bayer EA, Flint HJ (2005) Unconventional mode of attachment of the *Ruminococcus flavefaciens* cellulosome to the cell surface. J Bacteriol 187:7569–7578
93. Sleytr UB, Sára M, Pum D, Schuster B (2001) Characterization and use of crystalline bacterial cell surface layers. Prog Surf Sci 68(7–8):231–278
94. Zhao G, Ali E, Sakka M, Kimura T, Sakka K (2006) Binding of S-layer homology modules from *Clostridium thermocellum* SdbA to peptidoglycans. Appl Microbiol Biotechnol 70 (4):464–469
95. Ali MK, Kimura T, Sakka K, Ohmiya K (2001) The multidomain xylanase Xyn10B as a cellulose-binding protein in *Clostridium stercorarium*. FEMS Microbiol Lett 198:79–83
96. Feng JX, Karita S, Fujino E, Fujino T, Kimura T, Sakka K, Ohmiya K (2000) Cloning, sequencing, and expression of the gene encoding a cell-bound multi-domain xylanase from *Clostridium josui*, and characterization of the translated product. Biosci Biotechnol Biochem 64:2614–2624
97. Ezer A, Matalon E, Jindou S, Borovok I, Atamna N, Yu Z, Lamed R (2008) Cell surface enzyme attachment is mediated by family 37 carbohydrate-binding modules, unique to *Ruminococcus albus*. J Bacteriol 190(24):8220–8222
98. Montanier C, van Bueren AL, Dumon C, Flint JE, Correia M, Prates J, Gilbert HJ (2009) Evidence that family 35 carbohydrate binding modules display conserved specificity but divergent function. Proc Natl Acad Sci U S A 106(9):3065–3070
99. Raman B, McKeown CK, Rodriguez M, Brown SD, Mielenz JR (2011) Transcriptomic analysis of *Clostridium thermocellum* ATCC 27405 cellulose fermentation. BMC Microbiol 11(1):134
100. Wei H, Fu Y, Magnusson L, Baker JO, Maness PC, Xu Q, Ding SY (2014) Comparison of transcriptional profiles of *Clostridium thermocellum* grown on cellobiose and pretreated yellow poplar using RNA-seq. Front Microbiol 5:1–16
101. Gold ND, Martin VJJ (2007) Global view of the *Clostridium thermocellum* cellulosome revealed by quantitative proteomic analysis. J Bacteriol 189(19):6787–6795
102. Lochner A, Giannone RJ, Keller M, Antranikian G, Graham DE, Hettich RL (2011) Label-free quantitative proteomics for the extremely thermophilic bacterium *Caldicellulosiruptor obsidiansis* reveal distinct abundance patterns upon growth on cellobiose, crystalline cellulose, and switchgrass. J Proteome Res 10(12):5302–5314
103. Adav SS, Chao LT, Sze SK (2012) Quantitative secretomic analysis of *Trichoderma reesei* strains reveals enzymatic composition for lignocellulosic biomass degradation. Mol Cell Proteomics 11(7):1–15
104. Adav SS, Ng CS, Arulmani M, Sze SK (2010) Quantitative iTRAQ secretome analysis of cellulolytic *Thermobifida fusca*. J Proteome Res 9:3016–3024
105. Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, Cullen D (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci U S A 106(6):1954–1959
106. MacDonald J, Doering M, Canam T, Gong Y, Guttman DS, Campbell MM, Master ER (2011) Transcriptomic responses of the softwood-degrading white-rot fungus *Phanerochaete carnosa* during growth on coniferous and deciduous wood. Appl Environ Microbiol 77 (10):3211–3218

107. Johnson EA, Bouchot F, Demain AL (1985) Regulation of cellulase formation in *Clostridium thermocellum*. J Gen Microbiol 131(9):2303–2308
108. Zhang YH, Lynd LR (2005) Cellulose utilization by *Clostridium thermocellum*: bioenergetics and hydrolysis product assimilation. Proc Natl Acad Sci USA 102:7321–7325
109. Abdou L, Boileau C, De Philip P, Pagès S, Fiérobe HP, Tardif C (2008) Transcriptional regulation of the *Clostridium cellulolyticum* cip-cel operon: a complex mechanism involving a catabolite-responsive element. J Bacteriol 190:1499–1506
110. Servinsky MD, Kiel JT, Dupuy NF, Sund CJ (2010) Transcriptional analysis of differential carbohydrate utilization by *Clostridium acetobutylicum*. Microbiology 156:3478–3491
111. Kahel-Raifer H, Jindou S, Bahari L, Nataf Y, Shoham Y, Bayer EA, Lamed R (2010) The unique set of putative membrane-associated anti-sigma factors in *Clostridium thermocellum* suggests a novel extracellular carbohydrate-sensing mechanism involved in gene regulation. FEMS Microbiol Lett 308:84–93
112. Bahari L, Gilad Y, Borovok I, Kahel-Raifer H, Dassa B, Nataf Y, Bayer EA (2011) Glycoside hydrolases as components of putative carbohydrate biosensor proteins in *Clostridium thermocellum*. J Ind Microbiol Biotechnol 38:825–832
113. Petersen SH, Van Zyl WH, Pretorius IS (1998) Development of a polysaccharide degrading strain of *Saccharomyces cerevisiae*. Biotechnol Techniques 12:615–619
114. Ingram LO, Gomez PF, Lai X, Moniruzzaman M, Wood BE, Yomano LP, York SW (1998) Metabolic engineering of bacteria for ethanol production. Biotechnol Bioeng 58:204–214
115. Aro N, Ilmén M, Saloheimo A, Penttilä M (2003) ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. Appl Environ Microbiol 69:56–65
116. Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B (2009) Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol Biofuels 2:19
117. Kawaguchi H, Vertes AA, Okino S, Inui M, Yukawa H (2006) Engineering of a xylose metabolic pathway in *Corynebacterium glutamicum*. Appl Environ Microbiol 72:3418–3428
118. Hahn-Hägerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund MF (2007) Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 108:147–177
119. Ha SJ, Galazka JM, Rin Kim S, Choi J-H, Yang X, Seo J-H, Jin Y-S (2011) Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. Proc Natl Acad Sci 108(2):504–509
120. Alper H, Stephanopoulos G (2009) Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? Nat Rev Microbiology 7(10):715–723
121. Jin YS, Alper H, Yang YT, Stephanopoulos G (2005) Improvement of xylose uptake and ethanol production in recombinant *Saccharomyces cerevisiae* through an inverse metabolic engineering approach. Appl Environ Microbiol 71:8249–8256
122. Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler AA, Van Dijken JP, Pronk JT (2005) Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res 5:399–409
123. Jeffries TW, Jin YS (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol 63:495–509
124. Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50:484–524
125. Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yukawa H (2008) Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl Microbiol Biotechnol 77:1305–1316
126. Fierobe HP, Mechaly A, Tardif C, Belaich A, Lamed R, Shoham Y, Bayer EA (2001) Design and production of active cellulosome chimeras. Selective incorporation of dockerin-containing enzymes into defined functional complexes. J Biol Chem 276:21257–21261
127. Fierobe HP, Mingardon F, Mechaly A, Bélaïch A, Rincon MT, Pagès S, Bayer EA (2005) Action of designer cellulosomes on homogeneous versus complex substrates: controlled

incorporation of three distinct enzymes into a defined trifunctional scaffoldin. J Biol Chem 280:16325–16334
128. Fierobe HP, Bayer EA, Tardif C, Czjzek M, Mechaly A, Bélaïch A, Bélaïch JP (2002) Degradation of cellulose substrates by cellulosome chimeras: substrate targeting versus proximity of enzyme components. J Biol Chem 277:49621–49630
129. Wieczorek AS, Martin VJJ (2010) Engineering the cell surface display of cohesins for assembly of cellulosome-inspired enzyme complexes on *Lactococcus lactis*. Microb Cell Fact 9:69
130. Mingardon F, Chanal A, López-Contreras AM, Dray C, Bayer EA, Fierobe HP (2007) Incorporation of fungal cellulases in bacterial mini cellulosomes yields viable, synergistically acting cellulolytic complexes. Appl Environ Microbiol 73:3822–3832

Adv Biochem Eng Biotechnol (2016) 156: 139–154
DOI: 10.1007/10_2015_5006

Published online: 24 February 2016

Biotechnology of Anoxygenic Phototrophic Bacteria

Niels-Ulrik Frigaard

Abstract Anoxygenic phototrophic bacteria are a diverse collection of organisms that are defined by their ability to grow using energy from light without evolving oxygen. The dominant groups are purple sulfur bacteria, purple nonsulfur bacteria, green sulfur bacteria, and green and red filamentous anoxygenic phototrophic bacteria. They represent several bacterial phyla but they all have bacteriochlorophylls and carotenoids and photochemical reaction centers which generate ATP and cellular reductants used for CO_2 fixation. They typically have an anaerobic lifestyle in the light, although some grow aerobically in the dark. Some of them oxidize inorganic sulfur compounds for light-dependent CO_2 fixation; this ability can be exploited for photobiological removal of hydrogen sulfide from wastewater and biogas. The anoxygenic phototrophic bacteria also perform bioremediation of recalcitrant dyes, pesticides, and heavy metals under anaerobic conditions. Finally, these organisms may be useful for overexpression of membrane proteins and photobiological production of H_2 and other valuable compounds.

Keywords Biogas, Bioremediation, Carotenoids, Green sulfur bacteria, Hydrogen sulfide, Membrane proteins, Photosynthetic bacteria, Purple bacteria

Contents

N.-U. Frigaard (✉)
Department of Biology, University of Copenhagen, Strandpromenaden 5, 3000 Helsingør, Denmark
e-mail: nuf@bio.ku.dk

Abbreviations

BChl	Bacteriochlorophyll
E_0'	Standard reduction potential at pH 7 and 25 °C
EPS	Extracellular polymeric substances
FAP	Filamentous anoxygenic phototrophs
GSB	Green sulfur bacteria
PBR	Photobioreactor
PNSB	Purple nonsulfur bacteria
PSB	Purple sulfur bacteria

1 Introduction

Phototrophic organisms are defined by their ability to convert light energy into chemical energy in forms useful for growth and other metabolic processes [1–3]. Cyanobacteria and microalgae are the only phototrophic microorganisms that evolve O_2 as a result of this process. This is because they have an enzyme complex capable of oxidizing water to oxygen ($H_2O \rightarrow 2H^+ + ½O_2 + 2e^-$) and donating the electrons to a type II photochemical reaction center. These organisms are therefore oxygenic (i.e., oxygen-evolving) phototrophs. Phototrophs that do not oxidize water may, for example, oxidize hydrogen sulfide ($H_2S \rightarrow 2H^+ + S + 2e^-$) and donate the electrons to the photochemical reaction center in the organism. These organisms are therefore anoxygenic (i.e., not oxygen-evolving) phototrophs. In either case, these electron transfer reactions generate a transmembrane proton motive force used for ATP generation and other physiological purposes. The reductants generated by the reaction center are also used for biosynthetic purposes and cyclic electron transfer around the reaction centers to generate even more proton motive force.

The vast majority of anoxygenic phototrophic bacteria grow phototrophically only under anaerobic conditions [4]. Some may be capable of chemotrophic growth under aerobic conditions in the dark (typical of purple bacteria), but others are obligate anaerobic and are killed by O_2 (typical of green sulfur bacteria; GSB). Anoxygenic phototrophic bacteria rarely form visibly large accumulations in natural environments because of their anaerobic lifestyle, but occasionally various

purple bacteria may bloom and color ponds, coastal areas, and wastewater reservoirs red, purple, or reddish-brown.

Anoxygenic phototrophic bacteria may not be as extensively used for biotechnological applications as chemotrophic microorganisms. However, given their diversity and the rising interest in biological solutions to societal challenges, more applications should be explored. For example, the extensive intracellular membrane systems in purple bacteria may have practical applications (Sect. 8). Another underexplored area is the potential for production of proteins and metabolites that are O_2 sensitive. In the following, a survey is made of biotechnological applications of the anoxygenic phototrophic bacteria. The bioremediating properties of oxygenic and anoxygenic photosynthetic bacteria were recently reviewed [5].

1.1 Phototrophy and Photosynthesis

There are only two principal sources of energy for living organisms: energy from light (phototrophy) or energy from chemical compounds (chemotrophy) [3]. Photosynthesis usually refers specifically to photoautotrophy, i.e., growth based on CO_2 fixation where the required energy is derived from light. Thus, all photosynthetic organisms are phototrophic but not all phototrophic organisms are photosynthetic. The principal cellular component that allows phototrophy is the photochemical reaction center [1]. This is a large enzyme complex that is always membrane-bound and has the ability to convert excitation energy obtained from light into chemical energy by creating a light-induced charge separation. The chemical energy is used for generation of strong reductants and ATP that are used in CO_2 fixation, biosynthetic pathways, and other cellular activities (Fig. 1). There are only two types of photochemical reaction centers: type I and type II. Type I (also called iron-sulfur type) has a relatively low redox potential and reduces soluble, cytoplasmic ferredoxins with E_0' values between −0.5 and −0.6 V. Type II (also called quinone-type) has a relatively high redox potential and reduces membrane-bound isoprenoid quinones with E_0' values between +0.1 and −0.1 V. The oxygenic phototrophs (cyanobacteria, algae, and plants) have both types of reaction centers (known as photosystems I and II) whereas the anoxygenic phototrophic bacteria have only either type I or type II. Light-harvesting pigment-protein antenna complexes are associated with the reaction centers to increase the amount of light energy harvested by the cells [1, 2]. The excitation energy in the antenna pigments is channeled to the photochemical reaction center in the order of picoseconds. There is a large diversity of evolutionary unrelated light-harvesting antenna complexes, but common to most of them is the presence of chlorophylls or bacteriochlorophylls and carotenoids. Most of the pigmentation in phototrophic bacteria (>99%) is found in these light-harvesting antenna complexes.

In principle, phototrophy can also be supported by alternative enzymes: the rhodopsin-like proteins [2, 3]. These membrane-bound enzymes absorb light energy and, as a result, transfer protons across the membrane, thereby conserving the light energy as a transmembrane electrochemical proton gradient. Rhodopsins

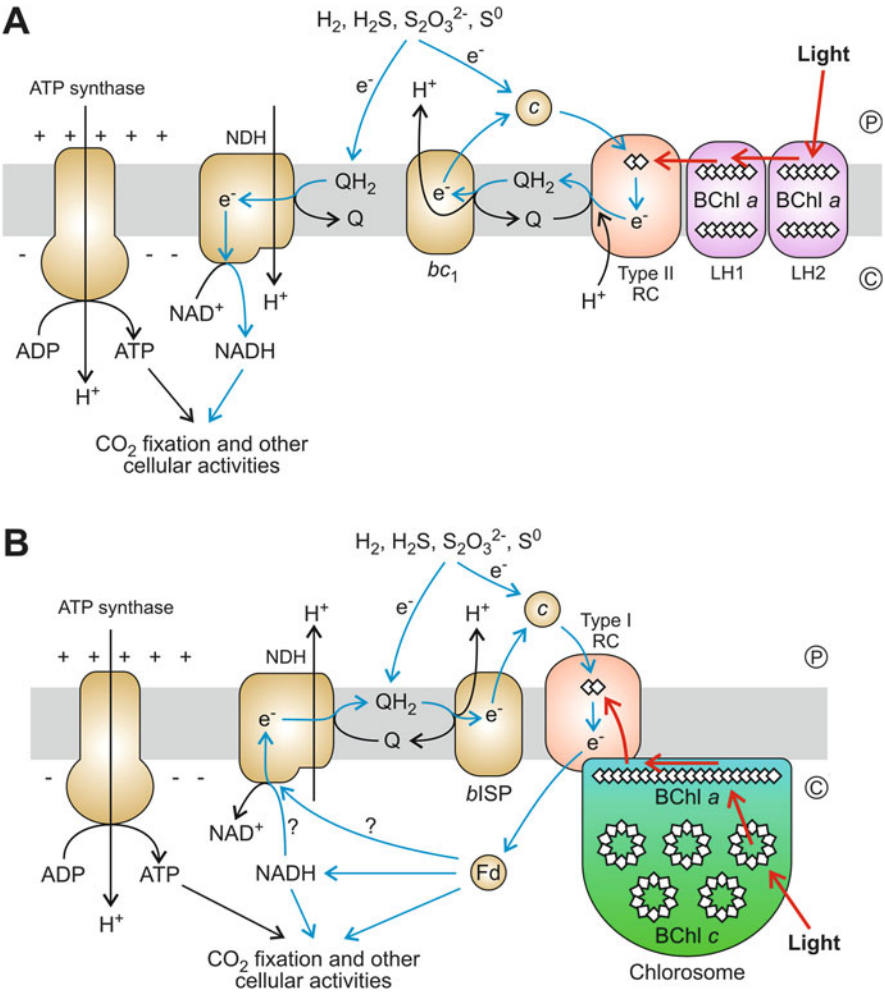


Fig. 1 Simplified models of the photosynthetic machinery in (**a**) purple bacteria and (**b**) green sulfur bacteria (GSB) during photoautotrophic growth on inorganic sulfur compounds. Light is harvested by the peripheral antennae (LH1, LH2, or chlorosomes) and the excitation energy is transferred to the reaction center (RC). Light-driven cyclic electron transfer occurs when electrons are continuously transferred between the reaction center and the membrane-bound cytochrome bc_1/*b*ISP in a light-dependent manner. This electron transfer generates a proton motive force, which fuels other physiological processes such as ATP synthesis. NADH is generated by reverse electron flow in purple bacteria under photoautotrophic growth conditions. If suitable organic substrates are available for photoheterotrophic growth in purple bacteria, NADH for cellular activities is instead obtained by oxidation of these organic substrates and the photochemical system may function exclusively in a cyclic mode to produce ATP. For details on oxidation of sulfur compounds (H_2S, $S_2O_3^{2-}$, and S^0) see Fig. 2. Pathways that carry electrons for cyclic electron transfer, CO_2 fixation, and other cellular activities are shown with *blue arrows*. Transfer of light and excitation energy is shown with *red arrows*. bc_1 cytochrome bc_1 complex, *BChl* bacteriochlorophyll, *b*ISP cytochrome *b* iron-sulfur protein, *C* (*circled*) cytoplasm, *Fd* ferredoxin, *NDH* NADH:quinone oxidoreductase, *P* (*circled*) periplasm, *Q* isoprenoid quinone (oxidized), *RC* photochemical reaction center. Modified from [1, 2]

do not transfer electrons and are not associated with large light-harvesting antennae as are photochemical reactions centers. Rhodopsins are well-known components of halophilic archaea where they support a phototrophic lifestyle under anaerobic conditions. Rhodopsin-like enzymes have recently been found in numerous very different marine microbes (archaea, bacteria, and eukaryotes) but the exact contribution to cell physiology is not always clear in these organisms. Phototrophy based on rhodopsins is not considered further here.

1.2 Types of Anoxygenic Phototrophic Bacteria

Purple bacteria belong to the phylum Proteobacteria and are characterized by having type II photochemical reaction centers and bacteriochlorophyll (BChl) *a* or BChl *b* as the major pigment [4, 6]. The light-harvesting (LH) antennae known as LH1 and LH2 are located in the cytoplasmic and intracytoplasmic membranes. As a group, the purple bacteria are very physiologically versatile and may grow photoautotrophically, photoheterotrophically, or chemotrophically in the dark by fermentation or by aerobic or anaerobic respiration. A subgroup of the purple bacteria, the purple sulfur bacteria (PSB), is characterized by a predominant photoautotrophic lifestyle where inorganic sulfur compounds such as sulfide serve as electron donors for photosynthesis. The other major subgroup of purple bacteria, known collectively as purple nonsulfur bacteria (PNSB), is characterized by a predominantly photoheterotrophic lifestyle where many organic compounds can be assimilated and these organisms are not as tolerant to sulfide as the PSB. PSB are Gammaproteobacteria whereas PNSB are Alfaproteobacteria and Betaproteobacteria.

GSB belong to the phylum Chlorobi and are characterized by having BChl *c*, *d*, or *e* organized into large light-harvesting organelles known as chlorosomes [4]. These organisms contain type I reaction centers and typically oxidize sulfur compounds for CO_2 fixation. Compared to the purple bacteria, the GSB have much more restricted growth requirements: they are strict anaerobic, obligate phototrophic, and obligate autotrophic.

Filamentous anoxygenic phototrophic (FAP) bacteria belong to the phylum Chloroflexi and are characterized by having BChl *a* organized into type II reaction centers and light-harvesting antennae similar to those found in purple bacteria [4]. There are two kinds of FAPs: the red FAPs have BChl *a* as the sole chlorophyll-like pigment, whereas the green FAPs, in addition to BChl *a*, also have BChl *c* or *d* organized in chlorosome structures similar to those found in GSB. The green FAPs are also known as green nonsulfur bacteria.

The only other groups of anoxygenic phototrophic bacteria known are the Heliobacteria, Chloracidobacteria, and the recently discovered Gemmamonatiales [7]. There are no known archaea that contain photochemical reaction centers.

Cyanobacteria are oxygenic phototrophs [8]. However, a few cyanobacteria are capable of anoxygenic photosynthesis and an anaerobic phototrophic lifestyle

where the organisms consume hydrogen sulfide as electron donor for photosynthesis, very similar to the phototrophic GSB and PSB described above. Microalgae are also oxygenic phototrophs but are not known to grow phototrophically under anaerobic conditions. However, this does not exclude the possibility that some microalgae have light-independent lifestyles under anaerobic conditions or in the dark.

1.3 Occurrence

In general, anoxygenic phototrophic bacteria thrive where anaerobic conditions intersect with light [4]. In natural environments these conditions occur in lakes and sediments, often in narrow bands limited by the extent of light penetration. If sulfide is present, typically either PSB or GSB or both accumulate. Purple bacteria in general are physiologically versatile and thrive in many man-made polluted environments such as wastewater lagoons where light coincides with anaerobic or microaerobic conditions and high loads of organic compounds.

1.4 Sulfur Metabolism and Sulfide Tolerance

Reduced inorganic sulfur compounds are abundant in anaerobic environments because of the degradation of sulfur-containing organic materials and H_2S production by sulfate-reducing bacteria. These compounds are oxidized by various chemotrophic bacteria (often collectively denoted "colorless sulfur bacteria") under aerobic conditions or by phototrophic bacteria under anaerobic conditions. The metabolism of sulfur compound oxidation in phototrophic bacteria is complex and not fully elucidated (Fig. 2) [9]. Sulfide and thiosulfate are often incompletely oxidized to elemental sulfur if the sulfide and thiosulfate are supplied in excess. This elemental sulfur is deposited outside or inside the cells as sulfur globules with diameters ranging from very small up to 2 μm. Intracellular sulfur globules are found in PSB of the family *Chromatiaceae* and extracellular sulfur globules are found in GSB, PNSB, and most PSB of the family *Ectothiorhodospiraceae*. Upon depletion of sulfide and other electron sources, the sulfur globules are oxidized completely to sulfate.

Although sulfide is metabolized by most, if not all, phototrophic bacteria, the levels of tolerance vary significantly [9]. Sulfide concentrations above 15–30 mg/L H_2S (0.5–1 mM) tend to inhibit the PNSB and favor the PSB and GSB. The highest sulfide concentrations tolerated by most PSB and GSB are about 100–150 mg/L H_2S (about 3–5 mM), where the GSB typically are the most tolerant. In extreme cases, up to 375 mg/L H_2S (11 mM) is tolerated.

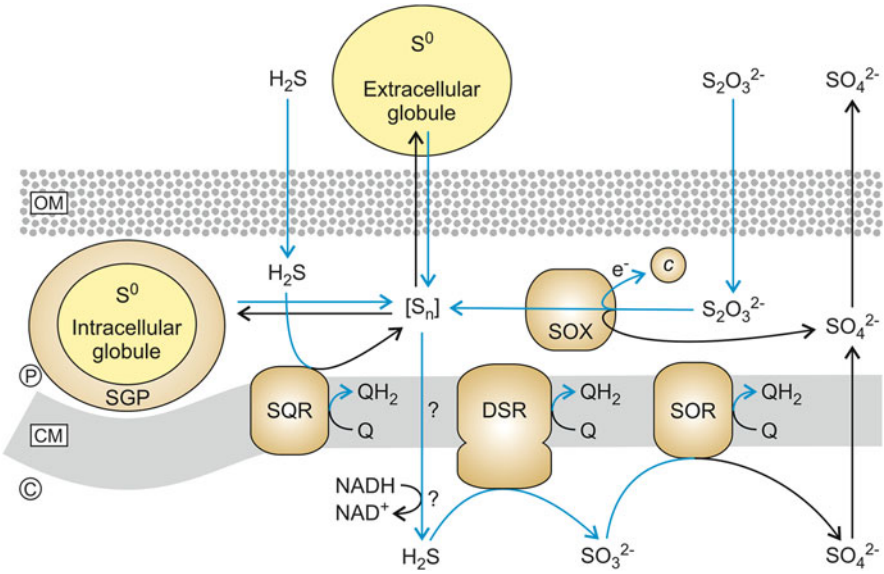


Fig. 2 Simplified model of the oxidative sulfur metabolism in phototrophic sulfur bacteria. Sulfur globules are shown in *yellow*. Intracellular sulfur globules in purple sulfur bacteria (PSB) are located in the periplasm and are associated with sulfur globule proteins (SGP). Pathways that carry electrons towards cellular electron carriers (isoprenoid quinones and cytochromes *c*) are shown in *blue*. *C* (*circled*) cytoplasm, *CM* (*boxed*) cytoplasmic membrane, *DSR* dissimilative sulfite reductase, *OM* (*boxed*) outer membrane, *P* (*circled*) periplasm, *Q* isoprenoid quinone (oxidized), *SGP* sulfur globule protein, [S_n] oligosulfide pool, *SOR* sulfite oxidoreductase (several types), *SOX* sulfur compound oxidizing system, *SQR* sulfide:quinone oxidoreductase. Modified from [9]

2 Removal of Hydrogen Sulfide from Wastewater Streams

Sulfide is a malodorous, corrosive, and toxic compound often present in liquid waste streams from domestic and industrial sources. The source is typically organically bound sulfur, which is liberated as sulfide or sulfate upon degradation, and inorganic sulfur (mostly sulfate), which is reduced to sulfide under anaerobic conditions by ubiquitous sulfate-reducing bacteria. Anoxygenic phototrophic bacteria offer an environmentally friendly biological approach to remove this sulfide [10]. Partial oxidation of H_2S to elemental sulfur (S^0) rather than complete oxidation to sulfate (SO_4^{2-}) is desirable for a number of reasons. (1) Elemental sulfur can be physically removed from the waste stream by sedimentation of the sulfur granules. This alleviates downstream problems with sulfate (such as reduction to sulfide by sulfate-reducing bacteria). In addition, this biogenic elemental sulfur has commercial value as fertilizer and chemical feedstock. (2) Oxidation of sulfide to elemental sulfur generates less H^+ than oxidation of sulfide to sulfate. This minimizes corrosion and the requirement for pH control. (3) Conversion to elemental sulfur requires less light than conversion to sulfate; this maximizes the sulfide removal per light input. For these reasons, conditions in the photobioreactor

(PBR) have to be carefully controlled (e.g., flow rate, light intensity) to avoid complete sulfide oxidation and to maximize the output of elemental sulfur. Alternatively, naturally occurring or genetically engineered strains of phototrophic sulfur bacteria could be used which are deficient in sulfate formation and only oxidize sulfide to elemental sulfur, regardless of the sulfide load [11].

For sulfide removal from waste streams, GSB have certain advantages over other anoxygenic phototrophic bacteria [10]. (1) GSB deposit elemental sulfur extracellularly, which enables recovery of the sulfur by sedimentation (most PSB accumulate the elemental sulfur intracellularly). (2) GSB oxidize more sulfide per light input than purple bacteria, which is important for practical design and economic reasons. (3) GSB have higher tolerance for sulfide and higher affinity for sulfide uptake than purple bacteria. (4) Sulfide uptake in purple bacteria is diminished when organic nutrients are available. For these reasons, if a defined microbial culture is to be used for photobiological sulfide removal, a GSB culture may be beneficial. In one such study, a pilot-scale fixed-film continuous-flow PBR (21 mL) with the GSB *Chlorobium limicola* strain DSM 257 was designed and successfully optimized for sulfide removal [12]. At loading rates of 111–286 mg/L/h sulfide, about 100% of the influent sulfide was consumed and 92–95% recovered as elemental sulfur [12].

In another pilot-scale study in Brazil, up-flow anaerobic PBRs were tested with a continuous flow of domestic wastewater containing 1–6 mg/L sulfide [13]. These systems relied on naturally developing microbial communities under the influence of natural light. Sulfide removal efficiencies reached 90% and the effluent contained less than 0.5 mg/L sulfide, which is below the national sulfide discharge standard in Brazil of 1 mg/L. Most of the sulfide was oxidized to elemental sulfur (S^0) although some sulfate (SO_4^{2-}) was also formed. The sulfide was oxidized by a mixed microbial community which was shown by molecular techniques to contain GSB, PSB of the *Chromatiaceae* lineage, and green FAPs of the *Chloronema* lineage. Sulfide removal by chemotrophic denitrification was unlikely to occur because the influent and effluent wastewater contained very little nitrate and nitrite, and microbes capable of preforming this process were not identified in the microbial community.

3 Removal of Hydrogen Sulfide from Gas Streams

Hydrogen sulfide is present in small amounts in biogas (typically 0.1–2%) and off-gases from wastewater treatment plants, for example. Besides being toxic and malodorous, H_2S in biogas causes corrosion and poisoning of the equipment using the biogas. Therefore, a number of chemical technologies are currently used to remove H_2S from biogas. A commercialized approach using chemotrophic sulfide-oxidizing bacteria in a fixed-film bioreactor under controlled oxygen conditions is also available [14]. Fixed-film or suspended-growth PBRs with anoxygenic phototrophic bacteria could be interesting alternatives for cost-effective H_2S

removal because of their ability to operate for long periods of time without requiring a biomass separation step and their ability to operate under high and variable sulfide loadings [14]. Illumination and design of the PBR is a key concern for practical application because light is rapidly attenuated in dense cell suspensions [15]. Studies suggest that illumination using light-emitting diodes (LED) appears to be a very cost-effective approach if the LED emission wavelength is matched to the pigment absorption by the cells [16].

A variety of reactor designs have been investigated which allow the influent gas to exchange H_2S with an aqueous medium containing suspended or immobilized sulfide-oxidizing bacteria. In one study, a continuous-stirred PBR with *Chlorobium thiosulfatophilum* was used to remove sulfide from a synthetic gas stream containing 2.5% H_2S [17]. At its optimum operation, the 1.25-L PBR consumed 118 mg/h H_2S with negligible formation of sulfate corresponding to a conversion rate of sulfide to elemental sulfur per PBR volume of about 100 mg/h/L H_2S. Another study with a larger PBR working volume (11.9 L) used a flat-panel gas-lift PBR design with a PBR thickness of 10 cm and LED illumination [16]. In this system, the sulfide conversion to elemental sulfur was only about 20 mg/h/L H_2S but the energy expenditure for illumination per amount of sulfide removed was the lowest reported in the literature because of the use of LED for illumination.

4 Degradation of Recalcitrant Dyes and Pesticides

Azo dyes are the most important group of synthetic colorants and are generally recalcitrant to biodegradation because of their xenobiotic nature. Degradation of azo dyes in wastewater streams is most efficient under anaerobic conditions because many anaerobic bacteria apparently have a broad-range ability to reduce the azo bond [18]. Azo dyes are decolorized by many PNSB and this degradation is dependent on the enzyme azoreductase [19, 20]. Pure culture studies with different isolates of *Rhodopseudomonas palustris* have shown that this organism efficiently decolorizes various azo dyes in concentrations of around 1 g/L dye [20, 21]. In these studies, decolorization and at least partial degradation of azo dyes occurred only under anaerobic conditions and in the light. In another study, different PNSB strains isolated from various water sources (*Rhodobacter adriaticus*, *Rhodobacter blasticus*, *Rhodobacter capsulatus*, *Rhodovulum strictum*, and *R. palustris*) decolorized up to 96% of the tested azo dyes after only 2 days of illuminated and anaerobic incubation [22].

To circumvent problems associated with wastewater treatment using suspended cultures of bacteria, Wang and colleagues constructed a PBR to treat azo dye-contaminated wastewater [23]. The naturally developed biofilm contained anoxygenic phototrophic bacteria related to the *Rhodopseudomonas*, *Rhodomicrobium*, and *Chlorobium* lineages. This PBR allowed the removal of most of the organic load and up to 90% of the azo dyes. Over a 30-day run, the predominant phototrophic bacteria in the biofilm changed from purple bacteria to a

mixture of purple and GSB, and finally to mainly GSB. During this period the decolorization of azo dye increased from 60% to 90%, suggesting that GSB may also be capable of efficiently decolorizing azo dyes.

Purple bacteria belonging to the *Rhodospirillum* and *Rhodopseudomonas* genera have also been investigated for use in anaerobic biodegradation of halogenated aromatic pollutants such as 3-chlorobenzoate [5, 24].

5 Removal of Toxic Metals and Radioisotopes

Microorganisms – dead or alive, free or immobilized – can be used to remove toxic metals and radioisotopes from the environment [25]. Among anoxygenic phototrophic bacteria, this ability has especially been studied in PNSB [5].

The PNSB *Rhodobacter sphaeroides* bioaccumulates heavy metals including cadmium (Cd), nickel (Ni), and lead (Pb) [26–28], and metals with radioactive isotopes, cesium (Cs) and strontium (Sr) [29]. In one study, *R. sphaeroides* strain S accumulated Cd^{2+} with a higher efficiency under aerobic-dark conditions (93%) than under anaerobic-light conditions (50%) over 7 days at an initial Cd^{2+} concentration of 5 mg/L [27]. However, the ability to bioaccumulate metal ions is dependent on the strain of the organism under investigation. *R. sphaeroides* strain SSI is a spontaneous mutant of *R. sphaeroides* strain S with an increased ability to produce extracellular polymeric substances (EPS) on the cell surface [29]. The SSI strain has been shown to remove efficiently toxic metals including cadmium, uranium (U), cobalt (Co), mercury (Hg), and chromium (Cr). The removal of these metals has been attributed to the high amount of EPS produced on the surface of strain SSI because the strong negative charges of EPS efficiently adsorb many different kinds of metal ions. In one experimental setup with immobilized cells of strain SSI and 5 mg/L of each Cs^{+} and Sr^{2+}, about 100% of the cesium and 50% of the strontium were removed in 3 days. Other strains of *R. sphaeroides* and other PNSB have also been shown to remove efficiently (>90%) copper (Cu^{2+}), zinc (Zn^{2+}), and Cd^{2+} from contaminated shrimp pond water [30].

Zinc consumption by live *R. capsulatus* strain B10 cells has been reported at levels of 164 mg Zn^{2+} per gram of cell dry weight [31]. This is among the highest zinc biosorption capacities reported for any microorganism and this suggests that *R. capsulatus* could be useful for zinc bioremediation. The unusually high zinc biosorption capacity was again mainly attributed to the physicochemical properties of the EPS on the cell surface.

6 Photobiological Production of Hydrogen

Hydrogen (H_2) has potential as an environmentally friendly fuel. Most, if not all, oxygenic as well as anoxygenic phototrophic microorganisms have the capacity to produce H_2 but the physiological mechanisms for H_2 production vary among these organisms [32]. Here, only H_2 production in anoxygenic phototrophic bacteria is considered.

Although H_2 production from phototrophic bacteria may not be economically viable by itself, H_2 could be a valuable byproduct from other application of these organisms such as wastewater or biogas cleanup [33]. Photobiological H_2 formation is catalyzed either by a hydrogenase ($2H^+ + 2e^- \leftrightarrows H_2$) or by nitrogenase as a byproduct from N_2 fixation ($8H^+ + N_2 + 8e^- \rightarrow H_2 + 2NH_3$). Most bacteria have the ability to re-uptake the produced H_2 so this ability may have to be eliminated if the H_2 production is to be useful.

In *Rhodospirillum rubrum* and other PNSB, photobiological H_2 production is primarily caused by nitrogenase and therefore H_2 production is induced under nitrogen limitation. Interestingly, if N_2 is completely removed (for example, by using an inert gas), nitrogenase produces exclusively H_2 and thus the H_2 productivity is increased. In a nitrogen-limited batch culture of *R. rubrum*, a continuous production of 0.48 L/day H_2 per gram cell dry weight was observed with lactate as electron donor [34]. *R. rubrum* and other purple bacteria also catalyze light-dependent H_2 production from CO with a net reaction corresponding to the water-gas shift reaction ($CO + H_2O \rightarrow H_2 + CO_2$) [35].

GSB also exhibit light- and nitrogenase-dependent H_2 production. In these bacteria, inorganic sulfur compounds (sulfide, sulfur, and thiosulfate) are electron donors for photosynthesis and H_2 production. Photobiological H_2 production from organic compounds using these bacteria was demonstrated in a syntrophic co-culture of the GSB *Chlorobium vibrioforme* and the acetate-oxidizing, sulfur-reducing bacterium *Desulfuromonas acetoxidans* [36]. In this co-culture, the chemotrophic partner oxidized acetate and reduced sulfur to H_2S, and the phototrophic partner oxidized H_2S back to sulfur and produced H_2 using nitrogenase. In this co-culture the productivity was 1.3 L/day H_2 per gram cell dry weight with acetate as electron donor.

An alternative approach to photobiological H_2 production in GSB might be engineered. In principle, the redox potentials of the type I photochemical reaction center (E_0' approx. −0.6 V) and the soluble ferredoxins (E_0' approx. −0.5 V) in GSB are low enough to allow reduction of H^+ to H_2 (E_0' −0.42 V). These strong cellular reductants are required for CO_2 fixation by the reductive TCA cycle found in GSB. However, this reducing power might also be used for photobiological H_2 production if a suitable hydrogenase could be inserted in these organisms that would accept electrons from the indigenous strong reductants. Then H_2 production would be independent of N_2 and nitrogenase.

7 Biosynthesis of Carotenoids and Other Terpenoids

Terpenoids (or isoprenoids) are compounds derived from one or more isoprene C_5 units and constitute a very large range of natural compounds. A prominent group is the carotenoids (C_{40} compounds) found in all phototrophic organisms and some chemotrophic bacteria, archaea, and fungi. Carotenoids are yellow, orange, and red pigments with broad applications in the food, feed, nutraceutical, cosmetic, and pharmaceutical industries because of their vibrant colors and health-promoting activities [37]. Most commercialized carotenoids, such as beta-carotene and astaxanthin, are produced in microalgae although genetically modified *Escherichia coli* and yeasts that produce commercially valuable carotenoids are also available [38]. Anoxygenic phototrophic bacteria naturally produce a range of carotenoids (such as okenone and lycopene derivatives) which may have commercial interest [37, 39] (Fig. 3).

The most important role of carotenoids in phototrophic organisms is in protection from light [40]. The detrimental effects of light are much more serious under aerobic conditions than under anaerobic conditions because excitation of (bacterio) chlorophylls under aerobic conditions causes formation of reactive oxygen species (ROS). Mechanistically this occurs by reaction of excited triplet-state chlorophyll (^{3}Chl*) with ground-state molecular oxygen (O_2), which results in formation of singlet oxygen ($^1O_2$). Singlet oxygen is extremely reactive and detrimental to the cell. Carotenoids quench ^{3}Chl* and thereby prevent formation of singlet oxygen. This means carotenoids cannot be completely removed from phototrophic organisms growing under aerobic conditions. However, phototrophic bacteria growing under anaerobic conditions do not have this requirement and therefore carotenoids are not essential for anoxygenic phototrophic bacteria. This in turn means the carotenoids of these bacteria in principle are freely available for biosynthetic engineering.

Genetic engineering has been used to synthesize the commercially valuable carotenoid lycopene in the purple non-sulfur bacterium *R. rubrum* [41]. Here, the indigenous carotenoid biosynthetic pathway was interrupted by targeted gene inactivation and as a result the cells accumulated lycopene as the sole carotenoid in a content of 2 mg/g cell dry weight. Although this is not high compared to the yield obtained in genetically engineered *E. coli* (33 mg/g cell dry weight lycopene as the sole carotenoid [42]), additional engineering of *R. rubrum* could surely increase the yield. Using a similar approach, lycopene and zeta-carotene have also been produced as the sole carotenoid species in GSB by genetic manipulation of *Chlorobaculum tepidum* [43]. Because carotenoids are not required in anaerobic phototrophic bacteria, the flux of isoprene precursors to carotenoid biosynthesis in these organisms could be redirected to any isoprenoid compound such as valuable plant-type terpenoids [44].

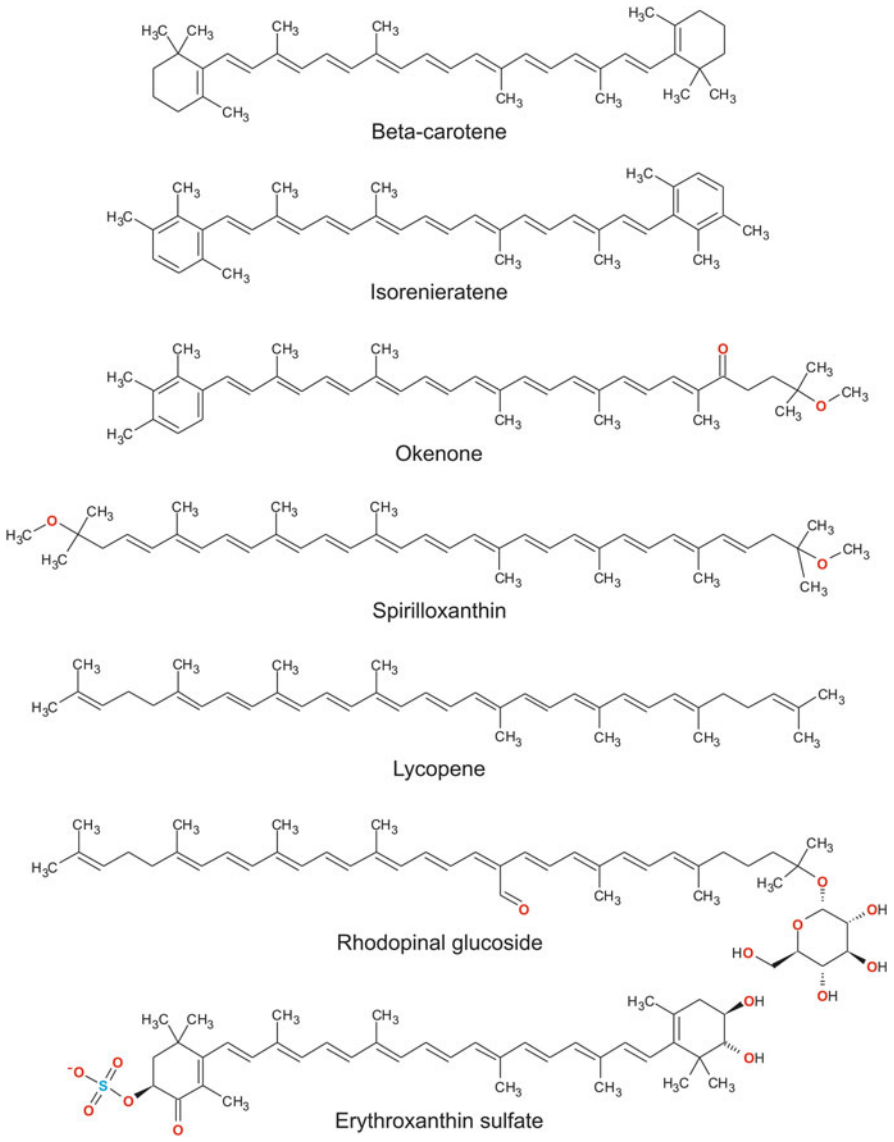


Fig. 3 Examples of carotenoids found in anoxygenic phototrophic bacteria. From [39] and Carotenoid Database (http://carotenoiddb.jp/)

8 Production of Functional Membrane Proteins

To study the structure and function of proteins, it is useful to overexpress the proteins in a foreign host organism to obtain amounts sufficient for experimentation. However, membrane proteins pose a challenge because they often denature in

the absence of a suitable membrane environment. For example, heterologous membrane proteins that are overexpressed in *E. coli* in a functional form are usually found in much lower titers than heterologous soluble proteins and tend to form inclusion bodies. To overcome this problem, the PNSB *Rba sphaeroides* has been developed as a host for overexpression of functional membrane proteins [45]. This system takes advantage of the large content of intracellular membranes in purple bacteria which hold the membrane-bound antennae and enzymes of the photochemical machinery. In *Rba. sphaeroides* these internal membranes form intracytoplasmic membrane (ICM) vesicles that sequester newly synthesized foreign proteins and enable easy purification following cell lysis. Using this system, challenging membrane proteins in a functional form have recently been prepared and examined: human aquaporin 9 (hAQP9), human tight junction protein occludin (Occ), *Rba. sphaeroides* cellulose synthase enzyme complex (BcsAB), and *Rba. capsulatus* cytochrome c_y [46].

References

1. Blankenship RE (2008) Molecular mechanisms of photosynthesis. Blackwell Science, Oxford, UK
2. Hohmann-Marriott MF, Blankenship RE (2011) Evolution of photosynthesis. Annu Rev Plant Biol 62:515–548
3. Bryant DA, Frigaard N-U (2006) Prokaryotic photosynthesis and phototrophy illuminated. Trends Microbiol 14(11):488–496
4. Blankenship RE, Madigan MT, Bauer CE (1995) Anoxygenic photosynthetic bacteria. Springer, The Netherlands
5. Idi A et al (2015) Photosynthetic bacteria: an eco-friendly and cheap tool for bioremediation. Rev Environ Sci Biotechnol 14(2):271–285
6. Hunter CN et al (2009) The purple phototrophic bacteria, Advances in photosynthesis and respiration. Springer, London
7. Zeng Y et al (2014) Functional type 2 photosynthetic reaction centers found in the rare bacterial phylum *Gemmatimonadetes*. Proc Natl Acad Sci U S A 111(21):7795–7800
8. Cohen Y, Gurevitz M (2006) The cyanobacteria—ecology, physiology and molecular genetics. Prokaryotes 4:1074–1098
9. Frigaard N-U, Dahl C (2009) Sulfur metabolism in phototrophic sulfur bacteria. Adv Microb Physiol 54:103–200
10. Hurse TJ, Kappler U, Keller J (2008) Using anoxygenic photosynthetic bacteria for the removal of sulfide from wastewater. In: Sulfur metabolism in phototrophic organisms. Springer, Dordrecht, pp 437–460
11. Holkenbrink C et al (2011) Sulfur globule oxidation in green sulfur bacteria is dependent on the dissimilatory sulfite reductase system. Microbiology 157(Pt 4):1229–1239
12. Henshaw PF, Zhu W (2001) Biological conversion of hydrogen sulphide to elemental sulphur in a fixed-film continuous flow photo-reactor. Water Res 35(15):3605–3610
13. Garcia GP et al (2015) Biological sulphide removal from anaerobically treated domestic sewage: reactor performance and microbial community dynamics. Environ Technol 36 (17):2177–2189
14. Syed M et al (2006) Removal of hydrogen sulfide from gas streams using biological processes – a review. Can Biosyst Eng 48:2.1–2.14

15. An JY, Kim BW (2000) Biological desulfurization in an optical-fiber photobioreactor using an automatic sunlight collection system. J Biotechnol 80(1):35–44
16. Kim YJ, Kim BW, Chang HN (1996) Desulfurization in a plate-type gas-lift photobioreactor using light emitting diodes. Korean J Chem Eng 13(6):606–611
17. Basu R, Clausen EC, Gaddy JL (1996) Biological conversion of hydrogen sulfide into elemental sulfur. Environ Prog 15(4):234–238
18. Pandey A, Singh P, Iyengar L (2007) Bacterial decolorization and degradation of azo dyes. Int Biodeter Biodegr 59(2):73–84
19. Bin Y et al (2004) Expression and characteristics of the gene encoding azoreductase from *Rhodobacter sphaeroides* AS1.1737. FEMS Microbiol Lett 236(1):129–136
20. Liu G-f et al (2006) Bacterial decolorization of azo dyes by *Rhodopseudomonas palustris*. World J Microbiol Biotechnol 22(10):1069–1074
21. Wang X et al (2008) Biodecolorization and partial mineralization of Reactive Black 5 by a strain of *Rhodopseudomonas palustris*. J Environ Sci (China) 20(10):1218–1225
22. Kim TTH et al (2003) Decolorization of azo dyes by purple non-sulfur bacteria. In: Annual Report of FY 2002, The Core University Program between Japan Society for the Promotion of Science (JSPS) and National Centre for Natural Science and Technology (NCST). pp 112–118
23. Wang X et al (2015) Formation characteristics of an anoxygenic photosynthetic bacterial biofilm in a photorotating biological contactor for azo dye wastewater treatment. J Chem Technol Biotechnol 90(1):176–184
24. Mutharasaiah K, Govindareddy V, Karigar C (2010) Photobiodegradation of halogenated aromatic pollutants. Adv Biosci Biotechnol 01(03):238–240
25. Dixit R et al (2015) Bioremediation of heavy metals from soil and aquatic environment: an overview of principles and criteria of fundamental processes. Sustainability 7(2):2189–2212
26. Seki H, Suzuki A, Mitsueda S-I (1998) Biosorption of heavy metal ions on *Rhodobacter sphaeroides* and *Alcaligenes eutrophus* H16. J Colloid Interface Sci 197(2):185–190
27. Watanabe M et al (2003) Biosorption of cadmium ions using a photosynthetic bacterium, *Rhodobacter sphaeroides* S and a marine photosynthetic bacterium, *Rhodovulum* sp. and their biosorption kinetics. J Biosci Bioeng 95(4):374–378
28. Buccolieri A et al (2006) Testing the photosynthetic bacterium *Rhodobacter sphaeroides* as heavy metal removal tool. Ann Chim 96(3–4):195–203
29. Sasaki K et al (2013) Simultaneous removal of cesium and strontium using a photosynthetic bacterium, *Rhodobacter sphaeroides* SSI immobilized on porous ceramic made from waste glass. Adv Biosci Biotechnol 04(01):6–13
30. Panwichian S et al (2011) Removal of heavy metals by exopolymeric substances produced by resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds. Electron J Biotechnol 14(4). http://dx.doi.org/10.2225/vol14-issue4-fulltext-2
31. Magnin JP, Gondrexon N, Willison JC (2014) Zinc biosorption by the purple non-sulfur bacterium *Rhodobacter capsulatus*. Can J Microbiol 60(12):829–837
32. Sakurai H et al (2013) Photobiological hydrogen production: bioenergetics and challenges for its practical application. J Photochem Photobiol C 17:1–25
33. Lazaro CZ, Varesche MBA, Silva EL (2015) Sequential fermentative and phototrophic system for hydrogen production: an approach for Brazilian alcohol distillery wastewater. Int J Hydrogen Energy 40(31):9642–9655
34. Zürrer H, Bachofen R (1979) Hydrogen production by the photosynthetic bacterium *Rhodospirillum rubrum*. Appl Environ Microbiol 37(5):789–793
35. Najafpour GD, Younesi H (2007) Bioconversion of synthesis gas to hydrogen using a light-dependent photosynthetic bacterium, *Rhodospirillum rubrum*. World J Microbiol Biotechnol 23(2):275–284
36. Warthmann R, Cypionka H, Pfennig N (1992) Photoproduction of H2 from acetate by syntrophic cocultures of green sulfur bacteria and sulfur-reducing bacteria. Arch Microbiol 157(4):343–348

37. Kirti K et al (2014) Colorful world of microbes: carotenoids and their applications. Adv Biol 2014:1–13
38. Sun Z et al (2014) Microalgae as the production platform for carotenoids. Recent advances in microalgal biotechnology. OMICS Group eBooks, Foster City, CA, USA, pp 1–17
39. Takaichi S (1999) Carotenoids and carotenogenesis in anoxygenic photosynthetic bacteria. In: The photochemistry of carotenoids. Kluwer, Dordrecht, pp 39–69
40. Fraser NJ, Hashimoto H, Cogdell RJ (2001) Carotenoids and bacterial photosynthesis: the story so far. Photosynth Res 70(3):249–256
41. Wang GS et al (2012) High-level production of the industrial product lycopene by the photosynthetic bacterium *Rhodospirillum rubrum*. Appl Environ Microbiol 78(20):7205–7215
42. Chen YY et al (2013) Chromosomal evolution of *Escherichia coli* for the efficient production of lycopene. BMC Biotechnol 13:6
43. Frigaard NU et al (2004) Genetic manipulation of carotenoid biosynthesis in the green sulfur bacterium *Chlorobium tepidum*. J Bacteriol 186(16):5210–5220
44. Englund E et al (2015) Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide. ACS Synth Biol 4(12):1270–1278
45. Laible PD, Mielke DL, Hanson DK (2009) Foreign gene expression in photosynthetic bacteria. In: The purple phototrophic bacteria. Springer, Dordrecht, pp 839–860
46. Erbakan M et al (2015) Advancing *Rhodobacter sphaeroides* as a platform for expression of functional membrane proteins. Protein Expr Purif 115:109–117

Adv Biochem Eng Biotechnol (2016) 156: 155–194
DOI: 10.1007/10_2016_11

Published online: 9 June 2016

Biological Processes for Hydrogen Production

Ed W. J. van Niel

Abstract Methane is produced usually from organic waste in a straightforward anaerobic digestion process. However, hydrogen production is technically more challenging as more stages are needed to convert all biomass to hydrogen because of thermodynamic constraints. Nevertheless, the benefit of hydrogen is that it can be produced, both biologically and thermochemically, in more than one way from either organic compounds or water. Research in biological hydrogen production is booming, as reflected by the myriad of recently published reviews on the topic. This overview is written from the perspective of how to transfer as much energy as possible from the feedstock into the gaseous products hydrogen, and to a lesser extent, methane. The status and remaining challenges of all the biological processes are concisely discussed.

Keywords Dark fermentation, Electrohydrogenesis, Hydrogen productivity, Hydrogen yield, Mesophiles, Photofermentation, Thermodynamics, Thermophiles

Contents

Ed W.J van Niel (✉)
Division of Applied Microbiology, Lund University, P.O. Box 124, 221 00 Lund, Sweden
e-mail: ed.van_niel@tmb.lth.se

Abbreviations

AcCoA	Acetyl-Coenzyme A
AD	Anaerobic digestion
BES	Bio-electrochemical systems
BHP	Biological hydrogen process
CBC	Calvin–Benson cycle
CEF	Cyclic electron flow
CEM	Cation exchange membrane
CSTR	Continuous stirred tank reactor
DF	Dark fermentation
DFE	Dark fermentation effluent
DOT	Dissolved oxygen tension
DW	Dry weight
E_{EMF}	Electromotive force (V)
E_{MEC}	Overall actual energy requirement of the system (V)
EMP	Embden–Meyerhof pathway
EOC	Excreted organic compounds
Fd	Ferredoxin
FHL	Formate hydrogen lyase
FNR	Ferredoxin:NAD(P)$^+$ oxidoreductase
H_2ase	Hydrogenase
HE	Hydroelectrogenesis
HRT	Hydraulic retention time (h)
I	Current (A)
I_V	Volumetric current density (A m^{-2})
LCA	Life cycle assessment
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
PBR	Photobioreactor
PF	Photofermentation
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin:oxidoreductase
P_{H2}	Partial hydrogen pressure (Pa)
PHB	Polyhydroxybutyrate
PS I	Photosystem I
PS II	Photosystem II

Q_{H2}	Volumetric hydrogen productivity (mol $H_2\,L^{-1}\,h^{-1}$)
r_{CAT}	Cathodic hydrogen recovery
RubisCO	Ribulose 1,5-biphosphate carboxylase/oxygenase
R_Ω	All the resistances in the system (Ω)
UA	Up-flow anaerobic reactor
W_{H2}	Energy content of hydrogen produced (J)
W_P	Energy of the power source (J)
W_S	Energy of the converted substrate (J)
Y_{H2}	Hydrogen yield (mol H_2/mol substrate)
η_A	Sum of contributions to the overpotential of the anode (V)
η_C	Sum of contributions to the overpotential of the cathode (V)
η_{TOT}	Overall energy recovery of the system

1 Introduction

In the light of creating a sustainable society, the interest in both hydrogen and biomethane is increasing. The global biogas market is expected to double between 2011 and 2022 from $ 17.3 to 33.1 billion [1]. There is increasing decentralized production for local demand (farmers and municipalities) and production for "greening" the natural gas grid. The global hydrogen market, on the other hand, is steadily increasing from about $ 87.5 billion (2011) to $ 118 billion in 2016 [2]. However, hydrogen is mainly produced thermochemically from petroleum and to a small extent through electrolysis of water, as industrial biological hydrogen processes (BHPs) are as yet non-existent. Today hydrogen is mainly used as an industrial reducing agent (oil, food, electronics, ammonia), for which a cost of about 1–2 € kg H_2^{-1} is set based on the estimated oil prices for 2020 [3]. The increasing demand for hydrogen is especially driven by ever stricter regulatory norms of removing sulfur from petroleum products. Hydrogen as an energy carrier is, as yet, only a niche market, mainly because of a lack of a comprehensible hydrogen fuel infrastructure and an effective hydrogen storage technology. Introducing CO_2 taxes is seen as a driver on the long road to a hydrogen economy [3].

Apart from water used as the source for hydrogen in biophotolysis, feedstocks for hydrogen and methane can be derived as wastes from forestry, agriculture, industry (e.g., food industry), and domestic waste. In addition, special energy crops can be cultivated which do not compete with edible crops. Regarding the biological production of gaseous fuels, anaerobic digestion is the most common and widely applied process. The product biogas, mainly a mixture of methane and carbon dioxide, may need to be purified depending on its use (vehicle fuel or the natural gas grid). Anaerobic digestion (AD) occurs naturally in places rich in organic waste, and is a straightforward process which can be applied, depending on the investment, with low-tech installations. Interestingly, hydrogen is a temporary intermediate in the fermentation process as hydrogen producers are essential members of the microbial consortium. Thus, in principle the fermentation can be

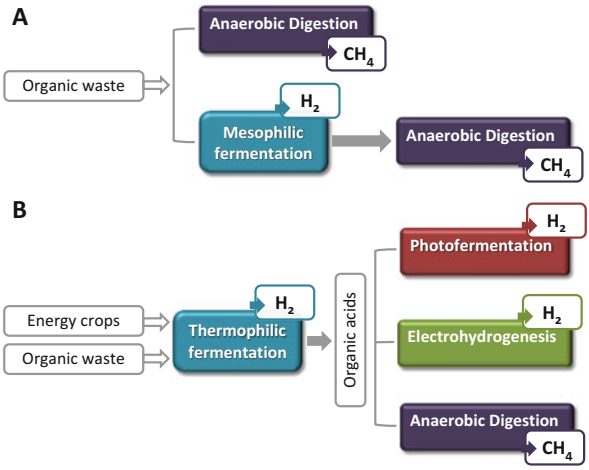


Fig. 1 Various configurations of hydrogen and/or biogas production from "cheap" feedstock (organic wastes) or dedicated energy crops. (**a**) Cheap feedstock may lead to simplification of gaseous energy carrier production, including emphasizing on hydrogen productivity, hence mesophilic dark fermentation. (**b**) Expensive feedstocks lead to emphasizing maximizing hydrogen yields, hence thermophilic dark fermentation combined with conversion steps of the organic acids that require either sunlight input (photofermentation) or electricity input (electrohydrogenesis). An alternative could be a hydrogen-methane two-step fermentation process

halted at hydrogen production by removing or inhibiting the methanogens. The drawback of this alternative process is that maximally only one-third of the energy content – on a hexose basis – is captured in the gaseous product. The remaining energy is left in the organic byproducts, but can be extracted in a second stage process consisting of photofermentation (PF), hydroelectrogenesis (HE), or methanogenesis (Fig. 1). Hence, a complete biomass conversion is accomplished by integration of two processes, i.e., a dark fermentation (DF) converting the organic feedstock to hydrogen and organic acids followed by a process that converts the organic acids to either hydrogen or methane. DF, a fermentation without light, comes in two variations depending on the type of bacteria used: (1) mesophilic, operating between 25 and 35 °C and (2) thermophilic, operating between 55 and 80 °C. The two-stage or hybrid hydrogen production process has been discussed earlier [4–6]. This process setup is required to maximize the energy yield contained in the biomass source to make the process sustainable (minimal waste!) and economically feasible.

The choice of mesophilic or thermophilic DF depends on the choice of feedstock:

1. If the feedstock is cheap then the hydrogen yield is less important; instead opt for high productivities for which mesophilic bacteria are the best choice
2. If specific energy crops or biomass pretreatment is necessary, then efficacy lies in high product yields rather than productivities; hence the choice falls on thermophilic bacteria

Where necessary, pretreatment of biomass increases accessibility of the microorganisms to the substrates [7]. The majority of raw biomass, especially lignocellulosics, consists of rigid materials which have to undergo a thermochemical treatment to destroy the delicate intertwined, fiber structure of the various polymers, i.e., lignin, cellulose, and hemicellulose. In this step, chopped up biomass is treated with steam using acid (sulfuric acid or phosphoric acid) or alkaline (lime or ammonia) water. Often this is followed by a hydrolysis step with a cocktail of commercial enzymes, including cellulases and xylanases. Updated cost analyses related to these different biological hydrogen processes (BHPs) have been published in the last 4–8 years [8, 9].

This chapter looks into the current status of each BHP process and highlights challenges that are still to be faced before an economical feasible process is possible. These challenges are of microbial, physical, and technical nature and solutions have to be found with minimal environmental impact. That is the reason why not one BHP process has moved far beyond the lab scale, and experience has been gained only with some pilot-scale installations. Biophotolysis is a standalone BHP process and can be carried out either aerobically or anaerobically. Therefore, it is not part of an integrated process (Fig. 1), but can deliver surplus algae or cyanobacteria biomass as a feedstock for one of the fermentation processes.

2 Background Information

Essential background information is provided here in order to follow the discussion of each of the processes below.

In principle, there are two different types of electron sources to make hydrogen, i.e., H_2O and organic compounds. The former is the sole original source in the biophotolysis process, whereas in the fermentation processes both electron sources are involved. This is demonstrated by the overall conversion reactions given below.

In biophotolysis, water is split, which demands a very high input of energy from solar radiation:

$$2\,H_2O + h\nu \rightarrow 2\,H_2 + O_2 \quad \Delta G^{0'} = +749\,\mathrm{kJ}\cdot\mathrm{mol}^{-1} \tag{1}$$

In the other BHP processes, sugar-based biomass is mainly used, consisting of both hexoses and pentoses. For the sake of convenience the reactions and hydrogen yields (Y_{H2}) are all based on the hexose glucose. Therefore, the stoichiometrically maximum yield of 12 H_2 per glucose according to [10]:

$$C_6H_{12}O_6 + 12\,H_2O \rightarrow 12\,H_2 + 6\,HCO_3^- + 6\,H^+ \quad \Delta G^{0'} = +3.2\,\mathrm{kJ}\cdot\mathrm{mol}^{-1} \tag{2}$$

is endergonic and thus not thermodynamically feasible. However, ideally it is possible to extract one-third of this total in a fermentation reaction yielding acetate as a byproduct:

$$C_6H_{12}O_6 + 4\,H_2O \rightarrow 4\,H_2 + 2\,HCO_3^- + 2\,CH_3COO^- + 4\,H^+$$
$$\Delta G^0 = -206.3\,\text{kJ} \cdot \text{mol}^{-1} \quad (3)$$

In mesophilic DF hydrogen can also be formed in the conversion of sugars to butyrate:

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,H_2 + 2\,HCO_3^- + C_3H_7COO^- + 3\,H^+$$
$$\Delta G^0 = -254.8\,\text{kJ} \cdot \text{mol}^{-1} \quad (4)$$

but at a lower stoichiometry, and is therefore not favored.

Conversion of the remaining two-thirds of the electrons stored in acetate to hydrogen is strongly endergonic:

$$CH_3COO^- + 4\,H_2O \rightarrow 4\,H_2 + 2\,HCO_3^- + H^+ \quad \Delta G^{0\prime} = +104.6\,\text{kJ} \cdot \text{mol}^{-1} \quad (5)$$

and thus needs an external energy source to push this reaction to the right. Sustainable external energy sources can be either solar radiation (photofermentation) or electricity from, e.g., windpower, solar cells, or microbial fuel cells (electrohydrogenesis).

Acetate can also be favorably converted to methane by acetoclastic methanogens:

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad \Delta G^{0\prime} = -31\,\text{kJ} \cdot \text{mol}^{-1} \quad (6)$$

All these metabolic conversions proceed under mild conditions, i.e., 30–80 °C and neutral to slightly acidic pH [11].

In the large body of BHP literature many different units are used for productivity. For the sake of comparison in this chapter the unit for volumetric hydrogen productivity (Q_{H2}) [mmol H_2 L reactor^{-1} h^{-1}] is used and [mol H_2 mol substrate^{-1}] for the hydrogen yield (Y_{H2}). Only the best results obtained so far have been gathered here to judge the order of magnitude of each BHP technology (Table 1). For detailed lists see the references to reviews mentioned below.

Table 1 Comparative overview of the best obtained hydrogen yields (Y_{H2}) and maximum potential capacities of volumetric hydrogen productivities (Q_{H2}) so far encountered among the various biotechnological techniques available

Process	Organism	Substrate	Y_{H2} mol $H_2 \cdot$ mol subst^{-1}	Max. Q_{H2} mmol $\cdot L^{-1} \cdot h^{-1}$	Reference
Direct biophotolysis	*Chlamydomonas reinhardtii*	H_2O	–	0.1–0.5	[12]
Indirect biophotolysis	*C. reinhardtii* *Anabaena variabilis*	Starch Starch	– –	2.00 1.68	[13] [14]
Photofermentation fed batch	*Rhodobacter sphaeroides*	DL-Malate	4.38	4.1	[15]
Mesophilic fermentation	Mixed culture on granular sludge	Sucrose	3.5	600	[16]
Thermophilic fermentation	*Thermoanaerobacterium thermosaccharolyticum*	Glucose	3.4–3.9	160	[17]
Electrohydrogenesis	Undefined consortia	Acetate (0.8 V)	3.8	5.2	[18]

3 Hydrogen Production Processes

3.1 Biophotolysis

With five decades of biophotolytic hydrogen production, investigations are still strong and ongoing, whereby two different research lines have been explored, i.e., direct biophotolysis and indirect biophotolysis with both algae and cyanobacteria. However, the majority of studies have remained at lab scale, of which only a few have progressed to pilot plant scale. These studies on photosynthetic metabolism, strategies for improvements and photobioreactor (PBR) development have been discussed recently in dedicated reviews and book chapters [19–23].

3.1.1 Oxygenic Photosynthetic Microbes

Biophotolysis is the only process where eukaryotes (algae) and prokaryotes (cyanobacteria) are exploited in BHP. Yet the hydrogen-producing algae and cyanobacteria share quite similar photosynthetic constitutions and pathways to channel electrons to hydrogen production (Fig. 2).

There are differences (Fig. 2) and one of the most obvious is that cyanobacteria can use nitrogenase to produce H_2 under non-nitrogen fixing conditions according to the following reaction:

$$2\,H^+ + 2\,e^- + 4\,ATP \rightarrow H_2 + 4\,ADP + 4\,Pi \tag{7}$$

which is an energy-demanding reaction. Hydrogen is a byproduct under nitrogen fixing conditions:

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,Pi \tag{8}$$

which is even more energy-expensive (fourfold) to gain H_2 and thus should be avoided.

Overall, hydrogen production rates observed with photosynthesis are relatively low (Table 1), especially for direct biophotolysis. Because oxygen irreversibly inhibits the hydrogenase, light-to-hydrogen conversion efficiencies are $<0.1\%$, which is considered impractical for commercial use [24]. Indirect biophotolysis indeed increased the Q_{H2} by an order of magnitude (Table 1). Yet the conversion efficiencies remained below 1%. A major breakthrough to increase hydrogen evolution has been obtained through creating conditions of sulfur limitation [25] as a means to deactivate PS II and thereby preventing oxygen generation. As a consequence, the environment becomes anaerobic, which induces the synthesis of an [FeFe]-hydrogenase that combines electrons and protons from the low active PS II and storage products [26]. Nevertheless, less than 10% of photosynthesis capacity is channeled to hydrogen production because of light saturation [11]. To improve

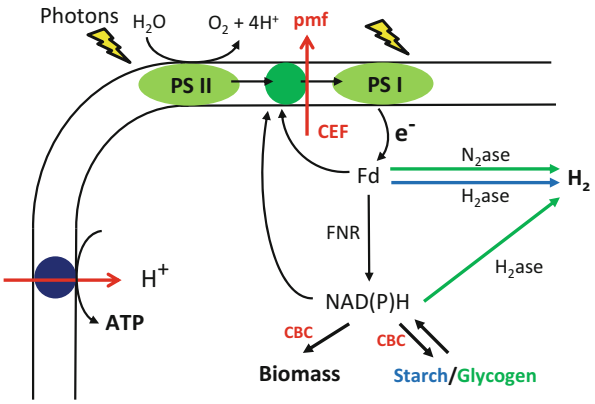


Fig. 2 Principle of direct and indirect biophotolysis in the oxygenic photosynthetic microbes. The major purpose of the photosystem II (PS II) in algae and cyanobacteria is to generate electrons through water splitting. These electrons are transferred via electron carriers in the electron transport chain to photosystem I (PS I) in the thylakoid membranes. PS I reduces the electron carrier ferredoxin (Fd), which is a cofactor for various enzymes. For direct hydrogen production, reduced Fd passes its electrons in algae to [FeFe]-hydrogenase (H_2ase) and in cyanobacteria to nitrogenase (N_2ase). Second, Fd can recycle the electrons in the electron transport chain around PS I (cyclic electron flow, CEF), which competes with hydrogen production during anaerobiosis. Finally, reduced Fd can donate its electrons to ferredoxin:NAD(P)$^+$ oxidoreductase (FNR) to generate NAD(P)H. The latter can be involved in cyanobacteria in direct hydrogen production by passing its electrons to an [NiFe]-hydrogenase. NAD(P)H is also important for biomass formation and starch (algae) or glycogen (cyanobacteria) production by donating its electrons to the electron transfer chain to produce ATP via the proton motive force (pmf) or to the Calvin-Benson cycle (CBC), using ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) as the key-enzyme to fix CO_2. Indirect biophotolysis can take place in the dark through fermentation of starch (glycogen), whereby the electrons are carried via NAD(P)H to a hydrogenase. *Blue arrows and text*: algae only; *green arrows and text*: cyanobacteria only

Q_{H2} further, researchers have looked into engineering better strains based on the understanding of the metabolism of photosynthesis and H_2 production.

Electrons liberated in PS II are distributed over at least three competing pathways, i.e., hydrogen production, cyclic electron flow (CEF), and the Calvin–Benson cycle (CBC) (Fig. 2). For direct biophotolysis, strategies to diminish CEF were indeed enhancing hydrogen production [27]. However, under direct biophotolysis conditions the CBC is essential for autotrophic growth [28], and thus deletion of this pathway might be lethal. Instead, it may be possible to lower the electron flux through the CBC by modifying the expression or engineering of RubisCO [26, 29]. Indirect biophotolysis relies on electrons being directed to storage product formation to enhance H_2 production. This was successfully accomplished in an engineered *C. reinhardtii* strain accumulating large quantities of starch [30]. Another approach for directing electrons to hydrogen production would be to engineer hydrogenases for higher affinities for Fd_{red} to better compete with the other pathways [31]. Likewise, introducing heterologous ferredoxins that show better affinities or protein engineering the interacting surfaces of the electron

donor and acceptor are potential strategies for both algae and cyanobacteria [32]. Finally, making chimeric complexes between electron carriers and electron acceptors is a recent approach that resulted in successes only in in vitro systems, but similar fusions have shown in vivo successes only in *Escherichia coli* so far [32].

3.1.2 Photobioreactor

For the design of a PBR the light regime and light conversion efficiency are very important factors [33]. The reactor requires a large surface area to volume ratio for optimal light availability per cell in the reactor. Therefore, the choices for closed systems are usually tubular and flat-panel reactors, and for open systems the pond or pool configuration. In the case of H_2 production, it is obvious that one should use closed, gas-tight systems to capture this gaseous product. For industrial-scale indirect biophotolysis a two-reactor in tandem system is likely to be used. As hydrogen production is disconnected from growth and oxygen production, the latter can take place in open systems, of which the raceway is a long-time favorite [22]. This system allows the best conditions for growth and carbon storage production and fewer variables need to be controlled (e.g., temperature and mixing conditions). To speed up growth, active supply of CO_2 into the liquid is required for meeting the carbon demand and maintaining a correct pH. Grown cells are subsequently centrifuged and pumped into the second, closed reactor and kept under sulfur deprivation, allowing the storage products to be fermented to H_2. For proper operation of the closed process it is essential to monitor many variables, including flow rates, pH, dissolved oxygen tension (DOT), H_2, and sulfur content. Still, outdoor tests with *C. reinhardtii* reached Q_{H2} of only about 0.024 mmol $H_2\,L^{-1}\,h^{-1}$ [34], whereas *A. variabilis* reached productivities as high as 1.68 mmol $H_2\,L^{-1}\,h^{-1}$ (Table 1). In theory, indirect biophotolysis leads to about 40% of the energy efficiency from light to H_2 of direct biophotolysis [35]. This is because of (1) more steps being involved to extract the captured energy, and (2) significant amounts of ATP are required for the nitrogenase (cyanobacteria only). Still, it more than compensates for the losses of direct biophotolysis with its inherent inhibitory nature of oxygen.

Because light conversion efficiencies tend to decrease at higher light intensities as a result of light saturation of the photosynthetic apparatus, light should be diluted by distribution over the entire reactor volume. Adequately mixing the culture therefore becomes essential to expose the cells only briefly to the light, plus it avoids sedimentation and nutrient gradients.

Because biophotolysis requires large surface areas, a detailed cost analysis is of the utmost importance to minimize material and operation costs. A strategic location of PBRs is part of this, as factors such as light environment, climate, land space, and availability of water should be considered. For upscaling, modular design is the most effective way to increase surface area, bringing flexibility of handling to the system, and minimizing efforts for mixing.

3.1.3 Conclusions and Challenges

The most crucial parameter of all photosynthetic processes is the photon conversion efficiency. Further, direct biophotolysis with oxygenic phototrophs is not a viable commercial option as the produced oxygen inhibits the hydrogenases. Therefore – next to sulfur deprivation – indirect biophotolysis is the best strategy to produce H_2, but requires a more complex reactor configuration and process operation. Interestingly, hydrogen production via direct biophotolysis can be further improved using designed co-cultures of the oxygenic photosynthetic microorganism with another microorganism that removes oxygen through respiration. For instance, co-cultivation of *C. reinhardtii* with *Bradyrhizobium japonicum*, a symbiotic rhizobium of the soybean *Glycine max*, resulted in improved Y_{H2} and 14-fold higher Q_{H2} [36]. This is an interesting field which needs to be further explored.

At present there is great uncertainty as to what scaling effects lie in store when progressing to pilot scale, as current calculations are based on data gathered from lab-scale experiments. As large surface areas are required for PBRs, because of low photon conversion efficiencies, it involves high costs for investment (material and land area) and operation. Yet, for further development of this BHP, abundant pilot-scale experience is required.

3.2 Photofermentation

The advantages of purple non-sulfur bacteria (PNSB) are (1) they do not produce oxygen, (2) they convert a broad variety of organic substrates, and (3) they harvest photons at a wide light spectrum (300–1,000 nm). Photofermentation has been extensively investigated with synthetic media, various organic waste streams, hydrolysates, and effluents from dark fermentations (DFE), in both indoor and outdoor situations, and was recently reviewed [21, 37]. Many different waste streams of the food industry, such as dairy food, molasses, olive mill waste (especially in the Mediterranean), and tofu (especially in Asia), can be directly converted by PNBS using light as an external energy source. The choice of feedstock is generally strain dependent, meaning that a screening for an adequate species needs to precede the optimization of the fermentation process. Still, many biological and technological parameters need to be optimized to arrive at a sustained process, and are briefly discussed below.

3.2.1 Feedstock

Most studies have been performed using artificial media, partly to optimize the system and partly to determine possible maximum productivities and yields without complications associated with complex feedstocks. One of the important

parameters of the feedstock is the carbon to nitrogen ratio (C/N ratio). It is essential that the concentration of the N-source is low enough to avoid repression of expression levels of nitrogenase. Ammonium is a strong inhibitor, but glutamate appeared to be an adequate alternative. A C/N-ratio of 25 for a feedstock containing mainly acetate and glutamate resulted in improved productivities and yields [38]. However, to reduce costs, it is essential to find cheap replacements for glutamate, most probably by using smart combinations of waste streams which are complementary in nutrients. Many feedstocks are short in particular nutrients, such as iron and molybdenum, which need to be added for optimal functioning of nitrogenase and proteins of the electron transport chain. In addition, the buffer capacity needs to be high enough to keep the pH between 6.5 and 8.0. This is of particular importance because it is very difficult to control the pH in large surface area bioreactors. Most probably the buffer capacity can best be increased with bicarbonate as phosphate is not a sustainable solution. However, this needs to be investigated as it might lead to higher CO_2 concentrations which can become inhibitory [39].

Even though PNSB can theoretically convert all 24 electrons in glucose to H_2, they prefer organic acids [40]. Moreover, in practice PNSB reach only a fraction of this maximum yield because of excretion of intermediates [41].

Raw feedstocks and hydrolysates are not transparent and contain particles that absorb precious light in the photobioreactor. For instance, the light penetration into the reactor to a depth of 1 cm is 51% for molasses dark fermentation effluent (DFE) compared to 89% for a clear artificial medium [42]. In addition, the absorption spectrum of the feedstock should not overlap too much with that of the PNSB. Therefore, a pretreatment step maybe required, such as filtration or decolorization. Clay treatment is a promising method as it removes the majority of light absorbing compounds though hardly affecting the preferred compounds [43]. Finally, the feedstock should be kept as anaerobic as possible. Oxygen does not kill the PNSB, but shifts its metabolism and thus decreases the hydrogen production rate and yield.

3.2.2 PNSB Strains

As in cyanobacteria, hydrogen production is catalyzed by a molybdenum nitrogenase (Mo-N_2ase), which is abundantly present in the cytoplasm, as a compensation for its slow reactivity (electron turnover ~5 s^{-1}). The latter explains its rate of H_2 production (approx. 1.3 mmol H_2 mg $protein^{-1}$ min^{-1}) being one order of magnitude lower than for hydrogenases, and matching the Q_{H2} of the hydrogen-utilizing oxygenic phototrophs [40]. Nitrogenase is expressed when the soluble N-source (NH_4^+) is below a certain critical concentration. In the presence of N_2 the Mo-N_2ase catalyzes the fixation of this gas molecule into ammonia, thereby producing hydrogen as a byproduct (8), but under non-fixing conditions of N_2 the energy demand, as with cyanobacteria, for hydrogen production by Mo-N_2ase is fourfold lower (7).

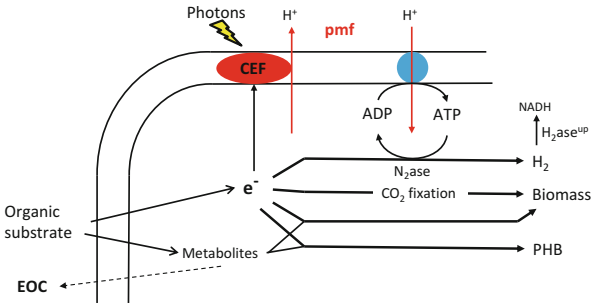


Fig. 3 Principle of photofermentation metabolism. Organic substrates donate electrons (e^-) in a pool of reductants, which are used for several purposes. (1) Electrons transferred to nitrogenase are converted together with protons to H_2, a reaction that requires ATP, making this reaction, in contrast to hydrogenases, quite irreversible (7). However, H_2 can partly donate electrons to NADH by an uptake hydrogenase (H_2ase^{up}). (2) Electrons together with light – in a cyclic electron flow (CEF) in the electron transport chain in the cell membrane – can create a pmf to enable ATP production. (3) Electrons are used for producing biomass via CO_2 fixation in the CBC and to some extent to produce polyhydroxybutyrate (PHB) for storage of carbon and electrons. Part of the organic substrates is C-source for biomass, PHB production, and excreted organic compounds (EOC). If the fermentation process is allowed enough time the EOC are taken up again and consumed [44]

Genetic modification has been another approach [23], in addition to determining optimal environmental conditions.

Several genetic engineering strategies have been carried out for improving hydrogen yields and productivities (Fig. 3), i.e., (1) removing the uptake hydrogenase, (2) removing the CBC pathway, and (3) increasing the expression of the proteins involved in directing electrons to nitrogenase and overexpressing the latter. Knocking out of the gene (Hup) coding for the uptake hydrogenase improved hydrogen production by *Rb. sphaeroides* as tested indoors in an artificial medium [45]. In addition, one of the highest Q_{H2} (approx. 2 mmol $H_2\,L^{-1}\,h^{-1}$) with a Y_{H2} of 3.1 mol H_2 mol acetate^{-1} was measured for the *Rb. capsulatus* hup$^-$ mutant on DFE of molasses in an outdoors flat panel reactor [46]. Deleting the RuBisCO genes in a *Rhodopseudomonas palustris*, possessing no uptake hydrogenase and containing constitutively expressed nitrogenase, indeed improved Y_{H2} [44]. However, it was more effective for succinate and butyrate (both twofold increase) as substrates than for acetate (only 1.3-fold increase), because of the metabolism of the former two substrates being connected to a higher CBC flux than for acetate. Conveniently, the constitutively expressed nitrogenase made growth and hydrogen production possible in the presence of NH_4^+ [47]. Overexpressing the Rnf complex in *Rb. capsulatus* [48] and NifA, encoding the specific transcriptional regulator of all *nif* genes, in *Rb. sphaeroides* [49] did improve H_2 production. The former study succeeded in increasing the electron flow to nitrogenase as it was rate-limiting in the wild type. In the latter study the expression of nitrogenase was made constitutive and perhaps increased its activity, whereby the H_2 production increased by 20%.

3.2.3 Light Radiation

Even though the majority of studies have been carried out indoors with artificial light, for cost-effective operation photobioreactors should be outside using sunlight. The major results of a wide selection of these studies are listed in recent reviews [21, 37]. So far, only a few studies have been performed outdoors, where additional issues affect sustained operation: (1) day and night rhythm, (2) temperature, and (3) light intensity. As sunlight is the light source, the culture needs to adjust to the day-night regime. Indeed, delay in growth and H_2 production for more than a week has been observed for outdoor conditions [50]. Second, sunlight contains infrared light and the biochemical reactions produce heat, and hence cooling is required to keep the temperature between 20 °C and 45 °C. This cooling is either accomplished internally [22] or by sprinkling water on the outer surface [36], although the latter may introduce cracks in the panels depending on the material.

3.2.4 Bioreactor and Operation Conditions

To allow as much light penetration per surface area, the best reactors are either of the tubular or flat panel type. Both types of reactors' configuration and operation have been discussed in detail [22]. The limitations of each reactor type for photofermentation are similar to those of biophotolysis. Light penetration is one of the most important parameters to gain high hydrogen yields, and therefore, the diameter of the tubular reactor should not be too big. To receive similar portions of light, high recirculation can be applied, which is also an appropriate way of mixing the culture [37].

High organic acid concentrations have a detrimental effect on the start up of the photofermentation process. Therefore, the substrate concentration requires dilution, which increases the water demand even though part of the water can come from recirculation of treated wastewater from the entire process. After a lag phase growth starts without hydrogen evolution, and once the culture reaches a critical mass, hydrogen production is observed. Optimal production is seen with a cell density of 0.5–0.7 g DW L^{-1} [51] and concentrations of 30–40 mM acetate [52], beyond which hydrogen production activity may decline again. Therefore, it is also important to regulate the optimal cell density for sustained operation.

3.2.5 Conclusions and Challenges

Comparison between different feedstocks revealed that PNSB prefer short-chain organic acids, particularly acetate, with which the highest yields and productivities are achieved [37]. This is an appropriate property for considering this process as a process step in tandem with the DF process. The best procedure would be to use a

combination of mutations in one strain to maximize the electron flow in the cell to nitrogenase and preventing H_2 being consumed.

The photofermentation process requires rigorous control (including light penetration, pH, temperature, substrate concentration, adequate mixing in reactors with high ratio of surface area to volume, and cell density). The optimum temperature of the process for PNSB is 30–35 °C [53], and consequently cooling is often required for much of the day. Interestingly, there are moderate thermophilic PNSB growing at 40–45 °C [54], but up till now they have not been tested for H_2 production. It would be of interest whether these thermophiles may give higher productivities and yields.

3.3 Electrohydrogenesis

Biocatalyzed electrolysis, as performed in microbial electrolysis cells (MECs) or bio-electrochemical systems (BESs), is the most recent technique applied to renewable hydrogen production [55, 56]. It is a variant of the microbial fuel cell (MFC), but, instead of producing electricity, biochemical conversion takes place through addition of a low voltage from an external power source. In general, similar to conventional batteries and water electrolysis cells, MECs consist of two chambers separated from each other by a semi-permeable membrane to prevent diffusion of hydrogen to the anode chamber. An MEC needs to be completely anaerobic as oxygen would interfere with either chamber. In principle, microorganisms oxidize organic compounds in the anode chamber, thereby transferring electrons to the anode. Gaseous carbon dioxide leaves the anode chamber and the protons diffuse through a cation exchange membrane (CEM) to the cathode where, together with the electrons supplied by the cathode, they form hydrogen (Fig. 4).

3.3.1 Electrochemistry

A small input of electrical energy is required to accomplish the endothermic conversion of acetate under anaerobic conditions (3). The upper limit of the electromotive force (E_{EMF}) of the MEC is set by the half reactions at the electrodes:

$$E_{EMF} = E_{CAT} - E_{AN}$$

In theory, 0.14 V is adequate for H_2 production through biocatalyzed electrolysis of acetate. This is according to the equilibrium potentials for the two half reactions, i.e., oxidation of acetate (1 mol L^{-1}) and proton reduction. Thus, the supplied electricity enables the conversion of, for instance, acetate at the anode:

$$CH_3COOH + 2\,H_2O \rightarrow 2\,CO_2 + 8\,H^+ + 8\,e^- \qquad E^{0'} = -0.28\,V \qquad (9)$$

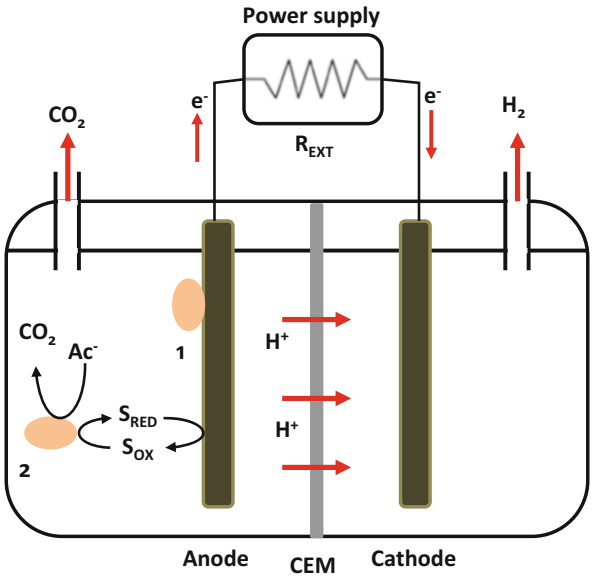


Fig. 4 Principle of the microbial electrolysis cell (MEC). Through oxidation of organic compounds (e.g., acetate Ac^-) microbes transfer electrons to the anode via (*1*) direct contact or (*2*) indirectly by an electron shuttle (reduced shuttle, S_{RED}). The shuttle is reoxidized at the anode and returns back into the culture (S_{OX}). The protons (H^+) diffuse through a cation exchange membrane (CEM) to the cathode where, together with the electrons from the cathode, it is converted to hydrogen. As the anodic reduction potential is higher than the cathodic one, a small voltage must be applied to drive the reaction. The power supply possesses a certain resistance (R_{EXT}) which contributes to energy loss

Together with the electrons of the external power source, the protons are converted to hydrogen at the cathode:

$$8\,H^+ + 8\,e^- \rightarrow 4\,H_2 \qquad E^{0'} = -0.42\,V \tag{10}$$

In practice, a higher voltage is required because of (1) the electrons being partly consumed by the bacteria for their growth and maintenance requirements, (2) the ohmic resistance of the electrochemical systems, and (3) the overpotentials of the electrodes. Hydrogen production is usually observed at the cathode at a voltage of >-0.2 V, corresponding with an applied voltage of at least 0.22 V instead of 0.14 V.

Ohmic voltage losses in MECs are determined by (1) the resistance to electron flow through electrical conductors (electrodes and external circuitry), (2) resistance to ion flow through ionic conductors (electrolyte and membrane), and (3) the reactor size and spatial configuration [57, 58]. Reducing electrode spacing, increasing electrolyte conductivity, and choice of the electrode material with low

resistivity are therefore pivotal. Even more important, removing the membrane and turning the MEC into a single chamber has a most profound effect on the ohmic loss [18].

Electrode overpotentials in MECs are related to (1) activation losses, (2) coulombic losses, and (3) concentration losses [57]. To overcome activation energy of a redox reaction, additional energy is required. These activation losses are inherent to the mode of transfer of electrons to or from a substance reacting at the electrode surface (see below). To minimize activation losses the catalyst reaction kinetics should be improved together with increasing the operating temperature and increasing surface areas [57–59]. Concentration losses are related to ionic transport between anode and cathode, and when considering protons, the pH is of utmost importance. Most processes use bacteria that ferment optimally at neutral pH, but at the anode where proton formation is high the pH might drop several units. Likewise, at the cathode, conversion of the protons to H_2 increases the pH to 11 [60]. At both electrodes, therefore, steep pH gradients may exist, which contribute to overpotentials. This can be prevented by enforcing the buffering capacity of the feedstock [61].

3.3.2 Bacteria Involved

The bacteria that transfer electrons to the anode are called electrigens and their mode of transfer is either direct or indirect. Direct electron transfer is accomplished by cells attached to the anode via (1) outer membrane c-type cytochromes and (2) nanowires. The dominating bacterial species attached to the anode surface are Gram-negatives belonging to the phylum Proteobacteria [62], possessing c-type cytochromes in the outer cell membrane. The connection of this cytochrome with external electron transfer was proved by *omcS*$^{-}$ mutants (deletion of one of the genes coding for outer membrane cytochromes) showing reduced current production [63]. Pili type IV are relatively big protein filaments (4–5 nm diameter and 20 μm long) and have been recognized as a means of cell-to-cell communication. These pili effectively function in biofilms as distributors and dissipaters of electrons, hence the name ‘nanowires.’ These nanowires enable electrons to be transferred from biofilms as thick as 75 μm to the anode [64].

Indirect electron transfer uses soluble exogenous mediators or electron shuttles that get reduced by the electrigen and diffuses to the anode where reoxidation takes place (Fig. 4). These shuttles are organic compounds which are either produced by the electrigens (e.g., riboflavins [65]) or can in principle be added to the anode culture (e.g., humic acids [66]). However, because of delaying diffusion processes, these shuttles introduce unnecessary energy losses to the system, and thus are not a preferred option for cost-effective MECs [67].

3.3.3 Calculating Efficiencies of the System

The conversion of acetate to H_2 is mediated by a voltage over two electrodes, according to (not considering the CO_2 formation)

$$1\,\text{Acetate} \xrightarrow{(1)} 8\,H^+ + 8\,e^- \xrightarrow{(2)} 4\,H_2 \tag{11}$$

so that two new efficiencies are introduced: (1) coulombic efficiency and (2) cathodic efficiency. The coulombic efficiency can be influenced by, e.g., the presence of microorganisms which can consume the produced hydrogen, such as methanogens. This is often the case when working with undefined microbial consortia and applying too low a voltage to the system [18]. In addition, coulombic losses are also produced by bacteria using part of the electrons for growth and maintenance requirements. Therefore, a balance should be found between bacterial growth and electrode potential for optimal performance of the system.

The hydrogen productivity is proportional to the volumetric current density (I_v) and the cathodic hydrogen recovery (r_{CAT}):

$$Q_{H2} \approx I_v \cdot r_{CAT}$$

and the hydrogen yield is proportional to the current (I):

$$Y_{H2} \approx I$$

Because $V = R \cdot I$, it is obvious that the system becomes more efficient the lower the overall resistance.

The overall energy recovery of the system (η_{TOT}) is estimated as a ratio of the energy content of hydrogen produced (W_{H2}) and the energy added to the system, i.e., energy of the converted substrate (W_S) and the energy of the power source (W_P):

$$\eta_{TOT} = W_{H2}/(W_P + W_S)$$

3.3.4 Factors Affecting Efficiency

Optimal performance of an MEC depends on a combination of parameters: (1) applied voltage, (2) electrode quality and surface area, (3) solution conductivity, (4) microbes, (5) substrate, and (6) cation exchange membrane (CEM). Each of these parameters are briefly discussed below.

1. The minimum voltage necessary is 0.14 V as it is the difference between the two half reactions (9) and (10). However, a voltage of at least 0.22 V is necessary in practice to overcome resistance in the system, but higher voltages up to 0.7 V has

been seen to increase the Q_{H2} [18]. All in all, the potential remains significantly below the value for electrolyzing water (1.6 V). Moreover, it should be noted that too high applied voltages can irreversibly damage biofilms, resulting in declining efficiency of the system [18].

2. Electrodes should have several qualities, including possessing high conductivity and high specific surface area, and should be non-corrosive, non-fouling, inexpensive, easy to manufacture, and scalable [59]. Both carbon and graphite electrodes meet the majority of these requirements, and are especially used as the material for the anode. To increase the surface area, brush-type electrodes are now the regular choice [18]. Graphite-based anodes require heat treatment prior to use as it leads to faster start up. For cathodes usually platinum electrodes are used in lab-scale experiments, but that would make the MEC technology too expensive for scaling up. Fortunately, microbial bio-cathodes have been developed that successfully catalyzed hydrogen production [68]. However, these cathodes have low cathodic hydrogen recovery yields (21%) and maximum hydrogen productivities are in the order of 0.067 mmol $H_2\,L^{-1}\,h^{-1}$.
3. Increase of the solution conductivity improves hydrogen production, but only to a certain limit as high values are detrimental to microbial activity [69]. Call and Logan [18] showed that increasing the solution conductivity increased the hydrogen production rates but decreased the total energy recovery. Clearly, an optimum should be determined here for each system at hand.
4. The majority of MEC studies mention the use of undefined microbial consortia, usually originating from sediments or wastewater treatment, leaving it up to a selection process as to which bacteria attach to the anode [55]. Thus far, pure culture studies were mainly performed with *Geobacter sulfurreducens* [56]. Interestingly, both options resulted in similar H_2 production rates and recovery yields. So far, no studies have been carried out to select better microorganisms. Most researchers remain with undefined consortia because of several advantages: (1) it improves system robustness, (2) no need to apply aseptic techniques against contaminations, and (3) greater potential for digesting a broader palette of organic compounds. However, care should be taken to avoid methanogen activity as it may remain persistent in the system once it has established itself [70]. To minimize methanogenic activity, several strategies can be followed, such as exposing the reactor to air between feeding cycles or applying polarity reversal at higher applied voltages for a short time [18, 70, 71].
5. Several MEC systems fed with different organics from sugars to fatty acids have been studied and a selection of the results is discussed in a review [56]. From these studies it became clear that acetate is the preferred substrate, as demonstrated by H_2 recovery yields >91% [72] compared to recovery yields of 10–28% with wastewater as substrate [73]. Note that for a high productivity the chamber should be well mixed for an optimal substrate flux to the biofilm, reducing diffusional gradients. This might add to energy-demanding mixing devices.
6. The membrane is traditionally applied coming from electrolysis of water, where the production of H_2 and O_2 should be kept separated. However, experience with

H_2 production in MECs revealed leakage of H_2 at the anode [55], indicating the fallible nature of the membrane. Furthermore, membranes hinder proton diffusion to the cathode, adding resistance to the system. Finally, membranes create a pH gradient across the membrane leading to substantial potential loss [74]. Therefore, removing the membrane altogether might improve operation. Indeed, the first studies with a single chamber MEC revealed a more than doubling of the hydrogen production rates at applied voltages of 0.3–0.8 V (Table 1, [18]), obtaining similar or higher hydrogen recoveries and higher energy recoveries. In addition, placing the electrodes close to each other meant that pH gradients were non-existent, adding to a lower ohmic loss. Thus, simplifying the design of MECs by removing the membrane is a way forward to cost-effective H_2 recovery. However, because H_2 is mixed with CO_2, a gas upgrading step is required.

Finally, performance optimization of MECs needs to take place. The challenge is to fine-tune the system pertaining to the type of substrate and microbial consortia applied.

Thus the overall actual energy requirement of the system (E_{MEC}) can be estimated [57]:

$$E_{MEC} = E_{EMF} - \sum \left(\eta_A + |\eta_C|\right) + I\left(\sum R_\Omega\right)$$

with η_A the sum of contributions to the overpotential of the anode, η_C the sum of contributions to the overpotential of the cathode, and IR_Ω the ohmic loss (R_Ω all the resistances in the system).

3.3.5 Conclusions and Challenges

Hydrogen production with MECs has undergone fast development in the last decade and a myriad of studies have demonstrated its potential to become an efficient and reliable technology. The knowledge obtained of MEC technology, including the microbiology and reactor configuration, can soon lead to real applications. Yet two major challenges are to be met in the near future before scaling up, i.e., low-cost cathode material and directions how to increase the Q_{H2}. The essential challenge is to find a solution for the expensive platinum cathode. Fortunately this expensive metal can be replaced by low-cost stainless steel and nickel alloys without loss in performance [75]. New electrodes have become available, consisting of combinations of materials (metals and carbon), although their manufacturing might be too costly for now [76]. The second major challenge, increasing Q_{H2}, can be met by optimizing MECs for high current densities with low overpotentials and low ohmic losses. This can be partly achieved by selecting improved anodic biofilms to enhance microbe–electrode interaction related to electron transfer [77].

Tests with the first MEC pilot scale (1 m^3) has revealed a longer initiation time to establish biofilms on the anode (~60 days) and maximum gas production was in the order of 0.32 mmol L^{-1} h^{-1}, although most of this was methane [78]. Even though this first reactor consisted of up-to-date technology (containing 24 modules, immersing brush anodes, and stainless steel cathodes), it again underlines the pivotal role of troubleshooting. It has been revealed that at a larger scale the operation conditions are crucial, especially at start up to initiate proper development of the microbial population.

Finally, both a thorough LCA and techno-economical evaluation is urgently required to determine the best options of this technology and how to implement it efficiently into other systems.

3.4 Dark Fermentation

About 73% of the research on dark fermentation (DF) has been carried out with mesophilic bacteria [79], whereas thermophilic DF has been researched to a lesser extent. Yet both share common process parameters that similarly affect the fermentation, such as partial hydrogen pressure (P_{H2}), pH, substrate concentration, and composition of the feedstock.

The P_{H2} is a key parameter as high hydrogen concentrations limit its own production because of a thermodynamic constraint (for the thermodynamics of hydrogen formation the reader is referred to reviews [80, 81]. If hydrogen is not removed effectively from the broth it easily accumulates at up to 12–70 times the equilibrium concentration because of liquid-to-gas mass transfer limitations [82]. As a consequence, the intracellular NADH/NAD^+ ratios rise, which shifts metabolism toward other reduced end-products rather than H_2 (Fig. 5) [24].

At the industrial scale, removal of H_2 using an inert gas (N_2) or CO_2 is not an option as it dilutes the H_2 gas, which drives up gas upgrading costs. In addition, CO_2 leads to acidification in the culture because of bicarbonate formation. As a consequence, more caustic agent is required to correct the pH. This unnecessarily increases the osmotic potential, thereby limiting hydrogen production [83]. Even though DF has been observed over a wide pH range [84], a slightly acidic pH (6–7) appears to be optimal for thermophilic H_2 production [85].

Increasing the substrate concentration is important for a cost-effective process, as relatively less water is required and it contributes to higher Q_{H2}. However, instead it has been observed that higher sugar concentrations led to decreases in Q_{H2} which can be because of the limitation of other nutrients, such as iron [86], or critical osmotic potentials [87].

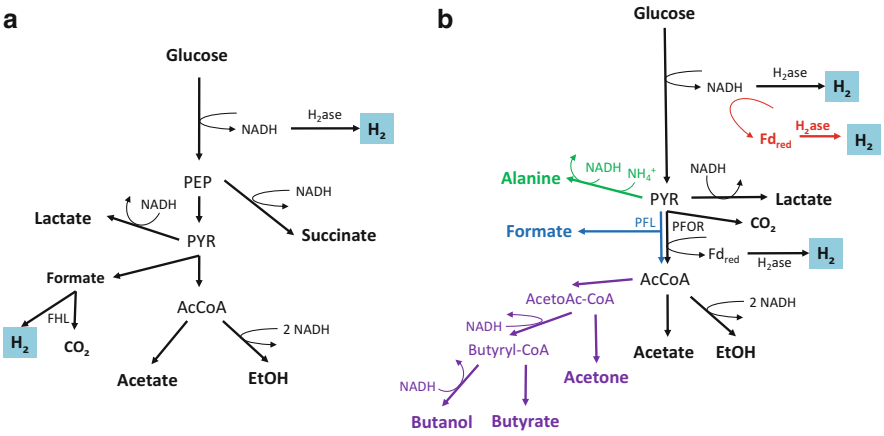


Fig. 5 Principle of hydrogen metabolism of dark fermentation. A variety of fermentative pathways exist because of facultative (**a**) and strict anaerobic (**b**) hydrogen producers. The common route for hexose metabolism is the Embden–Meyerhof pathway (EMP), although several hyperthermophilic archaea and bacteria employ both EMP and the Entner–Doudoroff pathway. (**a**) Optimal H_2 production in facultative anaerobes is via a hydrogenase (H_2ase) reoxidizing NADH combined with formate hydrogen lyase (FHL) which includes an [NiFe]-hydrogenase, thus producing CO_2 and acetate as by-products. (**b**) Strict anaerobic mesophiles have several catabolic pathways in common with the thermophiles (*black arrows*). Both possess pyruvate ferredoxin: oxidoreductase (PFOR) to catalyze the oxidation of pyruvate to acetyl-CoA (AcCoA), thereby delivering the electrons to ferredoxin. H_2 is produced via NADH and reduced ferredoxins (Fd_{red}) donating electrons to [FeFe]- and/or [NiFe]-hydrogenases. *Thermotoga maritima* contains a bifurcating hydrogenase using NADH and Fd_{red} simultaneously [88] (given in *red*). Strict anaerobic mesophilic hydrogen producers also possess other pathways, leading to less efficient hydrogen production (*purple arrows*). Among the thermophiles, *Thermotoga* species and *Pyrococcus furiosus* can also form and excrete alanine (given in *green*). *Caloramator celer* possesses pyruvate formate lyase (PFL) besides PFOR producing formate instead of H_2 and CO_2 [89] (given in *blue*). Depending on the microorganism, the alternative pathways to reoxidize NADH occur during conditions that create redox imbalances in the cell (leading to products such as succinate, lactate, ethanol, butanol, butyrate, acetone, alanine, and/or formate). *Abbreviations*: *PEP* phosphoenolpyruvate, *Pyr* pyruvate, *Ac-CoA* acetyl-CoA, *EtOH* ethanol

3.4.1 Mesophilic Fermentation

The biggest advantage of mesophilic DF is the capacity to reach very high volumetric hydrogen productivities (100–600 mmol $H_2\,L^{-1}\,h^{-1}$). Unfortunately, they are accompanied by relatively low Y_{H2} (<2.5 mol H_2 mol glucose^{-1}) (Table 1) [16] because of production of various other reduced byproducts (Fig. 5). For practical reasons, studies on mesophilic DF usually use undefined cultures as inocula originate from wastewater treatment, compost, or soil [79] (for a list of results see [21]). All these ecosystems contain both facultative and strict anaerobic hydrogen producers, often belonging to enterobacteriaceae and clostridia [84]. Mesophilic dark fermentations are relatively cheap and simple to operate with low or no contamination control, are very robust, and can take broad sources of feedstock

[90]. However, undefined inocula contain undesirable metabolic types such as methanogens. Therefore, a pretreatment of these inocula, such as acid shock or heat treatment, is carried out to minimize methanogenic activity. Reducing the HRT is an even better strategy to trim microbial diversity in the culture and clearly has been shown to increase the hydrogen yield [91]. Moreover, besides methanogens, with their low specific growth rates (0.017–0.02 h^{-1}), propionic acid bacteria are also removed, whereas hydrogenic bacteria remain [92, 93].

In the fermentation of sugars there are many by-products formed as NADH reoxidation is easily diverted from hydrogenase to other pathways (Fig. 5). To maximize NADH oxidation via the hydrogenase, the most practical solution is to remove hydrogen effectively from the culture broth. In the case of using pure cultures, knockouts of genes of competing pathways through metabolic engineering is an interesting option, which has indeed been shown to increase hydrogen production [94, 95].

3.4.2 Thermophilic Fermentation

Among thermophilic hydrogen production, three subclasses can be distinguished: moderate thermophiles ($50\ ^\circ C < T_{opt} < 64\ ^\circ C$), extreme thermophiles ($65\ ^\circ C < T_{opt} < 79\ ^\circ C$), and hyperthermophiles ($T_{opt} > 80\ ^\circ C$). The interest in thermophilic hydrogen production has increased in the last decade as there are several advantages attached to this process compared to mesophilic fermentation. Many thermophiles have been described to consume a wide range of sugars, including hexoses, pentoses, oligosaccharides, and polysaccharides such as cellulose and pectin (for extensive lists see [81, 96, 97]). According to a techno-economic evaluation, additional heat demand required for thermophilic DF did not incur significantly higher costs compared to mesophilic DF [98]. Instead, the production cost of DF is largely influenced by (1) the cost of media ingredients and (2) low substrate concentrations [98]. Yeast extract and phosphates are the most expensive components in the medium for which cheaper substitutes should be tested such as manure and urine. Alternatively, there are hydrogen producers that do not require a rich medium, as they can synthesize all amino acids and nearly all B vitamins [99]. A solution to using higher sugar concentrations is to apply osmotolerant strains, obtained through genetic engineering or evolutionary adaptation, which can stand higher substrate and product concentrations [100].

For an optimal DF process, an ideal hydrogen producer would be needed possessing superior properties, including (1) generating hydrogen at high Q_{H2} and Y_{H2}, (2) the ability to degrade a wide variety of biomass, (3) tolerating high sugar concentrations and fermentation products, (4) resisting inhibitors in the feedstock, (5) minimum requirement for a non-complex medium, (6) tolerating oxygen, and (7) easy to engineer genetically. However, so far none of the investigated organisms completely fulfil all these criteria, but thermophilic species of *Clostridium*, *Caldicellulosiruptor*, and the order of Thermotogales come very close, and, not surprisingly, are the most studied [81, 85, 101–104] in addition to *Cl. thermocellum*

[105]. Interestingly, the extreme thermophilic *Caldicellulosiruptor* species can degrade celluloses at the highest temperature so far found [106]. Working at higher temperatures lowers the chance of contamination, enabling one to work with pure cultures, and eliminates cooling of pretreated biomass. Another advantage of thermophilic hydrogen producers is the formation of less different by-products (Fig. 5). Thus, often only acetate is produced together with hydrogen near maximum theoretical yields (4 mol H_2 mol hexose^{-1}) under ideal growth conditions. Only under high P_{H2} or high osmolalities are reduced by-products such as lactate and ethanol produced [107]. Lactate formation is accompanied by less optimal growth and H_2 production and might not only be regulated by the redox ratio ($NADH/NAD^+$) but also by the energy status of the cell [108]. As several hydrogenic thermophiles are often isolated from terrestrial hot water springs with lignocellulosics as their primary substrates, they have adapted to low sugar concentrations and low osmolalities in general. Instead, they express a vast array of glycoside hydrolases to grow on (oligo)saccharides released during the rate-limiting breakdown of (hemi)celluloses. For that they adopt one of two strategies: either via secretion of exo hydrolases (e.g., *Caldicellulosiruptor* and *Thermotoga* spp.) or via a cellulosome attached to the outer cell surface (*Cl. thermocellum*) [109, 110].

3.4.3 DF Bioreactors

At lab scale the suspension culture in CSTR with sparging N_2 is the preferred system for fundamental research on hydrogen metabolism and factors influencing the Y_{H2} and Q_{H2}. However, this system has an upper limit for Q_{H2} of approximately 20 mmol H_2 L^{-1} h^{-1}, but can be increased threefold by increasing the cell density through immobilization [111]. Still, for an economically feasible process the rate should be an order of magnitude higher. The strategy foreseen to improve production yet keeping the operation costs low is based on increasing cell retention, using recirculation fluxes instead of sparging gas, and stirring to improve liquid-to-gas mass transfer rates. For that purpose, other bioreactor systems have been tested such as the up-flow anaerobic reactor (UA) [17], trickling filter [112], packed bed reactor [113], anaerobic sequencing blanket reactor [114], and membrane reactor [115]. These reactor types are further discussed in more detail elsewhere [116]. The most promising results obtained so far were obtained with the thermophile, *Thermoanaerobacterium thermosaccharolyticum* strain, forming biofilms on granular sludge in a UA reactor [17] (Table 1). However, granular sludge, originating from wastewater treatment, might give rise to contamination from hydrogenotrophic methanogens. This can be avoided by using other appropriate carrier material such as porous glass beads, as recently reported [117].

For optimizing the fermentation process it is important to avoid nutrient limitations, and consequently a feedstock needs to be supplied well-balanced in its elemental composition. Nearly all lignocellulosic-based feedstocks are low in vital elements such as nitrogen, phosphorus, sulfur, and trace elements. Therefore,

nutrients need to be added for allowing unlimited growth of the hydrogen producers. In lab-scale experiments yeast extract is often used, but this rich nutrient source is too costly for industrial application [98]. Cheap alternatives, such as manure, urine, and whey, need to be tested in combination with the carbon-rich feedstock.

3.4.4 Conclusions and Challenges

There are two choices of hydrogenic bacteria, either mesophiles or thermophiles. The practical advantage of mesophilic facultative anaerobes is a less stringent application of anaerobic conditions, making the process less expensive than thermophilic DF. Furthermore, if the feedstock is of low-grade organic waste, then it may be better to opt for high Q_{H2}. However, thermophilic DFs operating at $\geq$70 °C provide 'pasteurization conditions,' and thus are less inherent to contaminations (e.g., methanogens) and produce an effluent containing a smaller palette of by-products which complements most appropriately with the second process (either photofermentation or electrohydrogenesis). In addition, thermophilic DF leads to higher yields, which offers a wider choice of feedstocks from cheap waste to more expensive energy crops.

The biggest challenge for thermophilic DF is to increase the Q_{H2} by an order of magnitude to make it into a cost-effective process. The best way to tackle this might be a combination of several strategies: (1) (artificially) increasing cell densities (biofilm), (2) elevating osmotolerance (evolutionary adaptation), (3) designed co-cultures, and (4) applying an appropriate bioreactor configuration. These reactors should possess a proper manner of H_2 removal, thus excluding sparging gases to avoid expensive gas upgrading equipment. High cell densities of osmotolerant strains provide the solution for using high feed concentrations to reduce costs from water consumption and reactor material. Interestingly, applying designed co-cultures of two or more species, instead of pure cultures or undefined consortia, has been shown to create synergies based on complementary substrate utilization [118, 119], O_2 scavenging [120], extending optimal process conditions [121], kinship relation [122], and biofilm formation [123]. Finally, the ability to degrade lignocellulosic biomass either untreated, as recently shown for *Caldicellulosiruptor* species [124, 125], or in defined co-cultures [116], opens up new possibilities to explore whether consolidated bioprocess can be an economical viable replacement for the current proposal of a two-step pretreatment-DF.

Genetic engineering can be of interest to improve hydrogen producers through (1) eliminating pathways leading to undesirable by-product formation (such as lactate (e.g., [126]), (2) implementing new synthetic pathways to raise the Y_{H2} beyond the theoretical limits [127], and (3) constructing cells that are more robust against inhibitors and stresses (osmolality, inhibitors in hydrolysates) [128]. Most metabolic engineering has been carried out with mesophilic enterobacteria as they are relatively easy to manipulate genetically, but various challenges exist for strict anaerobic mesophilic and thermophilic hydrogen producers, including handling

under strict-anaerobic conditions, finding appropriate shuttle vectors and selection markers, and the presence of restriction modification systems preventing uptake of foreign DNA [85, 129]. Through trial and error, a few successes have been accomplished only recently.

4 Integrated Processes

The several possible combinations of BHP processes (Fig. 1b) are discussed below. With integration of these processes, new challenges are added on top of those of each single BHP process. Mostly they are related to the composition of the effluent of the dark fermentation (DFE) not being optimal for the second BHP. In general, all the obstacles related to tuning the two fermentation steps have to be dealt with before any integration can be realized. Few studies had looked beyond mere integration of two fermentation steps. One of the most intensive investigations, including mass, energy and exergy balances, and LCA, has been carried out for the DF-PF integration by the EU-funded project "Hyvolution" [130–133]. The outcome of the process simulations of this project may be similar for other integrated BHP processes. Of course, as a consequence of the simulations being based on experimental data that were available at that time, some conclusions possess limited validity. Nevertheless, it can be concluded that heat integration of effluent recirculation saves on the total required energy input and water demand (can be up to 90%) [132]. The latter is of particular importance when dealing with low substrate concentrations, and it provides a significant reduction in the environment impact of the process [133]. However, recirculation of fermentation effluents have the inherent problem of increasing osmolality. This is mainly because of the continuous correction of the pH with caustic agents (usually sodium or potassium hydroxide) in both fermentation processes. To prevent this, one should investigate the possibility of using other cheaper alternatives such as ammonia which can also be used as a nitrogen source. The outcome of the LCA study revealed that production of each process ingredient (phosphate, caustic agent, etc.) has nearly100% environmental impact, which is in great contrast with the impact of the DF-PF process itself which had a value tenfold lower than that of alternative hydrogen production processes, i.e., reformation of natural gas or the water gas shift reaction [133].

4.1 Integrated DF and PF

Recently an intensification of projects has taken place looking into the possibility of integration of mesophilic or thermophilic dark fermentation and photofermentation [21, 37, 134]. Demonstration of an integrated DF-PF system is currently lacking. Instead, researchers investigated the effect of the DFE on the photofermentation

process. The DF was run on either artificial media (glucose or sucrose as substrate) or pretreated biomass (wastewaters, potato starch, algal biomass, beet molasses), which have been reviewed in detail [4]. Use of artificial media and light is a means to determine the potential of the integrated system and how to tune the composition of the medium, considering each fermentation process step. One of the best results was obtained using a PBR with clay carriers and in situ optical fibers in addition to external light sources [135]. With this mesophilic-DF-PF system, maximum Y_{H2} values of 7.1 mol H_2 mol hexose^{-1} were obtained with a Q_{H2} of 1.2 mmol $H_2\,L^{-1}\,h^{-1}$ and nearly 90% carbon conversion. This study showed that elaborate light distribution significantly contributed to overall Y_{H2} and carbon conversion.

Using realistic feedstocks revealed new bottlenecks such as background color, particles, concentration levels of inhibitors and substrates, and buffering capacity. This may include redesigning the medium composition for the DF to be tuned with the criteria for PF. As an example, the ammonium concentration needs to be within a specific range in the initial feedstock, i.e., the minimum depends on the growth requirements in the DF and the maximum on the threshold value in the DFE that influences nitrogenase expression levels in the PNBS. Hence, it is required to know how much ammonium is consumed in the DF and the ammonium threshold value for the strain(s) used in the PF (average around 2 mM [36]). However, concentration variations are inherent to fermentation processes, and thus for the sake of process robustness it would be safer to remove the ammonium from the DFE by, e.g., electroseparation [136] or pretreatment with clinoptilolite (natural zeolite) [137] even though this brings in additional costs. Alternatively, ammonium concentration does not create any problems by applying ammonia-tolerant PNSB strains [47] which would be the most sophisticated solution.

For large-scale production, mild sterilization of the DFE might be necessary before it is added to the PF [37]. However, this is not required when the DF is thermophilic; although mild sterilization should be necessary for any additional components to the DFE, such as trace elements iron and molybdenum.

4.2 Integrated DF and MEC

Integration of the MEC with DF is an interesting strategy because (1) the MEC functions optimally with compounds that are typically byproducts of the DF, especially acetate [72], (2) both processes are near scaling up, and (3) both possess high Y_{H2}, at least when considering thermophilic DF, and thus complete conversion of sugars can be expected with this combination.

So far, only a few studies have fed DFE to an MEC [138], of which the best performance was seen with a hydrogen-ethanol fermentation reaching 83% conversion to hydrogen and 70% energy recovery, but with a Q_{H2} of approx. 2.3 mmol $H_2\,L^{-1}\,h^{-1}$ [139]. An interesting approach was reported by Wang et al. [140] through implementing an MFC in the DF-MEC process that was fed with DFE to produce electricity for driving the MEC. In this way, no external energy source was

necessary for the MEC and thus can be a starting point to increase further the energy efficiency of the DF-MEC process.

Before any scaling up is possible, the MEC needs to be improved in performance – as discussed above – plus optimization of the buffer capacity of the feedstock [139].

4.3 Integrated DF and AD

In this case, two different gases are produced and either used separately or mixed, as the latter, called hythane, forms a cleaner fuel (lower production of CO and greenhouse gases) than methane alone when used in combustion engines. In the last decade, the number of studies on the DF-AD process have developed close to a mature state which is ready for scaling up [141]. The process is quite promising as high total energy yields can be reached combined with nearly zero waste. To obtain high total product yields, the best option is to combine thermophilic DF with mesophilic or thermophilic AD. Just to illustrate this fact, several studies are compared with respect to the obtained product yields and productivities (Table 1). It is clear that the thermophilic processes have higher yields for both hydrogen and methane. Various organic acids are produced in the mesophilic DF (e.g., [142]), which require a more complex consortium composition for the methanogenic reactor. Working with pure cultures or designed co-cultures of thermophiles in the DF results mainly in acetate and low quantities of lactate in the DFE [143], which narrows the consortium composition of the AD to mainly acetoclastic methanogens. The study by Kongjan et al. [144], using an undefined consortium, but enriched in hydrogenic thermophiles, lies somewhere in the middle of these two extremes as it produced low quantities of butyrate and propionate.

It can be concluded from these studies that superior performance of the DF-AD process is related to a DF process that produces a DFE containing mainly acetate which simplifies, and thus improves yields of the AD [146]. In addition, the DF-AD process is superior over the single-stage AD process because of higher waste treatment efficiencies [147, 148]. In addition, according to [149] the DF-AD process adds only little production costs to the AD process, although at least 10% more energy is gained. However, the productivities of both the DF and AD remain quite low in the studies (see, e.g., Table 2). One way is to use higher substrate concentrations, but then the microorganisms in both the DF and AD need to be adapted to higher osmolalities, for instance by evolutionary adaptation.

Other challenges are related to adjusting the DFE to the AD. Most important would be the pH, as the DF runs at slightly lower pH (5–6.5) than the AD (pH 7–8),

Table 2 Comparison of a selection of thermophilic vs mesophilic fermentations of the DF-AD process

Process	TDF–TAD [144]	TDF–AD [143]	DF–AD [142]
Inoculum DF	Hydrogenic enrichment from a thermophilic methanogenic reactor	*C. saccharolyticus* 70 °C	Heat-treated mesophilic methanogenic sludge
Inoculum AD	Methanogenic granular sludge	Mesophilic granular sludge	Mesophilic methanogenic sludge
Y_{H2} (mol mol^{-1})	1.4	2.1–3.4	0.5–1.2
Q_{H2} (mMol h^{-1})	1.5	2.0–5.2	–
Y_{CH4} (mol mol^{-1})[a]	1.9–2.7	2.4	1.9
Q_{H4} (mMol h^{-1})	0.8–3.5	2.7–4.4	–

All studies used pretreated lignocellulosic biomass as feedstock
TDF thermophilic dark fermentation, *TAD* thermophilic anaerobic digestion
[a]Approximated 1 mmol CH_4 g $COD^{-1} \equiv 0.19$ mol CH_4 mol glucose^{-1} [145]

and micronutrients need to be added [143]. Ca^{2+} is preferred over Na^+ for correcting the pH as acetoclastic methanogens are relatively sensitive to the latter [150].

5 Conclusions

Research on BHP processes is a very active area as is reflected in a decade of impressive progress in understanding and genetically improving the metabolism and improving technical cultivation of hydrogen producers. Genomics, genome-wide metabolic models, and molecular technologies have recently matured and are now also entering the field of BHP (e.g., [151, 152]). This is a welcoming asset as BHP has a lot of biological challenges still needing to be tackled and systems biology brings a new approach for finding solutions. On the one hand, undefined consortia, mostly enrichment cultures, can be applied which are related to high Q_{H2} but low Y_{H2}. Its advantage is that no or little investment has to be made in the control of contamination. On the other hand, there is the possibility of using pure cultures, for which the challenge is to find ways to improve both Q_{H2} and Y_{H2}. This can be done by genetic engineering and/or evolutionary adaptation. The disadvantage is the high control on contamination prevention. Another strategy lies somewhere in the middle of these two extremes, i.e., by exploiting synergies between two or more species in optimized designed co-cultures, for which genetic engineering might not be required. Which of these three options are to be applied might depend on the costs of the feedstock and should be determined by a careful techno-economical evaluation.

There are also plenty of technological challenges to face before any cost-effective process is possible. The majority of the research has been carried out at lab scale, and several technologies have moved on to – or are on the brink of – scaling up. An important shift has taken place from artificial media to more realistic feedstocks. Likewise, research with integrated BHP systems is increasing as more researchers recognize it as the most suitable way for future biohydrogen production. Important here is the development of operation control of the two processes with all the recirculation flows, heat integration, and gas upgrading included.

The intention of these integrated BHP processes is to convert the biomass-carbon of the feedstocks to CO_2, a waste gas which can be applied as aerial fertilizer for greenhouse agriculture or algae ponds, or used in industrial processes based on critical carbon dioxide.

Most attention has been paid to integrative DF-PF processes, revealing there are still many challenges to meet for overall optimization. Further, a major drawback of photofermentation is its dependency on the diffuse nature of solar radiation, dilute streams of organic matter, and limited conversion efficiencies. Consequently, in the current state it requires a huge surface area and material investments [153]. Breakthroughs are needed in smart light distribution if it is to meet a viable industrial BHP process. Instead, the integration of thermophilic DF with MEC might be a better option for the near future, especially as it is concluded here that these two processes are tailor-made for each other.

For the near future, it can be foreseen that more pilot-scale plants of the dark fermentation process, the one most closely resembling a conventional fermentation process, should be operational. An earliest commercial production of such a process would fit best via coupling with existing anaerobic digestion plants for zero-waste production. This leads to a win-win situation as it creates an opportunity to build up essential experience with larger-scale biohydrogen production and to improve the anaerobic digestion process. In addition, decentralized small-scale hydrogen production creates new opportunities, such as jobs at the rural level and new ways of investment for plants and equipment [154].

The complexity of this area lies partly in that each BHP has advantages and disadvantages. One major obstacle related to that is the inverse relationship between Y_{H2} and Q_{H2}. Thus, improving on yield often directly affects productivity and vice versa. Tackling these challenges requires the work to be done by multidisciplinary teams. Furthermore, in a practical way it depends on the goal of producing H_2 and whether to opt for fast or for efficient production. The former process can be carried out in a simpler setup, whereas the latter requires more efficient control. Selection of the appropriate process is further related to the cost of the feedstock and whether the BHP process becomes part of a biorefinery process. In that respect, one of the most fundamental conclusions coming out of all the work is that the BHP process needs to be tailor-made to the specific waste [37].

Scale up and optimized reactor configurations are the next major step necessary to arrive at viable BHP processes. In addition, these activities should be accompanied with rigorous LCA and techno-economical evaluations to enable direct feedback for finding sustainable solutions. High integration, including heat integration

and water recirculation, can indeed pay off to make the process more cost- and energy-efficient. However, for the consequential osmolality increase in the system, sustainable solutions need to be found. Preliminary LCA has revealed that integrated processes themselves are highly sustainable, but their high environmental impact is connected to the additional nutrients from non-sustainable origin. Therefore, for lowering the impact of BHP processes, part of the focus should be on finding (cheap) renewable sources for all ingredients required for optimal operation of the fermentations.

Acknowledgments This work was financially supported by the Swedish Energy Agency (Energimyndigheten; project number 31090-2).

References

1. Navigant Research (2012) http://www.navigantresearch.com/newsroom/global-biogas-market-to-nearly-double-in-size-to-33-billion-by-2022
2. MarketsandMarkets (2014) http://www.marketsandmarkets.com/PressReleases/hydrogen.asp
3. Mansilla C, Avril S, Imbach J, Le Duigou A (2012) CO_2-free hydrogen as a substitute to fossil fuels: what are the targets? Prospective assessment of the hydrogen market attractiveness. Int J Hydrog Energy 37:9451–9458
4. Adessi A, de Philippis R, Hallenbeck PC (2012) Combined systems for maximum substrate conversion. In: Hallenbeck PC (ed) Microbial technologies in advanced biofuel production. Springer, New York, pp 107–126
5. Guwy AJ, Dinsdale RM, Kim JR, Massanet-Nicolau J, Premier G (2011) Fermentative biohydrogen production systems integration. Bioresour Technol 102:8534–8542
6. Liu Z, Zhang C, Lu Y, Wu X, Wang L, Wang L, Han B, Xing X-H (2013) States and challenges for high-value biohythane production from waste biomass by dark fermentation technology. Bioresour Technol 135:293–303
7. Bundhoo MAZ, Mohee R, Hassan MA (2015) Effects of pre-treatment technologies on dark fermentative biohydrogen production: a review. J Environ Manag 157:20–48
8. Sen U, Shakdwipee M, Banerjee R (2008) Status of biological hydrogen production. J Sci Ind Res 67:980–993
9. Escapa A, Gómez X, Tartakovsky B, Morán A (2012) Estimating microbial electrolysis cell (MEC) investment costs in wastewater treatment plants: case study. Int J Hydrog Energy 37:18641–18653
10. Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180
11. Show K-Y, Lee D-J, Chang J-S (2011) Bioreactor and process design for biohydrogen production. Bioresour Technol 102:8524–8533
12. Laurinavichene TV, Fedorov AS, Ghirardi ML, Seibert M, Tsyganko AA (2006) Demonstration of sustained hydrogen production by immobilized, sulphur-deprived *Chlamydomonas reinhardtii* cells. Int J Hydrog Energy 5:659–667
13. Winkler M, Hemschemeier A, Gotor C, Melis A, Happe T (2002) [Fe]-hydrogenases in green algae: photo-fermentation and hydrogen evolution under sulphur deprivation. Int J Hydrog Energy 27:1431–1439
14. Sveshnikov DA, Sveshnikova NV, Rao KK, Hall DO (1997) Hydrogen metabolism of mutant forms of *Anabaena variabilis* in continuous cultures and under nutritional stress. FEMS Microbiol Lett 147:297–301

15. Li X, Dai Z-Z, Wang Y-H, Zhang S-L (2011) Enhancement of phototrophic hydrogen production by *Rhodobacter sphaeroides* ZX-5 using fed-batch operation based on ORP level. Int J Hydrog Energy 36:12794–12802
16. Wu S-Y, Hung C-H, Lin C-N, Chen H-W, Lee A-S (2006) Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnol Bioeng 93:934–946
17. O-Thong S, Prasertsan P, Karakashev D, Angelidaki I (2008) High-rate continuous hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* PSU-2 immobilized on heat-pretreated methanogenic granules. Int J Hydrog Energy 33:6498–6508
18. Call D, Logan BE (2008) Hydrogen production in a single chamber microbial electrolysis cell lacking a membrane. Environ Sci Technol 42:3401–3406
19. Adessi A, De Philippis R (2014) Photobioreactor design and illumination systems for H_2 production with anoxygenic photosynthetic bacteria: a review. Int J Hydrog Energy 39:3127–3141
20. Adessi A, De Philippis R (2014) Photosynthesis and hydrogen production in purple non sulphur bacteria: fundamental and applied aspects. In: Zannoni D, de Philippis R (eds) Microbial bioenergy: hydrogen production, vol 38, Advances in photosynthesis and respiration. Springer, Dordrecht/Heidelberg/New York/London, pp 269–290
21. Azwar MY, Hussain MA, Abdul-Wahab AK (2014) Development of biohydrogen production by photobiological, fermentation and electrochemical processes: a review. Renew Sustain Energy Rev 31:158–173
22. Fernández-Sevilla JM, Acién-Fernández FG, Molina-Grima E (2014) Photobioreactors design for hydrogen production. In: Zannoni D, de Philippis R (eds) Microbial bioenergy: hydrogen production, vol 38, Advances in photosynthesis and respiration. Springer, Dordrecht/Heidelberg/New York/London, pp 291–320
23. Sakurai H, Masukawa H, Kitashima M, Inoue K (2013) Photobiological hydrogen production: bioenergetics and challenges for its practical application. J Photochem Photobiol C: Photochem Rev 17:1–25
24. Hallenbeck PC, Benemann JR (2002) Biological hydrogen production: fundamentals and limiting processes. Int J Hydrog Energy 27:1185–1193
25. Melis A, Zhang LP, Forestier M, Ghirardi ML, Seibert M (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol 122:127–135
26. Hemschemeier A, Fouchard S, Cournac L, Peltier G, Happe T (2008) Hydrogen production by *Chlamydomonas reinhardtii*: an elaborate interplay of electron sources and sinks. Planta 227:397–407
27. Tolleter D, Ghysels B, Alric J, Petroutsos D, Tolstygina I, Krawietz D, Happe T, Auroy P, Adriano JM, Beyly A, Cuiné S, Plet J, Reiter IM, Genty B, Cournac L, Hippler M, Peltier G (2011) Control of hydrogen photoproduction by the proton gradient generated by cyclic electron flow in *Chlamydomonas reinhardtii*. Plant Cell 23:2619–2630
28. Melis A (2007) Photosynthetic H_2 metabolism in *Chlamydomonas reinhardtii* (unicellular green algae). Planta 226:1075–1086
29. Marin-Navarro J, Esquivel MG, Moreno J (2010) Hydrogen production by *Chlamydomonas reinhardtii* revisited: rubisco as a biotechnological target. World J Microbiol Biotechnol 26:1785–1793
30. Kruse O, Rupprecht J, Bader KP, Thomas-Hall S, Schenk PM, Finazzi G, Hankamer B (2005) Improved photobiological H_2 production in engineered green algal cells. J Biol Chem 280:34170–34177
31. Ducat DC, Sachdeva G, Silver PA (2011) Rewiring hydrogenase-dependent redox circuits in cyanobacteria. Proc Natl Acad Sci U S A 108:3941–3946
32. Kontur WS, Noguera DR, Donohue TJ (2012) Maximizing reductant flow into microbial H_2 production. Curr Opin Biotechnol 23:382–389

33. Akkerman I, Janssen M, Rocha JMS, Reith JH, Wijffels RH (2003) Photobiological hydrogen production: photochemical efficiency and bioreactor design. Dutch Biological Hydrogen Foundation, Petten, pp 124–145
34. Gianelli L, Torzillo G (2012) Hydrogen production with the microalga *Chlamydomonas reinhardtii* grown in a compact tubular photobioreactor immersed in a scattering light nanoparticle suspension. Int J Hydrog Energy 37:16951–16961
35. Prince RC, Kheshgi HS (2005) The photobiological production of hydrogen: potential efficiency and effectiveness as a renewable fuel. Crit Rev Microbiol 31:9–31
36. Wu S, Li X, Yu J, Wang Q (2012) Increased hydrogen production in co-culture of *Chlamydomonas reinhardtii* and *Bradyrhizobium japonicum*. Bioresour Technol 123:184–188
37. Eroglu I, Özgür E, Eroglu E, Yücel M, Gündüz U (2014) Applications of photofermentative hydrogen production. In: Zannoni D, de Philippis R (eds) Microbial bioenergy: hydrogen production, vol 38, Advances in photosynthesis and respiration. Springer, Dordrecht/Heidelberg/New York/London, pp 237–267
38. Androga DD, Özgür E, Eroglu I, Gündüz U, Yücel M (2011) Significance of carbon to nitrogen ratio on the long-term stability of continuous photofermentative hydrogen production. Int J Hydrog Energy 36:15583–15594
39. Koku H, Eroglu I, Gündüz U, Yücel M, Türker L (2002) Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*. Int J Hydrog Energy 27:1315–1329
40. Harwood CS (2008) Nitrogenase-catalyzed hydrogen production by purple nonsulfur photosynthetic bacteria. In: Wall J, Harwood CS, Demain A (eds) Bioenergy. ASM Press, Washington, pp 259–271
41. Yilmaz LS, Kontur WS, Sanders AP, Sohmen U, Donohue TJ, Noguera DR (2010) Electron partitioning during light- and nutrient-powered hydrogen production by *Rhodobacter sphaeroides*. Bioenergy Res 3:55–66
42. Boran E, Özgür E, Yücel M, Gündüz U, Eroglu I (2012) Biohydrogen production by *Rhodobacter capsulatus* in solar tubular photobioreactor on thick juice dark fermenter effluent. J Clean Prod 31:150–157
43. Eroglu E, Eroglu I, Gündüz U, Yücel M (2008) Effect of clay pretreatment on photofermentative hydrogen production from olive mill wastewater. Bioresour Technol 99:6799–6808
44. McKinlay JB, Harwood CS (2011) Calvin cycle flux, pathway constraints, and substrate oxidation state together determine the H_2 biofuel yield in photoheterotrophic bacteria. mBio 2, e00323-10
45. Kars G, Gündüz U, Rakhely G, Yücel M, Eroglu I, Kovacs KL (2008) Improved hydrogen production by uptake hydrogenase deficient mutant strain of *Rhodobacter sphaeroides* O. U.001. Int J Hydrog Energy 33:3056–3060
46. Avcioglu SG, Özgür E, Eroglu I, Yücel M, Gündüz Y (2011) Biohydrogen production in an outdoor panel photobioreactor on dark fermentation effluent of molasses. Int J Hydrog Energy 36:11360–11368
47. McKinlay JB, Harwood CS (2010) Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. Proc Natl Acad Sci U S A 107:11669–11675
48. Jeong H-S, Jouanneau Y (2000) Enhanced nitrogenase activities in strains of *Rhodobacter capsulatus* that overexpress the *rnf* genes. J Bacteriol 182:1208–1214
49. Liu T, Li X, Zhou Z (2010) Improvement of hydrogen yield by hupR gene knock-out and nifA gene overexpression in *Rhodobacter sphaeroides* 6016. Int J Hydrog Energy 35:9603–9610
50. Boran E, Özgür E, Yücel M, Gündüz U, Eroglu I (2012) Biohydrogen production by *Rhodobacter capsulatus* Hup^- mutant in pilot solar tubular photobioreactor. Int J Hydrog Energy 37:16437–16445

51. Gebicki J, Modigell M, Schumacher M, van der Burg J, Roebroeck E (2010) Comparison of two reactor concepts for anoxygenic H_2 production by *Rhodobacter capsulatus*. J Clean Prod 18:S36–S42
52. Özgür E, Afsar N, de Vrije T, Yücel M, Gündüz U, Claassen PAM, Eroglu I (2010) Potential use of thermophilic dark fermentation effluents in photofermentative hydrogen production by *Rhodobacter capsulatus*. J Clean Prod 18:S23–S28
53. Stevens P, Vertonghen C, de Vos P, de Ley J (1984) The effect of temperature and light intensity on hydrogen production by different *Rhodopseudomonas capsulata* strains. Biotechnol Lett 6:277–282
54. Favinger J, Stadtwald R, Gest H (1989) *Rhodospirillum centenum*, sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. Ant Leeuwenhoek 55:291–296
55. Rozendal RA, Hamelers HVM, Euverink GJW, Metz SJ, Buisman CJN (2006) Principle and perspectives of hydrogen production through biocatalyzed electrolysis. Int J Hydrog Energy 31:1632–1640
56. Wrana N, Sparling R, Cicek N, Levin DB (2010) Hydrogen gas production in a microbial electrolysis cell by electrohydrogenesis. J Clean Prod 18:S105–S111
57. Logan BE, Hamelers B, Rozendal R, Schroder U, Keller J, Freguia S, Aelterman P, Verstraete W, Rabaey K (2006) Microbial fuel cells: methodology and technology. Environ Sci Technol 40:5181–5192
58. Clauwaert P, Aelterman P, Pham TH, de Schamphelaire L, Carballa M, Rabaey K, Verstraete W (2008) Minimizing losses in bio-electrochemical systems: the road to applications. Appl Microbiol Technol 79:901–913
59. Logan BE (2008) Microbial fuel cells. Wiley, Hoboken
60. Yuan Y, Zhou SG, Tang JH (2013) In situ investigation of cathode and local biofilm microenvironments reveals important roles of OH- and oxygen transport in microbial fuel cells. Environ Sci Technol 47:4911–4917
61. Torres CI (2014) On the importance of identifying, characterizing, and predicting fundamental phenomena towards microbial electrochemistry applications. Curr Opin Biotechnol 27:107–114
62. Aelterman P, Rabaey K, De Schamphelaire L, Clauwaert P, Boon N, Verstraete W (2008) Microbial fuel cells as an engineered ecosystem. In: Wall J, Harwood CS, Demain AL (eds) Bioenergy. ASM, Washington, pp 307–320
63. Holmes DE, Chaudhuri SK, Nevin KP, Mehta T, Methe BA, Liu A, Ward JE, Woodard TL, Webster J, Lovley DR (2006) Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfureducens*. Environ Microbiol 8:1805–1815
64. Lovley DR (2008) Extracellular electron transfer: wires, capacitors, iron lungs, and more. Geobiology 6:225–231
65. Marsili E, Baron DB, Shikare ID, Coursolle D, Gralnick JA, Bond DR (2008) *Shewanella* secretes flavins that mediate extracellular electron transfer. Proc Natl Acad Sci U S A 105:3968–3973
66. Sund CJ, McMasters S, Crittenden SR, Harrell LE, Sumner JJ (2007) Effect of electron mediators on current generation and fermentation in a microbial fuel cell. Appl Microbiol Biotechnol 76:561–568
67. Mahadevan R, Bond DR, Butler JE, Esteve-Nunez A, Coppi MV, Palsson BO, Schilling CH, Lovley DR (2006) Characterization of metabolism in the Fe(III)-reducing organism *Geobacter sulfurreducens* by constraint-based modeling. Appl Environ Microbiol 72:1558–1568
68. Rozendal RA, Jeremiasse A, Hamelers H, Buisman C (2008) Hydrogen production with a microbial biocathode. Environ Sci Technol 42:629–634
69. Liu H, Grot S, Logan BE (2005) Electrochemically assisted microbial production of hydrogen from acetate. Environ Sci Technol 39:4317–4320
70. Clauwaert P, Verstraete W (2009) Methanogenesis in membraneless microbial electrolysis cells. Appl Microbiol Biotechnol 82:829–836

71. Tice RC, Kim Y (2014) Methanogenesis control by electric oxygen production in microbial electrolysis cells. Int J Hydrog Energy 39:3079–3086
72. Cheng S, Logan BE (2007) Sustainable and efficient biohydrogen production via electrohydrogenesis. Proc Natl Acad Sci U S A 104:18871–18873
73. Wagner RC, Regan JM, Oh SE, Zuo Y, Logan BE (2009) Hydrogen and methane production from swine wastewater using microbial electrolysis cells. Water Res 43:1480–1488
74. Rozendal RA, Hamelers HVM, Buisman CJN (2006) Effects of membrane cation transport on pH and microbial fuel cell performance. Environ Sci Technol 40:5206–5211
75. Selembo PA, Merrill MD, Logan BE (2009) The use of stainless steel and nickel alloys as low-cost cathodes in microbial electrolysis cells. J Power Sources 190:271–278
76. Logan BE (2010) Scaling up microbial fuel cells and other bioelectrochemical systems. Appl Microbiol Biotechnol 85:1665–1671
77. Yi H, Nevin KP, Kim BC, Franks AE, Klimes A, Tender LM, Lovley DR (2009) Selection of a variant of *Geobacter sulfurreducens* with enhanced capacity for current production in microbial fuel cells. Biosens Bioelectron 24:3498–3503
78. Cusick RD, Bryan B, Parker DS, Merrill MD, Mehanna M, Kiely PD, Liu G, Logan BE (2011) Performance of a pilot-scale continuous flow microbial electrolysis cell fed winery wastewater. Appl Microbiol Biotechnol 89:2053–2063
79. Li CL, Fang HHP (2007) Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit Rev Environ Sci Technol 37:1–39
80. Amend JP, Shock EL (2001) Energetics of overall metabolic reactions of thermophilic and hyperthermophilic archaea and bacteria. FEMS Microbiol Rev 25:175–243
81. Kengen SWM, Goorissen HP, Verhaart M, Stams AJM, van Niel EWJ, Claassen PAM (2009) Biological hydrogen production by anaerobic microorganisms. In: Soetaert W, Vandamme E (eds) Biofuels. Wiley, Oxford, pp 197–222
82. Pauss A, Andre G, Perrier M, Guiot SR (1990) Liquid-to-gas mass transfer in anaerobic processes: inevitable transfer limitations of methane and hydrogen in the biomethanation process. Appl Environ Microbiol 56:1636–1644
83. Willquist K, Claassen PAM, van Niel EWJ (2009) Evaluation of the influence of CO_2 as stripping gas on the performance of the hydrogen producer *Caldicellulosiruptor saccharolyticus*. Int J Hydrog Energy 34:4718–4726
84. Wang J, Wan W (2009) Factors influencing fermentative hydrogen production: a review. Int J Hydrog Energy 34:799–811
85. Pawar SS, van Niel EWJ (2013) Thermophilic biohydrogen production: how far are we? Appl Microbiol Biotechnol 97:7999–8009
86. Van Ginkel S, Logan BE (2005) Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environ Sci Technol 39:9351–9356
87. Van Niel EWJ, Claassen PAM, Stams AJM (2003) Substrate and product inhibition of the hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnol Bioeng 81:255–262
88. Schut GJ, Adams MWW (2009) The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. J Bacteriol 191:4451–4457
89. Ciranna A, Larjo A, Kivistö A, Santala V, Roos C, Karp M (2013) Draft genome sequence of the hydrogen- and ethanol-producing anaerobic alkalithermophilic bacterium *Caloramator celer*. Genome Announc 1, e00471-13
90. Hallenbeck PC, Ghosh D (2009) Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol 27:287–297
91. Venkata Mohan S (2009) Harnessing of biohydrogen from wastewater treatment using mixed fermentative consortia: process evaluation towards optimization. Int J Hydrog Energy 34:7460–7474

92. Hussy I, Hawkes FR, Dinsdale R, Hawkes DL (2003) Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. Biotechnol Bioeng 84:619–626
93. Zhang ZP, Show KY, Tay JH, Liang DT, Lee DJ, Jiang WJ (2006) Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. Process Biochem 41:2118–2123
94. Abo-Hashesh M, Ghosh D, Tourigny A, Taous A, Hallenbeck PC (2001) Single stage photofermentative hydrogen production from glucose: an attractive alternative to two stage photofermentationor co-culture approaches. Int J Hydrog Energy 36:13889–13895
95. Oh Y-K, Raj SM, Jung GY, Park S (2011) Current status of the metabolic engineering of microorganisms for biohydrogen production. Bioresour Technol 102:8357–8367
96. Rittmann S, Herwig C (2012) A comprehensive and quantitative review of dark fermentative hydrogen production. Microb Cell Fact 11:115
97. Van Niel EWJ, Willquist K, Zeidan AA, de Vrije T, Mars AE, Claassen PAM (2011) Hydrogen production by thermophilic fermentation. In: Azbar N, Levin D (eds) State of the art and progress in production of biohydrogen. Bentham Ebooks, Sharjah, pp 137–159
98. Ljunggren M, Zacchi G (2010) Techno-economic analysis of a two-step biological process producing hydrogen and methane. Bioresour Technol 101:7780–7788
99. Willquist K, van Niel EWJ (2012) Growth and hydrogen production characteristics of *Caldicellulosiruptor saccharolyticus* on chemically-defined minimal media. Int J Hydrog Energy 37:4925–4929
100. Pawar SS (2014) *Caldicellulosiruptor saccharolyticus*: an ideal hydrogen producer? PhD thesis, Lund University, Lund
101. Bielen AAM, Verhaart MRA, van der Oost J, Kengen SWM (2013) Biohydrogen production by the thermophilic bacterium *Caldicellulosiruptor saccharolyticus*: current status and perspectives. Life 3:52–85
102. Cappelletti M, Zannoni D, Postec A, Ollivier B (2014) Members of the order Thermotogales: from microbiology to hydrogen production. In: Zannoni D, de Philippis R (eds) Microbial bioenergy: hydrogen production, vol 38, Advances in photosynthesis and respiration. Springer, Dordrecht/Heidelberg/New York/London, pp 197–224
103. Willquist K, Zeidan AA, van Niel EWJ (2010) Physiological characteristics of the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Microb Cell Fact 10:11
104. Zurawski JV, Blumer-Schuette SE, Conway JM, Kelly RM (2014) The extremely thermophilic genus Caldicellulosiruptor: physiological and genomic characteristics for complex carbohydrate conversion to molecular hydrogen. In: Zannoni D, de Philippis R (eds) Microbial bioenergy: hydrogen production, vol 38, Advances in photosynthesis and respiration. Springer, Dordrecht/Heidelberg/New York/London, pp 177–195
105. Rydzak T, Grigoryan M, Cunningham ZJ, Krokhin OV, Ezzati P, Cicek N, Levin DB, Wilkins JA, Sparling R (2014) Insights into electron flux through manipulation of fermentation conditions and assessment of protein expression profiles in *Clostridium thermocellum*. Appl Microbiol Biotechnol 98:6497–6510
106. Ivanova G, Rákhely G, Kovács KL (2008) Hydrogen production from biopolymers by *Caldicellulosiruptor saccharolyticus* and stabilization of the system by immobilization. Int J Hydrog Energy 33:6953–6961
107. Ljunggren M, Willquist K, Zacchi G, van Niel EWJ (2011) A kinetic model for quantitative evaluation of the effect of hydrogen and osmolarity on hydrogen production by *Caldicellulosiruptor saccharolyticus*. Biotechnol Biofuels 4:31
108. Willquist K, van Niel EWJ (2010) Lactate formation in *Caldicellulosiruptor saccharolyticus* is regulated by the energy carriers pyrophosphate and ATP. Metab Eng 12:282–290
109. Blumer-Schuette SE, Kataeva I, Westpheling J, Adams MWW, Kelly RM (2008) Extremely thermophilic microorganisms for biomass conversion: status and prospects. Curr Opin Biotechnol 19:210–217

110. Blumer-Schuette SE, Brown SD, Sander KB, Bayer EA, Kataeva I, Zurawski JV, Conway JM, Adams MWW, Kelly RM (2013) Thermophilic lignocellulose deconstruction. FEMS Microbiol Rev 38:393–448
111. Koskinen PEP, Lay C-H, Puhakka JA, Lin P-J, Wu S-Y, Örlyggson J, Lin C-Y (2008) High-efficiency hydrogen production by an anaerobic, thermophilic enrichment culture from an Icelandic hot spring. Biotechnol Bioeng 33:1168–1178
112. Van Groenestijn JW, Geelhoed JS, Goorissen HP, Meesters KPM, Stams AJM, Claassen PAM (2009) Performance and population analysis of a non-sterile trickle bed reactor inoculated with *Caldicellulosiruptor saccharolyticus*, a thermophilic hydrogen producer. Biotechnol Bioeng 102:1361–1367
113. Peintner C, Zeidan AA, Schnitzhofer W (2010) Bioreactor systems for thermophilic fermentative hydrogen production: evaluation and comparison of appropriate systems. J Clean Prod 18:S15–S22
114. Prasertsan P, O-Thong S, Birkeland N-K (2009) Optimization and microbial community analysis for production of biohydrogen from palm oil mill effluent by thermophilic fermentative process. Int J Hydrog Energy 34:7448–7459
115. Oh S-E, Iyer P, Bruns MA, Logan BE (2004) Biological hydrogen production using a membrane bioreactor. Biotechnol Bioeng 87:119–127
116. Ren N, Guo W, Liu B, Cao G, Ding J (2011) Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up productions. Curr Opin Biotechnol 22:365–370
117. Ngo TA, Bui HTV (2013) Biohydrogen production using immobilized cells of hyperthermophilic Eubacterium *Thermotoga neapolitana* on porous glass beads. J Technol Innov Renew Energy 2:231–238
118. Liu Y, Yu P, Song X, Qu Y (2008) Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. Int J Hydrog Energy 33:2927–2933
119. Li Q, Liu C (2012) Co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* for enhancing hydrogen production via thermophilic fermentation of cornstalk waste. Int J Hydrog Energy 37:10648–10654
120. Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y (1998) H2 production from starch by a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*. Biotechnol Lett 20:143–147
121. Lu W, Wen J, Chen Y, Sun B, Jia X, Liu M, Caiyin Q (2007) Synergistic effect of *Candida maltosa* HY-35 and *Enterobacter aerogenes* W-23 on hydrogen production. Int J Hydrog Energy 32:1059–1066
122. Zeidan AA, Rådström P, van Niel EWJ (2010) Stable coexistence of two *Caldicellulosiruptor* species in a de novo constructed hydrogen-producing co-culture. Microb Cell Fact 9:102
123. Pawar SS, Vongkompean T, Gray C, van Niel EWJ (2015) Biofilm formation by designed co-cultures of *Caldicellulosiruptor* species as a means to improve hydrogen productivity. Biotechnol Biofuels 8:19
124. Talluri S, Raj SM, Christopher LP (2013) Consolidated bioprocessing of untreated switchgrass to hydrogen by the extreme thermophile *Caldicellulosiruptor saccharolyticus* DSM 8903. Bioresour Technol 139:272–279
125. Kataeva I, Foston MB, Yang S-J, Pattathil S, Biswal AK, Poole FL II, Basen M, Rhaesa AM, Thomas TP, Azadi P, Olman V, Saffold TD, Mohler KE, Lewis DL, Doeppke C, Zeng Y, Tschaplinkski TJ, York WS, Davis M, Mohnen D, Xu Y, Ragauskus AJ, Ding S-Y, Kelly RM, Hahn MG, Adams MWW (2013) Carbohydrate and lignin are simultaneously solubilized from unpretreated switchgrass by microbial action at high temperature. Energy Environ Sci 6:2186–2195
126. Cha M, Chung D, Elkins JG, Guss AM, Westpheling J (2013) Metabolic engineering of *Caldicellulosiruptor bescii* yields increased hydrogen production from lignocellulosic biomass. Biotechnol Biofuels 6:85

127. Chittibabu G, Nath K, Das D (2006) Feasibility studies on the fermentative hydrogen production by recombinant *Escherichia coli* BL-21. Process Biochem 41:682–688
128. Nicolaou SA, Gaida SM, Papoutsakis ET (2010) A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng 12:307–331
129. Taylor MP, van Zyl L, Tuffin IM, Leak DJ, Cowan DA (2011) Genetic tool development underpins recent advances in thermophilic whole-cell biocatalysis. Microb Biotechnol 4:438–448
130. Claassen PAM, de Vrije T, Koukios E, van Niel EWJ, Eroglu I, Modigell M, Friedl A, Wukovits W, Ahrer W (2010) Non-thermal production of pure hydrogen from biomass: HYVOLUTION. J Clean Prod 18:S4–S8
131. Foglia D, Ljunggren M, Wukovits W, Friedl A, Zacchi G, Urbaniec K, Markowski M (2010) Integration studies on a two-stage fermentation process for the production of biohydrogen. J Clean Prod 18:S72–S80
132. Modarresi A, Wukovits W, Foglia D, Friedl A (2010) Effect of process integration on the exergy balance of a two-stage process for fermentative hydrogen production. J Clean Prod 18: S63–S71
133. Ochs D, Wukovits W, Ahrer W (2010) Life cycle inventory analysis of biological hydrogen production by thermophilic and photofermentation of potato steam peels (PSP). J Clean Prod 18:S88–S94
134. Eroglu E, Melis A (2011) Photobiological hydrogen production: recent advances and state of the art. Bioresour Technol 102:8403–8413
135. Chen C-Y, Yang M-H, Yeh K-L, Liu C-H, Chang J-S (2008) Biohydrogen production using sequential two-stage dark and photo fermentation processes. Int J Hydrog Energy 33:4755–4762
136. Redwood MD, Macaskie LE (2007) Method and apparatus for biohydrogen production. British patent application no. 0705583.3, UK
137. Androga DD, Özgür E, Eroglu I, Gündüz U, Yücel M (2012) Amelioration of photofermentative hydrogen production from molasses dark fermenter effluent by zeolite-based remova of ammonium ion. Int J Hydrog Energy 37:16421–16429
138. Lalaurette E, Thammannagowda S, Mohagheghi A, Maness P-C, Logan BE (2009) Hydrogen production from cellulose in a two-stage process combining fermentation and electrohydrogenesis. Int J Hydrog Energy 34:6201–6210
139. Liu BF, Ren NQ, Ding FXJ, Zheng GX, Guo WQ, Xu JF, Xie GJ (2009) Hydrogen production by immobilized *R. faecalis* RLD-53 using soluble metabolites from ethanol fermentation bacteria. Bioresour Technol 100:2719–2723
140. Wang AJ, Sun D, Cao GL, Wang HY, Ren NQ, Wu WM, Logan BE (2011) Integrated hydrogen production process from cellulose by combining dark fermentation, microbial fuel cells, and a microbial electrolysis cell. Bioresour Technol 102:4137–4143
141. Ueno Y, Fukui H, Goto M (2007) Operation of a two-stage fermentation process producing hydrogen and methane from organic waste. Environ Sci Technol 41:1413–1419
142. Giordano A, Cantù C, Spagni A (2011) Monitoring the biochemical hydrogen and methane potential of the two-stage dark-fermentative process. Bioresour Technol 102:4474–4479
143. Pawar SS, Nkemka VN, Zeidan AA, Murto M, van Niel EWJ (2013) Biohydrogen production from wheat straw hydrolysate using *Caldicellulosiruptor saccharolyticus* followed by biogas production in a two-step uncoupled process. Int J Hydrog Energy 38:9121–9130
144. Kongjan P, O-Thong S, Angelidaki I (2011) Performance and microbial community analysis of two-stage process with extreme thermophilic hydrogen and thermophilic methane production from hydrolysate in UASB reactors. Bioresour Technol 102:4028–4035
145. Van Haandel A, van der Lubbe J (2007) Handbook biological waste water treatment. IWA Publishing, London, pp 8–10

146. Willquist K, Nkemka V, Svensson H, Pawar SS, Ljunggren M, Hulteberg C, Murto M, van Niel EWJ, Karlsson H, Lidén G (2012) Design of a novel biohythane process with high H_2 and CH_4 production rates. Int J Hydrog Energy 37:17749–17762
147. Siddiqui Z, Horan NJ, Salter M (2011) Energy optimisation from co-digested waste using a two-phase process to generate hydrogen and methane. Int J Hydrog Energy 36:4792–4799
148. Yang ZM, Guo RB, Xu XH, Fan XL, Luo SJ (2011) Hydrogen and methane production from lipid-extracted microalgal biomass residues. Int J Hydrog Energy 36:3465–3470
149. Lee Y, Chung J (2010) Bioproduction of hydrogen from food waste by pilot-scale combined hydrogen/methane fermentation. Int J Hydrog Energy 35:11746–11755
150. Kyazze G, Dinsdale R, Hawkes F, Guwy A, Premier G, Donnison I (2008) Direct fermentation of fodder maize, chicory fructans and perennial ryegrass to hydrogen using mixed microflora. Bioresour Technol 99:8833–8839
151. McKinlay JB (2014) Systems biology of photobiological hydrogen production by purple non-sulfur bacteria. In: Zannoni D, de Philippis R (eds) Microbial bioenergy: hydrogen production, vol 38, Advances in photosynthesis and respiration. Springer, Dordrecht/Heidelberg/New York/London, pp 155–176
152. Munro SA, Zinder SH, Walker LP (2011) Comparative constraint-based model development for thermophilic hydrogen production. Ind Biotechnol 7:63–82
153. Ljunggren M, Zacchi G (2009) Techno-economic evaluation of a two-step biological process for hydrogen production. Biotechnol Prog 26:496–504
154. Zannoni D, Antonioni G, Frascari D, de Philippis R (2014) Hydrogen production and possible impact on global energy demand: open problems and perspectives. In: Zannoni D, de Philippis R (eds) Microbial bioenergy: hydrogen production, vol 38, Advances in photosynthesis and respiration. Springer, Dordrecht/Heidelberg/New York/London, pp 349–356

Adv Biochem Eng Biotechnol (2016) 156: 195–234
DOI: 10.1007/10_2016_5

Published online: 19 July 2016

Biogas Production: Microbiology and Technology

Anna Schnürer

Abstract Biogas, containing energy-rich methane, is produced by microbial decomposition of organic material under anaerobic conditions. Under controlled conditions, this process can be used for the production of energy and a nutrient-rich residue suitable for use as a fertilising agent. The biogas can be used for production of heat, electricity or vehicle fuel. Different substrates can be used in the process and, depending on substrate character, various reactor technologies are available. The microbiological process leading to methane production is complex and involves many different types of microorganisms, often operating in close relationships because of the limited amount of energy available for growth. The microbial community structure is shaped by the incoming material, but also by operating parameters such as process temperature. Factors leading to an imbalance in the microbial community can result in process instability or even complete process failure. To ensure stable operation, different key parameters, such as levels of degradation intermediates and gas quality, are often monitored. Despite the fact that the anaerobic digestion process has long been used for industrial production of biogas, many questions need still to be resolved to achieve optimal management and gas yields and to exploit the great energy and nutrient potential available in waste material. This chapter discusses the different aspects that need to be taken into consideration to achieve optimal degradation and gas production, with particular focus on operation management and microbiology.

Keywords Biogas, Feedstocks, Microbiology, Process operation, Reactor concepts

A. Schnürer (✉)
Department of Microbiology, Swedish University of Agricultural Sciences, P.O. Box 7025, 750 07 Uppsala, Sweden
e-mail: anna.achnurer@slu.se

Contents

Abbreviations

Acetyl-CoA	Acetyl coenzyme A
COD	Chemical oxygen demand
CODH	Carbon monoxide dehydrogenase
CSTR	Continuous stirred tank reactor
DIET	Direct electron transfer
ED	Enter–Doudoroff
EGSB	Expanded granular sludge blanket
EMP	Embden–Meyerhof–Parnas
FHS	Formyltetrahydrofolate synthetase
H_4MPT	Tetrahydromethanopterin
HS-CoM	Coenzyme M
mcrA	Methyl coenzyme A
MFR	Methanofuran (MFR)
PA	Partial Alkalinity
SAO	Syntrophic acetate oxidation
SAOB	Syntrophic acetate oxidising bacteria
SGBR	Static granular sludge reactor
TA	Total Alkalinity
TS	Total solids
UASB	Upflow anaerobic sludge blanket
VS	Volatile solids
W–L pathway	Wood–Ljungdahl pathway

1 Introduction

Methane-rich biogas is produced through biological conversion of organic matter in the absence of oxygen. The process occurs in natural environments, but can also be implemented for controlled production on either small or large scale. This makes it interesting when designing flexible and sustainable energy solutions for industrial applications and also for farms and even single households, as typically seen in developing countries [1, 2]. Methane can also be produced by thermal gasification, but in that case comprises a lower proportion of the gas than in biogas [3]. For biological production of gaseous fuels, anaerobic digestion is the most common and widely applied process.

Biogas is a versatile renewable energy carrier which can be used to replace fossil fuels in power and heat production and can also, after purification, be converted to vehicle fuel [4]. Methane-rich biogas can also replace natural gas as a feedstock for production of other biochemicals [4, 5]. In a number of studies, production of biogas has been shown to offer significant advantages over other forms of bioenergy production and it has been rated one of the most energy-efficient and environmentally beneficial technologies for bioenergy production [1, 6, 7]. Moreover, during the anaerobic digestion (AD) process, nutrients are retained, making the digestion residue suitable as an organic fertiliser which can replace fossil energy-requiring mineral fertilisers [8, 9]. Anaerobic digestion for full-scale biogas production has been in operation for many years [4], but many questions still need to be resolved, on a technological and microbiological level, to achieve an economically feasible process. Moreover, application of this process is currently expanding to include not only energy and nutrient recovery but also production of value-added chemicals through mixed culture biotechnologies [10]. Depending on the character of the organic material to be degraded, different approaches can be used as regards digester design and operation management strategies. The biogas production process involves a series of successive metabolic reactions and requires combined activity of several groups of microorganisms with differing metabolic capacities and growth requirements. To obtain a stable and efficient biogas process, all these conversion steps and microorganisms must work in a synchronised manner, and it is important to meet the requirements of all microorganisms involved. In this chapter, different aspects that need to be taken into consideration to reach optimal degradation and gas production are discussed, with specific focus on operation management and microbiology.

2 Methane Production Process

Anaerobic digestion of organic material is a complex microbiological process requiring the combined activity of several groups of microorganisms with differing metabolic capacities [11]. The microorganisms engaged in the process are mainly

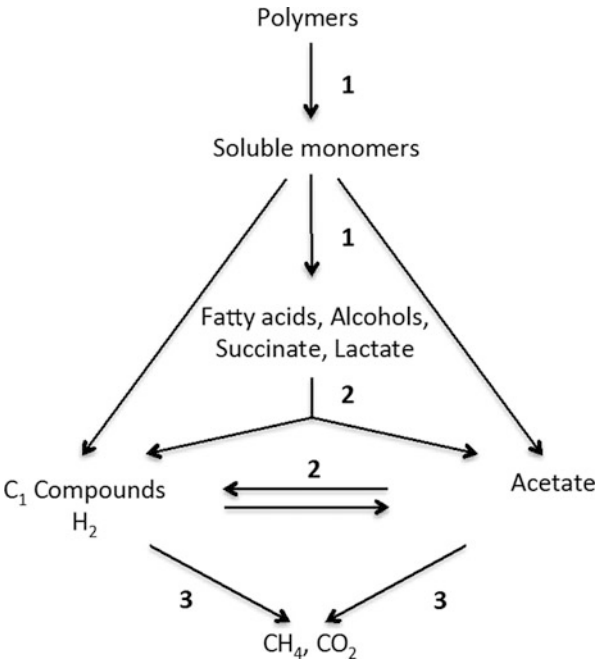


Fig. 1 Methane production through different degradation steps and trophic groups of microorganisms. 1. Hydrolysis and acidogenesis; 2. Acetogenesis and syntrophy; 3. Methanogenesis. Figure modified from Schink [22] and Angelidaki et al. [11]

assigned to the Bacteria and Archaea domains, but fungi also take part in the degradation. It is clear that the community is comprised of both generalists and specialists, together forming a microbial community which by intricate interactions converts large organic macromolecules into a variety of smaller organic components and finally into methane. The degradation process can be divided into four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. These are performed by the combined action of three physiological groups of microorganisms: hydrolytic-acidogenic bacteria (and most likely fungi), syntrophic acetogenic bacteria and methanogenic archaea (Fig. 1).

During the first two steps, polymers (lipids, proteins, carbohydrates, etc.) are converted to soluble monomers (long-chain fatty acids, glycerol, amino acids, sugars, etc.), which are subsequently further converted via various fermentation reactions to short-chain fatty acids, alcohols, hydrogen and carbon dioxide. In the next step the acids and alcohols are degraded through anaerobic oxidation by proton-reducing syntrophic acetogens to form hydrogen, carbon dioxide and acetate, which are used by the methanogens in the final step for the production of biogas.

2.1 Hydrolysis/Acidogenesis

In the hydrolysis and acidogenesis steps, complex polymeric compounds are broken down into soluble monomers. The hydrolysis is mediated by extracellular enzymes, either secreted to the bulk solution or attached to the cell wall. The hydrolysis rate varies depending on the character of the polymeric compound being broken down, but this step is often considered the rate-limiting step, particularly when plant-based lignocellulosic materials or sludge from wastewater treatment plants are used as feedstock for biogas production [12–14]. The low rate is caused by the intricate structure of lignocellulose, which comprises lignin, cellulose and hemicellulose closely associated in a structure recalcitrant to microbial degradation. The microbes responsible for lignocellulose degradation use either free extracellular enzyme systems or cell-anchored enzyme systems including cellulosomes, a large enzyme complex [12, 14, 15]. This complex contains numerous catalytic modules, including a dockerin domain, as well as a carbohydrate-binding module (See also Chap. 5 in this volume). The presence of a cellulosome distinguishes the anaerobic cellulose-degrading bacteria from their aerobic counterparts, which instead secrete different enzymes with cellulolytic capacity [15]. Proteins and lipids, often found together in waste of animal origin, are converted by proteases and lipases, respectively, and the degradation rate depends on the chemical structure, but also the solubility [11]. The monomers resulting from the different hydrolysis reactions are further oxidised mainly through various fermentation reactions through the Embden–Meyerhof–Parnas (EMP) or Enter–Doudoroff (ED) pathways. The biochemical pathways of sugar oxidation are diverse, but in most cases end up with pyruvate as a key intermediate. In the next step, pyruvate can be used as an internal electron acceptor for re-oxidation of NADH, resulting in production of C2–C6 products such as acetate, propionate, butyrate, lactate, valerate and caproate, and to some extent hydrogen/formate. Pyruvate can also be further oxidised through anaerobic respiration to acetate by acetogenic bacteria (acetogenesis step). In the presence of a hydrogen-scavenging partner such as methanogens, some bacteria can also reoxidise NADH by the formation of hydrogen, redirecting the fermentation towards production of comparatively more oxidised end products such as acetate and carbon dioxide, and inherently increased ATP yield [16].

Amino acids are in principle degraded anaerobically in two different ways [17]. The Stickland reaction represents one degradation pathway. Here, pairs of amino acids are degraded by coupled oxidation/reduction reactions. One amino acid is used as an electron donor and the other as an electron acceptor. The amino acid acting as the electron donor is oxidised to a volatile carboxylic acid that is one carbon atom shorter than the original amino acid. For example, alanine with its three-carbon chain is converted to acetate. If the hydrogen partial pressure is sufficiently low, fermentation of amino acids can also proceed through an alternative pathway involving uncoupled oxidation and release of electrons as hydrogen. Irrespective of the degradation pathway, the amino group in the amino acid is released as ammonia and the sulphur in cysteine and methionine results in sulphide.

Triglycerides are degraded into glycerol and long-chain fatty acids, i.e. carboxylic acids containing >12 carbon atoms. The composition of long-chain fatty acids varies between different raw materials, but palmitic and oleic acid are in general the most abundant saturated and unsaturated long-chain fatty acids, respectively [18]. LCFA are further degraded to acetate and hydrogen by β-oxidation by syntrophic acetogenic bacteria (see below).

2.2 Acetogenesis and Syntrophy

Acetogenesis is performed by a phylogenetically diverse bacterial group (acetogens) and is characterised by the reduction of carbon dioxide (CO_2) to the acetyl moiety of acetyl-co-enzyme A (CoA) through the acetyl-CoA pathway, also called the Wood–Ljungdahl (W–L) pathway [19, 20] (Fig. 2). The W–L pathway serves two functions: as an electron-accepting, energy-conserving pathway and as a pathway for carbon assimilation. Acetogens can use a wide variety of carbon sources, electron donors and acceptors and grow as autotrophs or heterotrophs. One-carbon compounds used for growth include, for example, H_2+CO_2, carbon monoxide (CO), formate, methanol and methyl groups from many methoxylated aromatic compounds. In addition sugars, aldehydes and organic acids can be used by acetogens (Table 1). Various electron acceptors can also be used, including CO_2, nitrate, sulphate and protons, with the latter being most important in the biogas process [21]. When acetogens grow as lithotrophs with H_2+CO_2, one molecule of CO_2 is reduced to CO by the enzyme carbon monoxide dehydrogenase (CODH), which becomes the carbonyl group of acetyl-CoA, and another molecule of CO_2 is reduced to formate, which serves as the precursor of the methyl group of acetyl-CoA (Fig. 2).

Under heterotrophic growth conditions, sugars are converted to pyruvate through the EMP pathway and the pentose phosphate pathway. Carbon dioxide and electrons, generated from the decarboxylation of pyruvate by a pyruvate ferredoxin oxidoreductase, and external CO_2 are shuttled into the W–L pathway (Fig. 2). When CO is the growth substrate, one molecule of CO must be converted to CO_2, which is then reduced to formate for conversion to the methyl group of acetyl-CoA, and another molecule of CO can be incorporated directly into the carbonyl group. Acetogens that oxidise organic acids typically produce hydrogen or formate as electron carriers, a process that is limited by unfavourable thermodynamics [16].

The reactions can only proceed if the partial pressure of these products is kept low, for example through consumption by methanogens. For some acids, such as propionate, the removal of acetate can also be of crucial importance. The difficult thermodynamic situation for the oxidation of volatile fatty acids is clearly illustrated by the positive values of Gibbs free energy (Table 2). The energetic situation for the methanogens is more favourable, however, and combining these reactions allows both organisms to obtain energy for growth. This type of symbiosis, in which

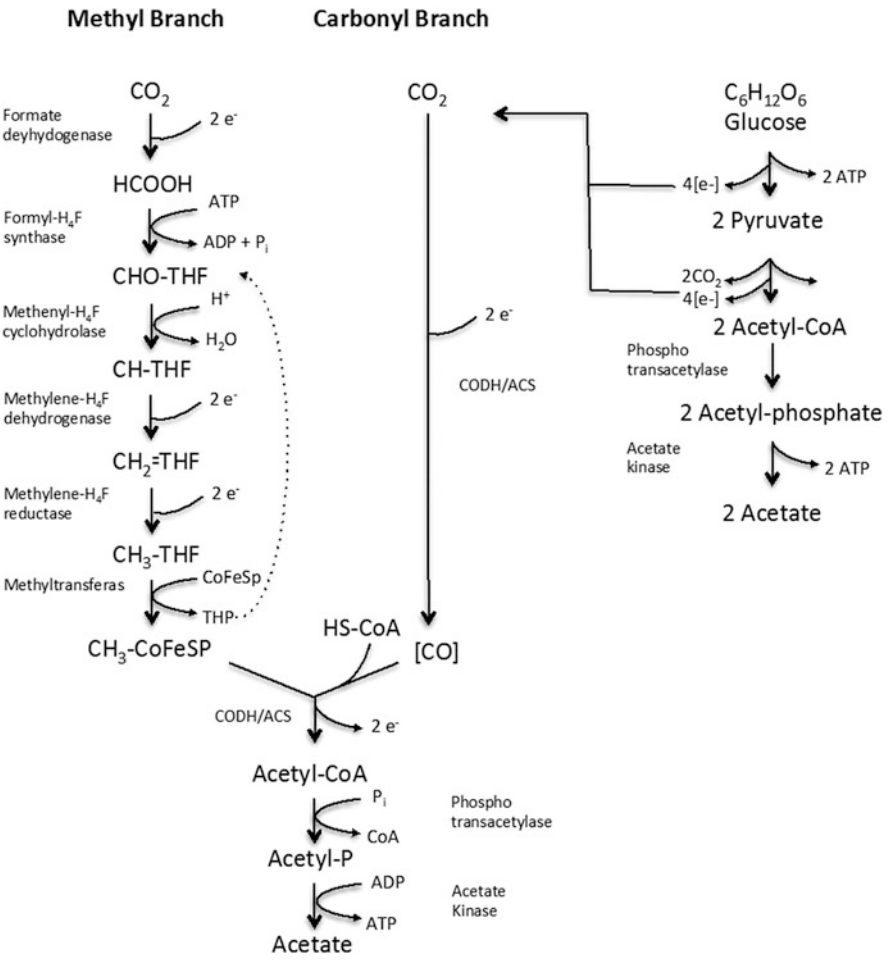


Fig. 2 *Left*: The Wood–Ljungdahl pathway (also called the Acetyl-CoA pathway). The pathway comprises two branches, the methyl and the carbonyl branch, and involves a series of reactions resulting in the reduction of two carbon dioxide molecules to form acetate finally. During the process no net ATP is formed and energy production is dependent on chemiosmotic processes coupled to the translocation of protons or sodium ions. *Right*: The two molecules of CO_2 that are reduced in the W–L pathway can also be derived through oxidation of an exogenous carbon source, such as glucose. In this process, two ATP molecules are produced through substrate level phosphorylation. *ACS* acetyl-CoA synthase, *CODH* carbon monoxide dehydrogenase, *CoFeSP* corrinoid iron sulphur protein, *THF* tetrahydrofolate

neither organism can operate without the other but together they exhibit metabolic activities which they could not accomplish on their own, is called syntrophy [16, 22].

The actual energy available to each member of the syntrophic consortium depends on the in situ concentration of substrate, intermediates and products

Table 1 Some electron donors and electron acceptors used by acetogenic bacteria Ragsdale and Pierce [21]

Electron donors	Electron acceptors
CO	CO_2
H_2, formate	Fumarate
Methyl chloride	Nitrate
Pyruvate	Thiosulfate
Lactate	Dimethylsulfoxide
Glycolate, glyoxylate	Pyruvate
Oxalate	Acetaldehyde
Methoxyacetate and methoxylated aromatics	H^+
Alcohols	
Hexoses, pentoses	
Betaine, acetoin	
Cellobiose	

Table 2 Standard Gibbs free energy changes for oxidation of some fatty acids and methanogenesis (taken from Worm et al. [16])

Reaction	ΔG^o (kJ/reaction)
Fatty acid oxidation	
Acetate-$+4H_2O \rightarrow H_2+2HCO_3^- + H^+$	+105
Propionate$^-+3H_2O \rightarrow$ acetate-$+HCO_3^- + H^+ + 3H_2$	+76
Butyrate$^-+2H_2O \rightarrow 2$ acetate$^- + H^+ + 2H_2$	+48
Methanogenesis	
$4H_2+HCO_3^- + H^+ \rightarrow CH_4+3H_2O$	−136
Acetate$+H^+ \rightarrow CH_4+CO_2$	−35
4Formate$+4H^+ \rightarrow CH_4+3CO_2+2H_2O$	−145
Syntrophic oxidation of acetate	
Acetate$^-+H_2O \rightarrow CH_4+HCO_3^-$	−31

[23]. These concentrations can vary during growth and thus also change the thermodynamic situation for a specific reaction. Another important factor for the efficiency of the process is the distance between bacteria and methanogens. Felchner-Zwirello et al. [24] showed that a decrease in the interbacterial distance between propionate degraders and methanogens from 5.30 to 0.29 μm caused an increase in the maximum possible hydrogen flux from 1.1 to 10.3 nmol mL^{-1} min^{-1}. The maximum possible hydrogen flux was always higher than the hydrogen formation and consumption rate, indicating that reducing the interspecies distance by aggregation is advantageous. Hydrogen transfer is considered a key factor for syntrophy, because many syntrophic relationships are dependent on hydrogen as an electron shuttle [25]. Hydrogen is a small molecule which can easily diffuse. It functions well as an electron carrier and also an electron donor, and is thus efficiently removed. Hydrogen partial pressure in syntrophic co-cultures has been

shown to be as low as 10^{-5} Pa and calculations suggest that, under environmental conditions, syntrophic reactions can reach -10 to -30 kJ mol^{-1}, a very small amount of energy [23]. As mentioned above, several studies also suggest that, in addition to hydrogen transfer, interspecies formate transfer can happen in methanogenic syntrophy [26]. Acetate, a key intermediate in biogas processes, has also been shown to act as an electron carrier for syntrophic partners [25]. Moreover, transfer of electrons by direct cell-cell contact, without production of hydrogen, has been shown to be possible through so-called pili or nanowires or the formation of multispecies aggregates [25–27]. Such electron transfer is called direct electron transfer (DIET).

2.3 *Methanogenesis*

Methanogens catalyse the terminal step in the anaerobic food chain by converting products from acidogenesis/acetogenesis into methane. If the methanogens maintain a low concentration of products such as hydrogen and acetate, numerous classical primary fermentations are shifted to the formation of hydrogen, carbon dioxide and acetate and many fewer reduced side-products such as fatty acids are produced. Based on substrate and pathway used, methanogens are typically classified into two groups: the hydrogenotrophs and the methylotrophs [28, 29]. The hydrogenotrophs use formate or hydrogen as an energy source, and CO_2 is reduced to methane. Some methanogens within this group can also use certain alcohols as an electron donor. Moreover, this group contains methanogens with the ability to use hydrogen obligately and reduce methanol and methylamines instead of carbon dioxide. The ability to use hydrogen and formate is common among methanogens, but the ability to utilise alcohols is less common. The hydrogenotrophic methanogens are either obligate and use only hydrogen/formate as an electron donor or are more flexible and also use other energy sources. Methylotrophic methanogens are more versatile and substrates for methanogenesis include hydrogen and carbon dioxide, acetate, methyl compounds such as methanol and methylamines, and carbon monoxide. Here the methyl group is reduced to methane. Methanogens have a metabolism involving unique enzymes and co-enzymes [28, 29]. During methanogenesis from carbon dioxide, the CO_2 is first reduced to a formyl group, attached to the carrier methanofuran (MFR) (Fig. 3). The formyl group is then further transferred to the carrier tetrahydromethanopterin (H_4MPT), followed by dehydration to form methenyl-H_4MPT. This methenyl group is then reduced further to a methylene and finally to a methyl group, which is further transferred to a third carrier, the sulphydryl-containing coenzyme M (HS-CoM). Finally, the methyl group is reduced to methane and the coenzyme is regenerated. In methanogenesis from methanol, methylamines, etc., the substrate enters the pathway as methyl-S-CoM (Fig. 3). Electrons for reduction of methyl-S-CoM to methane come either from hydrogen or methyl disproportionation, e.g. oxidation of another methyl-S-CoM to carbon dioxide [28]. During growth on acetate, the

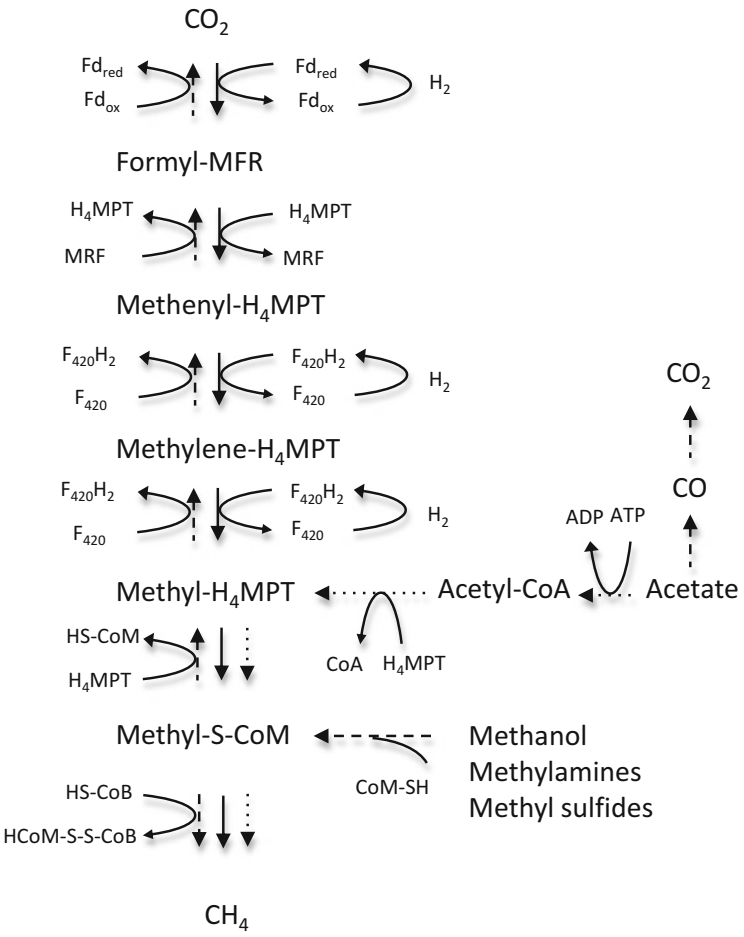


Fig. 3 Pathway of methanogenesis. *Solid black lines* represent hydrogenotrophic methanogenesis, *dashed lines* methylotrophic, and *dotted lines* the aceticlastic. During methylotrophic methanogenesis the methylated compounds enter at the methyl-S-CoM level. During aceticlastic methanogenesis, the methyl group of carbon enters at the level of methyl-H_4MPT. All pathways share the final step. Electrons from the oxidation of the carbonyl carbon are used for the reduction of the methyl group to methane. *MFR* methanofuran, *H_4MPT* tetrahydromethanopterin, *HS-CoM* coenzyme M, *HS-CoB* coenzyme B, *Fd* ferredoxin, *F_{420}* coenzyme F_{420} (reduced form $F_{420}H_2$). Figure adapted from Welte and Deppenmeir [84] and Costa and Leigh [28]

methyl group enters the pathway as methyl-H_4MPT, and the carboxyl carbon is oxidised to provide an electron for methyl reduction (Fig. 3). Irrespective of pathway used, energy is most likely only generated by proton or sodium motive forces, as methanogens cannot use substrate level phosphorylation for ATP production [28, 29].

3 Microbial Composition

The microbial communities engaged in methanogenesis have been extensively investigated, initially mainly through isolation and cultivation work, but in recent years also using various molecular techniques to determine the diversity and dynamics of the microbial community and the level of expression [30–33, 139].

The knowledge we have today about the physiological capacities of the microorganisms involved in the biogas process to a great extent derives from traditional microbiological methodologies, i.e. the isolation and cultivation of pure strains and species. The invention of techniques for cultivation of strict anaerobes can thus be considered a breakthrough in the area of biogas [34] (Fig. 4). For many years these methods represented the main tools for generating knowledge about the organisms engaged in methane production. However, with the powerful tools available today, knowledge of these isolated organisms has expanded to include detailed information about their genomic structure and gene expression, as well as valuable insights about mechanisms within microbial metabolism (Fig. 5). However, as with other complex environments, the majority of microorganisms in the anaerobic digestion process have not yet been cultivated, as is obvious from recent amplicon sequencing studies targeting 16S rDNA and the whole metagenome [35–39]. Thus, an increasing number of microbes in the biogas process have been found, but not all have been identified, or have been identified as 'Candidatus', such as the candidate phylum Cloacimonetes (WWE1) and the candidatus genus Cloacimonas [39, 40].

Traditionally, cultivation of anaerobic species from the biogas process is mainly done by manipulating the level of macronutrients and micronutrients in a low redox medium and by changing the cultivation conditions. The removal of oxygen from the medium is performed by purging and replacing the gas phase with O_2-free gases, together with the addition of reducing agents such as hydrogen sulphide and cysteine. Strict anaerobic microorganisms such as methanogens and different syntrophic bacteria have been isolated using this rather tedious method. However,

Fig. 4 Anaerobic cultivations. *Left*: Single bacterial colonies growing in an anaerobic agarshake. *Right*: Cultivation of cellulose degrading bacteria in anaerobic liquid medium in serum bottles. Photo: Li Sun

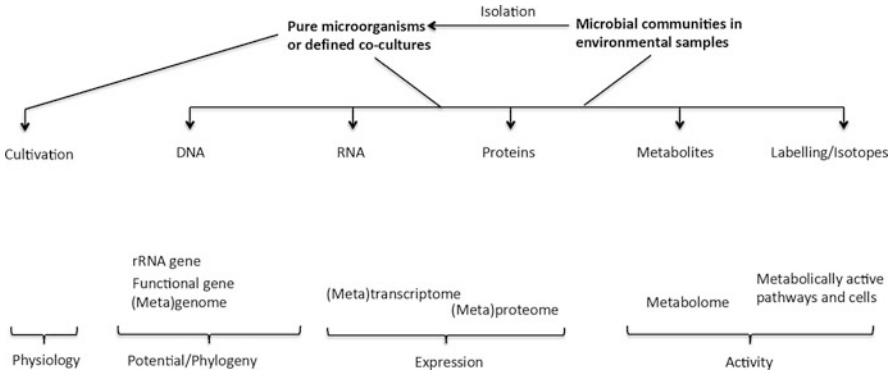


Fig. 5 An array of different approaches, both cultivation based and molecular methods as well as labelling techniques, using environmental samples, defined co-cultures or pure microbial strains, can be used to generate information with regard to physiology, phylogeny, expression and activity. Isolation and cultivation can be used to study physiology and generate information with regard to substrate utilization, etc. Analysis of DNA reveals information with regard to phylogeny and shows genetic potential of both pure strains and whole communities, and analysis of RNA (transcriptome) shows actual expression of different genes. Both DNA and RNA can also be used to target and quantify specific genes, either rRNA or functional genes. Proteomics and metabolomics reflect the functional proteins expression and activity, and labelling techniques such as fluorescence probes and stable or radioactive isotopes can be used to generate information on specific pathways, to show correlation between activity and cells and to visualize cells. Using a combination of these approaches can generate valuable information needed to link community structure with function of anaerobic digesters. Figure adopted from Vanwonterghem et al. [33]

identification of strict anaerobic microorganisms is highly challenging, because of the requirement for low oxygen concentrations and the high degree of commensalism and mutualism in the communities, making them difficult to isolate and cultivate [41–43]. Significant numbers of novel cultivation methods have been introduced over the past 10 years, all leading to a significant increase in microbial recovery [41]. Strategies that have been successful include the use of metabolites/signal substances in the media, prolonged incubation times and co-cultivation [41]. In addition, to facilitate and simplify the isolation, less laborious methods for cultivating and isolating anaerobic microorganisms, such as a six-well plate system, have been developed [44]. This technique has been successfully used to isolate a number of strict anaerobes, including the first methanogenic representative of the class Thermoplasmata, *Methanomassilicoccus* [45, 46]. This organism was first isolated from human faeces but has lately also been observed in different biogas processes and is suggested to be important for maintained methane production at high organic loads [47–49].

As mentioned above, studies concerning microbial communities in anaerobic digestion processes have recently been expanded to include culture-independent molecular methods [30, 139]. As the function of the anaerobic microbiome depends on a highly complex interplay rather than on the potential of individuals, studies using culture-based methods for the whole community are essential. Functions

related to competition and interaction between microorganisms, which is typical of the biogas process, are difficult to determine when using isolated microorganisms only [33]. Different techniques also need to be used depending on the research question [30], i.e. microbial diversity (what is there?), microbial dynamics (how does the community change over time?), microbial quantification and microbial functioning (what are the roles of the different groups in the anaerobic community)? Many studies are also devoted to finding links between microbial community structure and interactions with the function of the anaerobic digester [33].

To optimise the anaerobic digestion process and steer it in a desired direction, it is important to have knowledge and understanding of the anaerobic microbiome, including metabolic capacities of the microorganisms, the level of functional redundancy within the community and the fundamental mechanisms for interspecies interactions. Methods applied to generate knowledge in this area include community analysis based on the extraction of DNA, RNA, protein, phospholipid fatty acids or metabolites [32, 33, 50–52]. Based on generated genomic data, a variety of molecular methods have also been developed for in-depth investigations of the microbial community structures within anaerobic digestion processes (Fig. 5).

The 16S rRNA gene is the most commonly used marker gene, both for description of the whole bacterial and archaeal communities [53, 54] and for detection of specific key groups or species, such as methanogens [55], syntrophic bacteria [56–58] and lignocellulose-degrading bacteria [59]. Communities with specific functions have also been analysed by targeting different functional genes, such as methyl coenzyme A (mcrA), a key enzyme for methanogenesis [60, 61], formyltetrahydrofolate synthetase (FHS), a key enzyme for acetogenesis [62] and glycosidase hydrolase, a cellulose-degrading enzyme commonly found in anaerobic bacterial communities [63]. Besides characterisation of genes, mRNAs, proteins and metabolites using dedicated assays, microscopic analysis of microorganisms continues to be a valuable option, particularly when using labelled probes specifically targeting microbial groups or species of interest [30, 52]. Such methods provide different levels of information concerning spatial organisation and taxonomic composition. Use of the various methods described above has generated valuable information about the structure of the microbiome in the biogas process and its activities.

3.1 Bacterial Communities

In the biogas process, microbial communities appear to undergo large shifts in species diversity over the short term, but show surprising robustness and consistency over the long term. Microbial dynamics are strongly related to operating parameters such as substrate composition, process temperature, retention time and organic loading rate. Many recent studies have investigated correlations between microbial composition and digester performance in terms of microbial community

structure, diversity and activity, and degradation pathways [31, 33, 52, 64–66]. However, the majority of these studies have been unable to show consistent relationships, particularly for methane production. Large-scale shifts in communities have been observed, particularly in the bacterial community, without any change in function of the digester. Changes in performance without any significant changes in the microbial community present have also been observed. However, some recent investigations using molecular tools have successfully correlated specific microorganisms to digester performance, including methane production and fatty acid degradation and operating parameters such as temperature and ammonia concentration [53, 58, 62, 64, 67–69]. The complexity of the biogas system was clearly illustrated in a recent publication examining a total of 43 rural digesters in China [70]. Correlation-based network analysis of the prokaryotic communities in all digesters suggested strong within- and between-domain correlations between different groups of microorganisms in the biogas digestion system. It also showed that the prokaryotic communities of biogas digesters are well organised by some functional modules. Moreover, significant positive correlations were observed between members within modules, suggesting mutualistic interactions such as exchange of metabolic intermediates and syntrophic interactions [70]. It is clear that more research is needed in this area to link fully the microbiology with the function of the process and to reach a point when microbial management could be accomplished [31].

In general, members of the Firmicutes and Bacteriodetes dominate in the biogas process, but representatives from the phyla Proteobacteria and Chloriflexi are also commonly found, although in lower abundance [31, 32, 39, 53, 54, 70, 71] (Fig. 6). The bacteria within these phyla in total possess a great ability to degrade a wide range of complex organic macromolecules, most likely explaining their high prevalence in biogas reactors. In addition, representatives belonging to the other phyla such as Acidobacteria, Actinobacteria, Fibrobacteres, Spirochaetes, Thermotogae, Planctomycetes, Synergistetes, Tenericutes, candidate phylum Cloacimonetes, Thermi and Verrucomicrobia have also been found, but typically at comparatively lower levels. Despite the overall dominance of a few phyla, a high degree of variation is often seen with regard to variance between, but also within, these phyla. This diversity is driven by the composition of the substrate used for the biogas process and by the operating conditions applied, including strongly regulating parameters such as retention time, organic loading rate and temperature [31, 53, 70]. Ammonia level is another parameter with a strong impact on microbial community structure [53, 62, 70, 72]. Recent studies have also suggested core populations and co-occurrence patterns within and between different groups in the digesters, again varying with substrate and process conditions [53, 70, 73].

Within Firmicutes, the classes Clostridia, degrading both cellulose and protein, and Bacillus, typically degrading fat and carbohydrates, dominate. The class Clostridia also contains species capable of performing both acetogenesis and syntrophic acid degradation. Known syntrophs belonging to this class include the genus *Syntophomonas*, degrading different fatty acids including long-chain fatty acids [16], and acetate oxidisers belonging to the families Thermoanaerobacteriaceae and

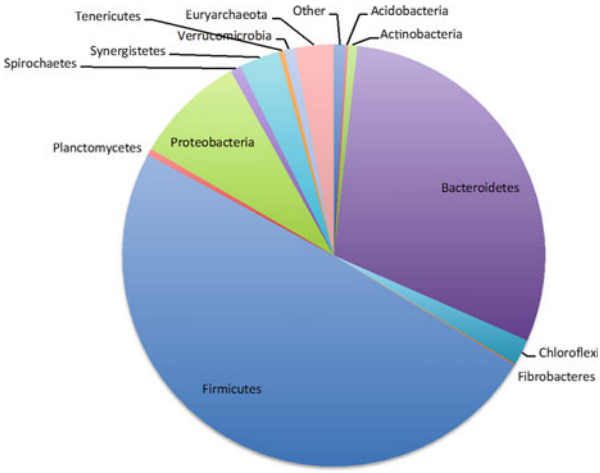


Fig. 6 A typical biogas community is dominated by members belonging to the bacterial phyla Firmicutes and Bacteriodetes. The methanogenic community is mainly represented by the phylum Euryarchaeota and at a comparably lower abundance than the bacteria

Clostridiaceae [74]. Firmicutes, especially the class Clostridia, have been shown to increase in response to increasing temperatures and increasing ammonia levels arising because of the degradation of proteins [53, 70, 72].

The phylum Bacteriodetes is typically dominated by the class Bacteriodia and this phylum has also been shown to have hydrolytic activity [14, 75]. De Vrieze et al. [53] suggest that this phylum appears in biogas digesters operating under "easy" conditions, i.e. low levels of volatile fatty acids, mesophilic conditions and low ammonia and salt concentrations. This suggestion is in line with results reported by Sun et al. [39], who identified an increase in this phylum in response to straw addition and a decrease in response to increased temperature in biogas reactors digesting manure. However, representatives of the Bacteroidetes have also been reported to ferment sugars to acetate and propionate. Moreover, Proteobacteria are often found in relatively high abundance in upflow anaerobic sludge blanket (UASB) systems and this phylum contains many members that can convert sugars, fatty acids and aromatic compounds such as benzoate and ethanol [14, 53, 76, 77]. The phylum includes syntrophs belonging to the genera *Syntrophus*, *Pelobacter*, *Smithella*, *Syntrophorhabdus* and *Syntrophobacter*. In addition, genes necessary for degradation of cellulose have been found in bacteria belonging to this phylum [78, 79]).

The candidate phylum Cloacimonetes has been found to represent as much as 10% of the community and this uncultured cluster has been found in several anaerobic digesters [39, 70]. The genome of one species from this phylum, namely '*Candidatus* Cloacamonas acidaminovorans', was recovered in a metagenomic study which suggested that it is a syntrophic bacterium capable of degrading propionate and amino acids [80]. In a recent study, evidence emerged suggesting that this group of bacteria is also involved in anaerobic digestion of cellulose, through an extracellular cellulose hydrolysis process and/or fermentation of organic substrates originating from cellulose [81]. The phylum Fibrobacter also contains

lignocellulose-degrading members, so far only represented by two cultured species [14, 82]. Thermotogae, containing hyperthermophilic, thermophilic and mesophilic members, are known to degrade carbohydrates with production of hydrogen and are typically seen in higher abundance in thermophilic biogas plants [14, 59]. Representatives of this phylum have also been suggested to be involved in syntrophic acetate oxidation (SAO) [83]. The phylum Chloriflexi has been shown to dominate in digesters operating with municipal wastewater [54] whereas manure digesters are more typically dominated by Firmicutes [70, 76]. Similarly to representatives from the phylum Synergistetes, many bacteria belonging to the Chloriflexi are able to perform syntrophic metabolism in association with hydrogenotrophic methanogens [72].

3.2 Archaeal Communities

The methanogenic community, representing typically a few percent of the whole microbial community in biogas digesters but still performing a critical role, is dominated by members of the domain Archaea and exclusively the kingdom Euryarchaeota. The methanogens are divided into seven different taxonomic orders: Methanobacteriales, Methanomicrobiales, Methanocellales, Methanopyrales, Methanococcales, Methanosarcinales and the recently discovered seventh order Methanoplasmatales [84]. Among these seven orders, three are frequently found in biogas digesters. These are Methanobacteriales, Methanomicrobiales and Methanosarcinales [11, 67]. Methanoccocales are seldom found in biogas digesters, but have been found, for example, in granular sludge [85]. Methanopylares, containing hyperthermophilic species, and Methanocellales, typically found in rice paddy soils, have not yet been detected in biogas processes. However, Methanomassiliicoccaceae, order Methanoplasmatales, have recently been found in biogas digesters operating at relatively high organic load and high ammonia levels [47, 48, 86].

As mentioned above, depending on substrate and pathway, methanogens are divided into two groups: the hydrogenotrophs and the methylotrophs. Hydrogenotrophic methanogens belong to the orders Methanobacteriales, Methanococcales, Methanomicrobiales and Methanosarcinales. Methylotrophs are represented mainly by members of the order Methanosarcinales and the families Methanosarcinaceae and Methanosaetaceae. Members of the family Methanosarcinaceae are comparatively more versatile, having the ability to grow on several different substrates, such as acetate, hydrogen and methanol, whereas the Methanosaetaceae use only acetate [87]. In addition to representatives from the order Methanosarcinales, *Methanosphaera* species belonging to the order Methanobacteriales have been shown to perform methylotrophic methanogenesis [88]. Methanomassiliicoccaceae from the order Methanoplasmatales are obligate hydrogen-consuming methanogens, but they reduce methanol and methylamines instead of carbon dioxide [45]. The methanogenic population dominating in a

specific methane production process depends on many parameters, including type of process, operating parameters such as organic load, hydraulic retention time and environmental conditions such as pH, temperature, and ammonia and acetate concentrations. Stable biogas reactors operating at low ammonia levels are often reported to have a comparatively higher abundance of aceticlastic methanogens. It is commonly accepted that aceticlastic methanogens contribute as much as 70% of the methane produced in biogas digesters. Experimental data generally support this assumption, especially for digesters operating with municipal sewage sludge or manure, which are typically characterised by low ammonia levels and low organic loading rate [39, 54, 76]. Two methanogens, *Methanosarcina* and *Methanosaeta* sp., use acetate for growth. These methanogens have differing characteristics. For example, *Methanosarcina* generally exhibits higher growth rate but requires acetate concentrations above 1 mM, whereas *Methanosaeta* species typically dominate below that range because of their higher affinity for acetate [87, 89]. High ammonia and acetate levels suppress the growth of *Methanosaeta* sp., although *Methanosarcina* has been reported in high abundance even in high ammonia digesters [87]. *Methanosarcina* has also been reported to grow better under high loading rates, most likely because of higher acetate turnover. In general, *Methanosarcina* is reported to be a very robust methanogen because of its ability to tolerate common stress factors in biogas reactors, including temperature variations, high organic loading rates, high concentrations of ammonia and other salts and low pH [67, 87]. The ability of *Methanosarcina* sp. to use several different substrates, not only acetate as in the case of *Methanosaeta* sp., most likely improves its competitiveness. However, in processes dominated by *Methanosaeta* sp., a decrease in the numbers of this methanogen has been suggested to be an early warning of process instability [31].

Although acetate is considered an important precursor for methanogenesis, a growing number of studies report a clear dominance of hydrogenotrophic over aceticlastic methanogenesis [67]. This dominance of hydrogenotrophic methanogens suggests that hydrogen and/or formate is available in large amounts in some types of biodigesters and is the main methanogenic substrate, rather than acetate. The abundance of hydrogenotrophic relative to aceticlastic methanogens typically increases at elevated process temperatures and levels of compounds, such as high ammonia levels, causing selective inhibition of acetate-utilising methanogens. High ammonia levels are believed to result in the appearance of microbial competitors for acetate, promoting the development of SAO as the main mechanism for methanogenesis [90]. Syntrophic acetate oxidising bacteria (SAOB) are considered to be slow growers, which can be a disadvantage in the competition for acetate with the aceticlastic methanogens. Nevertheless, a majority of the SAOB possess relatively high ammonia tolerance, a feature that probably provides them with a competitive advantage in ammonia-stressed systems. The oxidation of acetate, instead of direct cleavage as performed by the aceticlastic methanogens, results in production of hydrogen/formate, which can be further utilised by hydrogenotrophic methanogens.

Several investigations of biogas processes operating at high ammonia levels have demonstrated a correlation between ammonia and the abundance of hydrogenotrophic methanogens, as well as SAO [53, 67, 90, 91]. At mesophilic conditions (~37°C), a positive correlation has been observed between ammonia levels and numbers of the genus *Methanoculleus*, belonging to the order Methanomicrobiales [31, 67, 70, 90]. In particular, the level of *Methanoculleus bourgenis* has been shown to be positively correlated with high ammonia levels. However, other hydrogenotrophic methanogens are also reported to be abundant at elevated ammonia levels, including *Methanomassiliicoccacea luminyensise* and *Methanoculleus marisnigri* as well as members of the Methanobacteriales and Methanosarcinales [67, 70, 90] (Fig. 7). Interestingly, *Methanosarcina* sp. has frequently also been reported at relatively high levels in various processes operating at high ammonia levels and with methanogenesis proceeding mainly through SAO. This has led some authors to suggest that this methanogen is engaged as a hydrogen consumer during SAO, or is even able to mediate the entire process from acetate [11, 90].

A decrease in abundance of Methanosarcinales and Methanobacteriales and an increase in abundance of Methanomicrobiales in response to increasing temperature has also been shown and is most likely caused by redirection of various fermentation reactions towards increased hydrogen production [39, 92]. Methanobacteriales species have also been reported as the hydrogenotrophic partner during SAO in thermophilic conditions [90]. Dominance of hydrogenotrophic methanogenesis has also been observed for a number of bioreactors operating under lower ammonia and temperature conditions and with various substrates and operating conditions [67].

Despite the importance of methanogens for the overall degradation process, contradictory results have been reported in the literature regarding the possibility of following this specific group as a way to determine process performance. However, it is obvious that there are some general trends regarding abundance in reactors operating under different conditions. The structure of the methanogenic community compared with that of the bacterial community is most likely shaped to a higher degree by operating parameters such as temperature, ammonia and acetate level, etc., rather than the type of substrate used by the biogas plant.

3.3 Anaerobic Fungi

Anaerobic fungi have been reported to be present in biogas processes. Anaerobic fungi are best known in the rumen of herbivores, where they are key players in the degradation of lignocellulosic plant fibre [93]. Anaerobic fungi belonging to the phylum Neocallimastigomycota utilise various carbohydrates through fermentation reactions and produce molecular hydrogen, carbon dioxide, acetate, formate, lactate and ethanol as metabolic waste products [93]. Hydrogen is produced through hydrogenosomes, organelles containing hydrogenases [93, 94]. In the rumen,

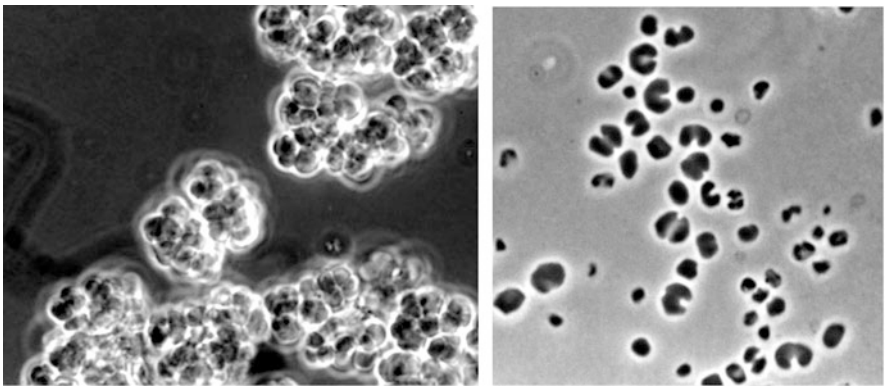


Fig. 7 *Methanosarcina* sp (*left*) and *Methanoculleus* sp (*right*) represent two commonly detected methanogens in biogas processes. Photo: Anna Schnürer

anaerobic fungi exist in a relationship with methanogenic archaea and the latter have been shown to increase the enzymatic activity in the fungi through the removal of hydrogen, which is followed by a shift in metabolic activity within the fungi towards higher production of methanogenic precursors [95]. Experimental studies on cellulose degradation have shown that co-culture of methanogens and fungi increases the rate of cellulose breakdown dramatically compared with fungal monocultures [96, 97]. The fungi can attach to the most lignified plant tissues and this is followed in turn by the ingress of cellulolytic bacteria, which then gain access to the interior of otherwise less fermentable plant material [98]. The capacity of the fungi to produce a wide range of enzymes and to degrade even the lignified walls of plant cells has made them interesting for different biotechnological approaches, including development of biogas reactors [93, 99, 100]. In a recent study, different anaerobic fungi were also demonstrated to be present in several German biogas plants [100]. Fungi belonging to the subphyla Agaricomycotina, Mucoromycotina, Pezizomycotina, Pucciniomycotina and Saccharomycotina and to the class Neocallimastigomycetes were identified in that study using molecular techniques.

4 Substrate and Operation

4.1 Substrates

To achieve a stable and efficient biogas production process, the material added to digesters must have a good balance of both macro- and micronutrients [11]. Some materials work well as a single substrate, whereas others can only be used in mixtures with other substrates. To overcome the drawbacks of a single material, simultaneous co-digestion using two or more substrates in a mixture is a feasible alternative to mono-digestion [101]. Co-digestion can result in favourable nutrient

and water content and in dilution of potential inhibitors. To ensure sufficient microbial activity, some materials and mixtures of materials may also have to be complemented with process additives such as iron, trace metals, buffering chemicals, anti-foaming agents, etc. [102–104]. The chemical composition of the material used as substrate affects the biogas yield and the methane content of the gas, as well as the biodegradability and degradation kinetics [105–107, 183]. The actual yield can be estimated by a theoretical calculation (Buswell formula, Table 3) or by performing a so-called biomethane potential test, where the substrate is added to an inoculum and the methane production is monitored using a controlled batch cultivation procedure [108]. Optimally, the substrate should have a composition that meets the nutritional requirements of the microorganisms involved and also results in high biogas and methane yield and a high quality digestate in terms of high nutrient composition and low levels of contaminants. These different requirements are sometimes difficult to meet, as some materials with high methane potential, e.g. fat and protein, can cause problems with inhibition of the biogas process [109].

Protein-rich materials such as slaughterhouse waste and various food wastes have a relatively high methane yield potential and result in high levels of plant-available ammonium nitrogen (NH_4^+-N) in the residue [90]. However, degradation of proteins results in the release of ammonium, in equilibrium with ammonia. A high content of ammonium provides the process with alkalinity and increases the value of the digestate as a fertilising agent, but unfortunately it also causes inhibition of the process, specifically of the methanogenic community [90, 110]. Temperature and pH indirectly affect the level of inhibition, because these parameters shift the equilibrium between ammonium (NH_4^+) and ammonia (NH_3) towards the latter, which has been shown to be the actual cause of the inhibition. Inhibition has been reported to occur at varying NH_3 concentrations ranging from 53 to 1450 mg L^{-1} and is most likely caused by differences in substrate composition, reactor design and operating parameters such as hydraulic retention time and temperature. High levels are often associated with reactor instability, indicated by reduced methane production rate and high effluent concentrations of volatile fatty acids, believed to be caused by selective inhibition of aceticlastic methanogens, as mentioned above [110]. However, adaptation to high

Table 3 Theoretical methane potential of some organic polymers calculated using the Buswell formula

Compound	~Methane yield (m^3/kg VS[d])	Composition of the biogas, CH_4:CO_2 (%)
Carbohydrate[a]	0.42	50:50
Lipid[b]	1.01	70:30
Protein[c]	0.50	50:50

$(C_nH_aO_b+(n–a/4–b/2)H_2O\rightarrow(n/2–a/8+b/4)CO_2+(n/2+a/8–b/4)CH_4)$

[a]Calculation made using cellulose

[b]Calculation made using gelatin

[c]Calculation made using glycerol trioleate (figures taken from [11])

[d]Volatile solids

ammonia levels has long been emphasised in the literature, as reviewed in Westerholm et al. [90]. Allowing the microbial community to acclimatise to the prevailing conditions can allow efficient biogas production even at elevated ammonia levels. Adaptation to high ammonia levels has been shown to be correlated with a shift in the methane-producing pathway, with significant contributions by SAO to methane formation [90]. For optimal degradation of proteins in an anaerobic digestion process, the carbon:nitrogen (C/N) ratio has been suggested to be set between 15:1 and 25:1 [111]. If the ratio is too low, the process risks suffering from ammonia inhibition, whereas if it is too high the bacteria may experience nitrogen limitation. A high C/N ratio poses a greater risk of process problems arising if the majority of the carbon is easily accessible, e.g. as is that in starch. In that case, the carbon is quickly degraded and there is a risk of acidification. In addition to ammonia, protein-rich substrates are also a common source of formation of sulphide, which is not only toxic for various microbial populations but also forms complexes with metals, resulting in decreased bioavailability of trace elements essential for microbial activity [112]. Sulphides are also undesirable in methanogenic processes because of direct toxic effects [109] and contamination of the biogas, causing bad odours and corrosive effects on pipes and gas engines. Sulphides can be reduced by iron addition.

Materials rich in lipids, such as fats, oils, fish waste and slaughterhouse waste, are also energy-rich and have high methane potential. However, lipids result in the release of long-chain fatty acids, which can cause a drop in pH, foaming and, at high levels, toxicity to the microbial community [101, 109, 113]. Degradation of LCFA is considered the slowest degradation step during degradation of lipids, and a difference between the rate of the hydrolysis step and the oxidation of LCFA can result in product imbalance and accumulation over time [114]. Acclimatisation to inhibitory levels of long-chain fatty acids has been shown to be possible by repeated exposure of the process, followed by periods of recovery [115, 116]. An alternative strategy to access the high biogas potential in lipid-rich waste is to use a stepwise start-up strategy to allow development of a specialist microbial community [117].

As mentioned earlier, materials with a high degree of lignocellulose, such as straw and crop residues, are difficult to degrade and thus give somewhat restricted biogas production and slow degradation. Some plant-based materials in this category also contain low levels of trace metals and alkalinity and need to be mixed with complementary materials to function as a substrate for biogas production [118]. The degradation of this type of material and biogas production can be somewhat improved by pre-treatment to break up the intricate structure of the lignocellulose [118–123]. Different pre-treatment methods have been evaluated and shown to increase the biogas production including, for example, mechanical, thermal and biological treatments. It should, however, be noted that many pre-treatments are energy- and cost-intensive, sometimes limiting large-scale application. However, materials with a high level of easily accessible carbon, such as fruit residues, potato and sugar beet, undergo rapid initial conversion, which can instead cause acidification in the process if they are added in large amounts [124].

4.2 Operating Parameters

Important operating parameters include stirring, process temperature, organic loading rate and retention time [108]. Reactor stirring is a parameter of critical importance, as mixing increases the contact between substrate and microorganisms, and thus the degradation rate. Mixing mode and mixing intensity have consequently been shown to have direct effects on biogas yield [125]. Mixing ensures even distribution of the substrate and thus good nutrient supply to the microorganisms active in the biogas process. However, if too harsh, mixing can disrupt microbial aggregates, resulting in less efficient degradation. Biogas production can proceed at different temperatures, typically mesophilic (35–42°C) or thermophilic (46–60°C). Biogas production can also proceed at psychrophilic temperature (15–25°C) [126]. Temperature, together with substrate, is the most strongly determining parameter for stability and process performance. As mentioned above, the temperature impacts strongly on community structure, but also on microbial diversity, degradation pathways and degradation rate [108, 127]. In general, anaerobic digestion at thermophilic temperatures gives higher methane production rates and higher methane yield, but this is not always the case [108, 128]. Moreover, thermophilic digestion results in comparatively higher reduction of pathogens [108, 129] and gives lower viscosity, resulting in less energy consumption for stirring [130]. Disadvantages with higher temperatures include lower microbial diversity, with an accompanying risk of a less stable process and less efficient degradation of certain chemical compounds, such as phenols [108, 128]. Moreover, a higher process temperature needs a higher energy input in the form of heating. Processes operating at mesophilic temperature are generally considered to be more stable and less sensitive to inhibitory components such as ammonia. The microbial community, specifically the methanogens, are sensitive to temperature variations and experience from large-scale operation shows that temperature fluctuations should not exceed ±2–3°C for best results and to avoid instability [131]. However, biogas production is possible at a wide range of temperatures, even in the range between mesophilic and thermophilic temperatures, and it is also possible to shift from mesophilic to thermophilic temperature and vice versa [90, 132, 133]. To ensure stable operation, the organic loading rate, defined as the amount of organic material added per reactor volume (active) and day, should not be too high. The optimal load depends on a number of factors, including substrate characteristics and the operating temperature [108]. The load to the digester can either be the daily amount added on one occasion, spread out over several occasions or continuously fed into the reactor. The feeding approach affects the degradation kinetics, formation of intermediates and biogas production, but usually does not affect the final biogas yield [134]. The feeding regime has also been shown to affect the activity and structure of the microbial community and its functional stability [65, 135]. Repeated pulse feeding with addition of substrate every second day, compared with daily feeding, has been shown to result in a bacterial community that is more tolerant to organic shock load and high ammonia [135]. Dynamic feeding has lately also been suggested as an

approach to allow flexible electricity supply from biogas [134]. An increase in organic loading rate typically results in a decrease in retention time which, if too short, might cause wash-out of microorganisms and inefficient degradation. The retention time should be sufficiently long to ensure good degradation of a specific material under specific operating parameters. The retention time varies depending on the type of process, e.g. high or low rate with 15–40 days and 1h-1 day, respectively, being commonly used. Substrates rich in sugar and starch are typically easily broken down and require shorter retention times. For the degradation of these materials, no hydrolysis is necessary and the degradation starts directly at the second degradation step, fermentation. However, much longer times are required for microbial degradation of fibre- and cellulose-rich plant matter. For such materials it is often the hydrolysis step and not methanogenesis that limits the rate of decomposition. A typical sign of too low organic loading rate is either accumulation of degradation intermediates or a low degree of degradation.

4.3 Monitoring

It is important to monitor carefully the biogas process, as many different parameters can result in instability and failure of the process [52, 108, 131]. Monitoring makes it possible to detect problems in a timely manner and rectify them before things have gone so far that the process deteriorates (Fig. 8). Some microorganisms, such as methane producers, are extremely sensitive and may stop growing and/or be washed out of the process if they do not thrive. For example, the process temperature must be closely monitored, as some microorganisms engaged in degradation are sensitive to temperature fluctuations. Alkalinity and pH are other parameters of high relevance. The optimal range of pH for methane production is around 6.5–7.5, but the range varies with different substrates and operating parameters. With an acid substrate or an easily degradable substrate resulting in rapid acid production, a pH change can occur in the reactor. The magnitude of this pH change depends on the available alkalinity (buffer capacity) in the reactor, which also regulates how fast the pH is restored to optimal levels. The total alkalinity (TA) gives a measure of the combined effect of several different buffering systems [136]. The partial alkalinity (PA) represents the buffering capacity of the carbonate system, and also includes the ammonium-ammonia system. The intermediate alkalinity (IA) is the difference between TA and PA and mainly includes the buffering capacity of the volatile fatty acids. The stability of the process can be evaluated by calculating the IA/PA ratio, with a value of 0.3 or less indicating a stable process [108]. A value between 0.3 and 0.8, on the other hand, indicates a risk of instability and a value above 0.8 suggests instability.

Other parameters of importance for monitoring include the concentration of fatty acids and ammonium (see section on substrate) and the carbon dioxide and methane content of the gas. Accumulation of volatile fatty acids is highly undesirable, as it is a sign of an inefficient process and also represents a significant loss of biogas. These

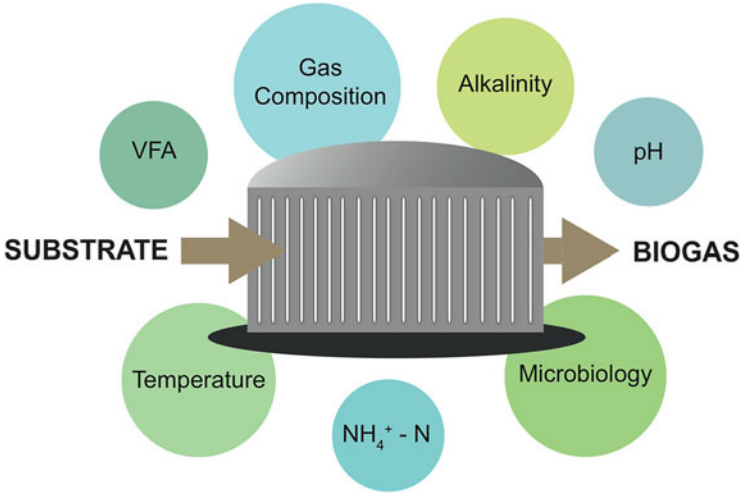


Fig. 8 Many different parameters impact on the performance of a biogas process, such as substrate composition, organic load, retention time, process temperature and stirring efficiency. To secure a stable and efficient process a monitoring program can be used. Important parameters in this regard are total gas production, gas production kinetics and gas composition (methane, carbon dioxide, hydrogen sulphide and hydrogen). Other important parameters to monitor are temperature, pH, alkalinity, ammonium ($N\text{-}NH_4^+$) and volatile fatty acids (VFA). Different microbiological methods have also recently been evaluated as early warning indicators

compounds represent intermediates from the acidogenesis and acetogenesis steps and typically the degradation proceeds through syntrophic reactions involving acid degraders and methanogens. Accumulation is believed to be a consequence of lower activity of methanogens compared with acid producers and to be caused either by organic overloading or by inhibition of the methanogenic microbial communities. Alternatively, accumulation can be a consequence of direct inhibition of the acid-degrading community. The feedstock itself can also have high levels of acids. For monitoring, the propionate:acetate ratio is reported to be a useful early indicator of imminent process failure [137], with an increasing ratio indicating a higher risk. With increasing volatile fatty acid concentration there is also a significant risk of a pH drop, particularly in processes with low buffering capacity.

The composition of the gas is of great interest for monitoring, as a change in gas composition, i.e. increasing levels of carbon dioxide, may be a sign of process instability. However, if the input material varies over time, a change in gas composition can also just reflect the character of the substrate. To detect a deviation from the 'normal' variation, it is thus important to consider the gas composition and carbon dioxide content over a longer period and look for increasing/decreasing trends. Hydrogen is another interesting monitoring parameter, and in a balanced anaerobic digester the hydrogen concentration is normally low. However, an imbalance between hydrogen-producing bacteria and hydrogenotrophic methanogens leads to hydrogen accumulation. Higher hydrogen concentrations,

as mentioned earlier, redirect the electron flow and result in the production of reduced compounds such as butyrate, propionate, lactate, or ethanol, and consequently result in lower methane yield. For technical reasons, the hydrogen level is usually not monitored in industrial scale processes. However, a hydrogen-sensitive palladium metal oxide semiconductor (Pd-MOS) sensor in combination with a membrane for liquid-to-gas transfer for monitoring of dissolved hydrogen was suggested to be possible to adjust for large-scale applications [138]. Moreover, analysis of hydrogen sulphide is also important for evaluating the gas quality. High levels of hydrogen sulphide in the gas indicate risk for formation of complexes with metals in the liquid, which can decrease the bioavailability of trace elements essential for microbial activity [109].

Microbial analysis represents a new and interesting approach for the monitoring of biogas plants. Such techniques are mainly investigated in lab-scale reactors and have so far not been used in full-scale biogas plants to any large extent but represents promising approaches for successful process control. Analysis of the whole microbial community or specific microbial bioindicators can allow identification of risks for process failure before conventionally used process chemical parameters [139]. By analysis of relevant microorganisms critical for crucial degradation, steps can be taken to manage the process towards high stability and efficiency [31, 33, 52]. Microbial analysis as a tool for monitoring includes both general and specific approaches, for example analysis of metagenome and metabolome [140], the whole bacteria and archaeal community [66, 141], and specific groups of key organisms such as methanogens [67, 142, 143] and cellulose degraders [59]. Other approaches investigated for monitoring include stable isotope analysis of gases to determine the pathway used for production of methane [144, 145] and analysis of the metabolic quotient (MQ) and the cDNA/DNA quota to determine methanogenic activity [184]. The MQ corresponds to the ratio of the predicted to the actual concentration of methanogens and the cDNA/DNA ratio reflects the activity of the methanogens. These studies and their results all represents steps towards the implementation of microbial ecology-based engineering to optimize performance of the anaerobic digesters.

4.4 Process Additives

Different chemical and biological components to enhance methanogenesis and/or improve stability are used and under evaluation [146]. For example, addition of macro and micronutrients trace have been evaluated in a number of studies and often shown positive results [146, 147]. Macronutrient, such as P, N, S, are essential for microbial growth and can in some materials be a limiting factor. Micro nutrient, such as trace elements, are essential for enzymatic activity and addition of trace elements has been shown to circumvent accumulation of degradation intermediates and lower the risk of process instabilities, for example those produced by ammonia inhibition [102, 147, 148]. Addition of trace metals has given positive results with

various types of substrate, such as food waste, crop material and stillage [49, 103, 108]. Cobalt, nickel, molybdenum and selenium are suggested to be critical to process performance, but other metals can also be important [146, 147]. With materials resulting in sulphide formation, such as protein-rich materials, metals can also be precipitated in the form of metal sulphides. Inclusion of iron in addition to trace metals has been shown to give positive effects in such cases, primarily because of removal of sulphide by the iron, allowing higher bioavailability of trace elements [146]. Addition of iron also improves the gas quality by lowering the amount of hydrogen sulphide in the gas phase. Iron can be added directly to the reactor liquid in different forms to precipitate the sulphides and hence reduce the undesired precipitation of trace elements [108]. Sulphides can also be reduced in biogas by aeration of headspace, resulting in oxidation of reduced sulphides to elemental sulphur.

Addition of conductive materials, such as granular activated carbon, carbon cloth, graphite, biochar and magnetite (Fe_3O_4) particles to methanogenic reactors has in some cases been shown to improve methanogenesis and degradation of different organic acids [26, 149]. The background to such addition is that DIET has been shown to be mediated by the presence of conductive materials, functioning as wires and electrically connecting the syntrophic organisms (reviewed in [26, 150]).

If necessary, alkalinity and pH can be adjusted in the biogas process by adding various stabilising agents such as carbonates and bicarbonates, combined with sodium or potassium, calcium carbonate (lime) and hydrochloric acid [108]. Other alkaline substances, such as lime, ammonia, lye and urea, can be used but are more easily overdosed and may not always contribute to increased alkalinity in the process. The exact amount of buffering substance that must be added to alter the alkalinity may vary between different biogas processes and is dependent on several factors, such as the bicarbonate content, temperature, pH, fatty acid concentration, ammonia content, etc.

Foaming is a common cause of process problems and common factors triggering foam formation include organic loading and substrate composition, such as content of proteins or lipids. The most commonly applied solution to suppress foaming is the addition of antifoams [151], e.g. surface active chemicals which destroy the foam by causing bubble coalescence. Recently Kougias et al. [151] showed that natural oils such as rapeseed oil and oleic acid efficiently suppress foaming.

Recently, different attempts to enhance the biogas process by direct addition of microorganisms or enzymes have been made, some with successful results [146]. Bioaugmentation has been made mainly to enhance the hydrolytic step of the biogas process and improve the degradation of lignocellulose. For example, addition of the cellulose-degrading bacterium *Clostridium cellulolyticum* was recently shown to increase degradation efficiency of wheat straw [152]. Bioaugmentation with the aim of improving the hydrolysis was also shown to be successful using an anaerobic fungus, *Piromyces rhizinflata*, in a two-stage process for biohydrogen and biogas production using corn silage [153] and with the fermentative bacterium *Acetobacteroides hydrogenigenes* for methane production from corn straw [154].

Moreover, addition of a consortium comprised of 16 isolated strains of cellulose degrading bacteria were shown to improve the methane yield from maize silage with as much as 38% [155]. Attempts have also been made to improve the stability and efficiency of methane production at high ammonia levels and here addition of a pure methanogenic culture (*Methanoculleus bourgensis*, strain $MS2^T$) was proposed to enhance successfully the methane yield in an ammonia-stressed continuous biogas digester [156]. Improved methane yield was also obtained after addition of the hydrogen producing acetogen *Enterobacter cloacae* [157] and improved lipid degradation was obtained with the lipolytic bacterium *Clostridium lundense* [158]. Most studies using direct addition of enzymes to the biogas process have shown negative results. However, addition of proteases was shown to give an increase in methane yield in batch tests with maize silage, chicken dung and cow manure, but no effect was seen during semi-continuous operations [74]. Application of a commercially available enzyme mixture, prepared by fungal fermentation, resulted in increased biogas production yield (10–15%) and an increased methane content of the biogas (5–10%) in a semi-continuous process using grain silage and maize silage as substrate [159].

5 Digester Technologies

Anaerobic digestion can be set up with various configurations, for example continuous or batch mode and one- or two-stage systems (Fig. 9). Digester types are usually categorised into two groups, low and high rate, but classification of the process can also be based on the characteristics of the incoming material, i.e. low (<15%), medium (15–20%) or high (20–40%) total solids concentration [160]. Total solids contents lower and higher than 15% characterise wet and dry anaerobic digestion systems, respectively. Materials with high and medium total solids concentration are typically treated in low rate digesters with comparatively long retention times and large digester volumes. For more diluted materials, high rate digesters with short retention times and high dilution rates are typically used [161, 162].

5.1 Low and Medium Rate Reactors

A common reactor design for low rate continuous digestion includes the continuously stirred tank reactors (CSTR) for wet digestion and plug flow (PL) reactors for dryer materials. The most commonly used approach for commercial scale biogas production in Europe is the CSTR, using a continuous or semi-continuous feeding system. The process is typically used for materials with a dry solids content between a few percent and up to about 10% and commonly used waste streams include sludge from wastewater treatment plants, slaughterhouse waste, food waste,

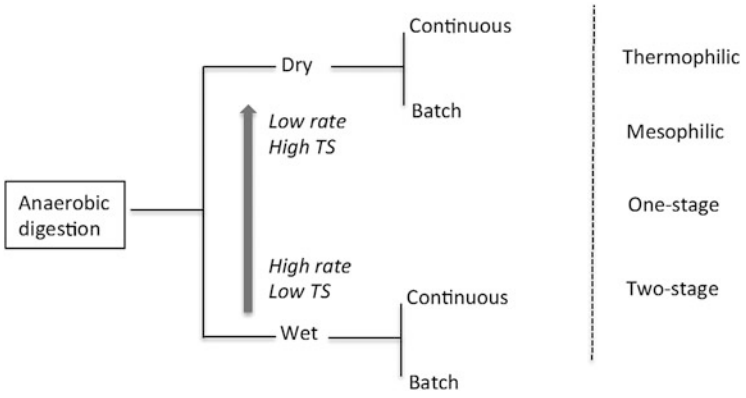


Fig. 9 Anaerobic digestion can proceed with different types of process configurations, mainly depending on the character of the material to be treated in the system. Dry materials are typically digested in a low rate digester although more diluted materials are used in high rate systems. The digestion either proceeds in a continuous process or in batch, set up in a one- or a two-stage configuration. The operation proceeds typically at either a thermophilic or mesophilic temperature

manure or other industrial waste streams, crops and crop residues [4, 163]. During operation of a CSTR, materials are typically continuously pumped into the reactor or fed in a semi-continuous manner, digestate being taken out in similar amounts and biogas being produced continuously. Solid materials, such as solid manure, crops or crop residues, are, however, more typically fed into the reactor in batch mode, for example through semi-continuous feeding. The feeding frequency for a CSTR is often set based on practical considerations, i.e. how often the operator can feed the system, but also depends on the characteristics of the feeding material. The hydraulic retention time should typically be set to 15–40 days, but can also be shorter or longer depending on substrate availability and operating parameters. The organic loading rate commonly ranges between 2 and 6 g L^{-1} day^{-1} volatile solids. The degree of degradation varies greatly depending on the characteristics of the material, but a common range is 30–70% volatile solids reduction [108].

Dry anaerobic digestion is an attractive method for stabilisation of organic waste with a high solids concentration (>15%). Different types of reactor concepts can be applied for this type of digestion and it can be operated in both batch and continuous mode [160]. The batch reactor concept involves a single-mode or sequential batch system, with several reactors connected in series. An increasing number of dry anaerobic digestion systems have been installed in Europe in recent years, because they provide some advantages over the wet digestion systems, for example they require less reactor volume, which reduces the material cost and need for heating [160]. Moreover, the digestion residue produced has comparatively lower water content and thus also higher nutrient content per unit wet weight, making it attractive as a fertilising agent. Many different types of feedstocks have been successfully used for biogas production in the dry digestion process, such as different crops and crop residues, manure and the organic fraction of municipal organic waste. The performance of the process is robust and allows equal or higher

loading rates than the CSTR process [160]. In line with this, the retention times are usually shorter than with the CSTR, but still result in a greater volatile solids reduction. Different feedstocks require different loading systems depending on their consistency [160]. Addition of water or a more diluted co-digestion material is sometimes needed to improve stirring of the material and conditions suitable for microbial growth and nutrient transportation. Water is seldom added from external sources but process liquid (leachate) is often recycled back to the process and sprayed on or mixed into the material [35]. For anaerobic microorganisms to be able to grow, a water content of >0.91 has been suggested. Some dry digestion systems available in the market are coupled to a second digester, for example an upflow anaerobic sludge blanket (UASB, see below). The leachate from the first reactor, which contains a high level of organic acids, is transferred to the UASB, where methanogenesis occurs.

5.2 High Rate Reactors

In high rate reactors, biomass is retained by the formation of granules or flocculated sludge or by attachment to a support material [162, 164, 185, 186]. This concept allows decoupling of the hydraulic retention time and sludge retention time, thus allowing high organic loading rates without risk of wash-out of biomass and at relatively small reactor sizes. Self-immobilisation of the microorganisms not only improves the ability of the biomass to withstand high-strength wastewaters and shock loads, but also increases the tolerance to toxic compounds. As anaerobic microorganisms grow slowly because of low energy yield per unit substrate utilised, detainment of biomass is of particular importance when using diluted waste streams for anaerobic digestion and energy production. Thus, high rate reactors are typically used for energy, nutrient and water recovery from various municipal and agro-food industrial wastewater streams [165], such as wastewater from slaughterhouses [166, 167] and dairy industries [168], municipal wastewaster [164], manure [169] and wastewaster from pulp and paper industries [170]. The operating costs are low, the effluent quality is relatively good and the footprint is small. Different types of reactor concepts have been developed and are used depending on the chemical composition and organic content of the wastewater and the purpose of the process [182, 185, 186].

UASB reactors represent the first generation of granule-based high-rate reactor systems and were developed back in the late 1970s ([171]; see also Chap. 13). To date, this reactor type is the most commonly used design for commercial wastewater treatment applications. In a UASB reactor, wastewater is pumped upwards through the reactor under strict anaerobic conditions at rates between 1 and 5 m h^{-1}. Inside the reactor, anaerobic microorganisms grow and form cell aggregates (granules) of varying size, typically 0.5–5 mm diameter [172]. Biodegradable organic matter is converted to biogas by the microbial communities making up the granules at organic loading rates of about 10–20 kg chemical oxygen demand (COD) per m^3 reactor volume and day, with potential COD removal

efficiencies of above 90%. The conventional sludge bed UASB reactor has a low mixing intensity and, as a consequence, rather poor substrate-biomass contact. Optimisations in this regard have led to the development of the expanded granular sludge blanket (EGSB) reactor and static granular sludge reactor (SGBR) [164, 173–175]. These reactors have been modified to improve the treatment performance through improving the contact between the wastewater and the granular sludge by internal recycling (EGSB) or by operation in downflow mode without flow recirculation (SGBR). Compared with the UASB reactor, these processes can typically operate at higher upflow velocity and/or at higher organic loads. For this reason, the EGSB reactor has been suggested as an attractive alternative for treatment of low strength wastewaster, particularly at ambient temperatures, because of the efficient biomass-substrate contact induced by the high upflow velocity [164, 174]. The hydrolysis rate of suspended solids drops with decreasing temperature, but nevertheless anaerobic reactor systems with stable methanogenesis have been successfully operated at temperatures as low as 4–5°C [174].

For optimal operation of high rate upflow anaerobic treatment systems, development of a granular sludge with high strength, high biological activity and a narrow settling distribution is necessary. The granulation process is complex and affected by many parameters, including physico-chemical and microbiological factors, the inorganic and organic composition of the wastewater, operating parameters such as load, retention time, temperature and pH, the microbial composition and the impact on the formation and characteristics of the granular sludge [172, 175–178] investigated granules from full-scale reactors and compared them in terms of basic composition, size distribution, density, settling velocity, shear strength and extracellular polymer substances content. The results suggest that granule properties are influenced relatively more by wastewater type than by reactor design or operating conditions such as pre-acidification level. The formation of granules involves transportation and irreversible adhesion of a cell to the surface of an inert material or another cell, followed by multiplication to develop the granule. The cell transportation can proceed through non-active processes such as advection or diffusion or active processes using, for example, flagella. The granular structure contains cavities and holes, which are suggested to function as transport channels for gases, substrates and metabolites. In this process, extracellular polymer substances such as polysaccharides, proteins, lipids, phenols and nucleic acids play a very important role [175]. High levels of divalent cations (e.g. Ca^{2+}, Mg^{2+} and $Fe^{2+)}$ and organic and inorganic nuclei (e.g. clay minerals) have also been shown to be important for initiating granule formation [172, 179]. Divalent cations have been suggested to stimulate the formation of granules by attracting negatively charged bacteria and have also been suggested to increase the amount of protein and polysaccharides in the sludge [179]. Granules harbour all the different metabolic groups of microorganisms engaged in anaerobic degradation, including hydrolytic, acidogenic, acetogenic and methanogenic microorganisms [180, 181]. Regarding the placement of different microbial groups, various investigations have shown differing results, but often with hydrolytic and acidogenic bacteria situated in the outer layers of granules and methanogens in the centre [175].

6 Concluding Remarks

Biogas production represents a highly interesting process for recovery of both energy and nutrients from various organic waste streams and dedicated crops. The process has also become of great interest for production of value-added chemicals through mixed culture biotechnologies and is also suitable for use in combined bioenergy production systems and other industrial networks. The process can also be used for "energy on demand" production, i.e. by varying the feeding regimes and substrate compositions, a flexible biogas production can be achieved. The multifunctionality of this process and the fact that it can be operated at different scales assures its place in a future more sustainable society. Knowledge of digester technologies and process biology has expanded rapidly in recent years, and could soon reach a point where the process can be set up and managed under even more controlled conditions than is possible today. However, new microbial methods and models for monitoring the efficiency and stability of the process need to be developed to steer and manage the process towards higher efficiency and for controlled production. Another important factor for future expansion of biogas production is the development of small-scale, cheap and efficient technologies for use at farm scale to reach the full potential and to access the high gas potential in the agricultural sector. Small-scale solutions are also of importance in non-industrialised countries, where this process can be of great importance for the nation as a whole and for individual families.

References

1. Lozanovski A, Linder JP, Bos U (2014) Environmental evaluation and comparison of selected industrial scale biomethane production facilities across Europe. Int J Life Cycle Assess 19:1823–1832
2. Rajendran K, Aslanzadeh S, Taherzadeh MJ (2012) Household biogas digesters – a review. Energies 5:2911–2942
3. Lan W, Chen G, Zhu X, Wang X, Xu B (2015) Progress in techniques of biomass conversion into syngas. J Energy Inst 88:151–156
4. Weiland P (2010) Biogas production: current state and perspectives. Appl Microbiol Biotechnol 85:849–860
5. Vanholme B, Desmet T, Ronsse F, Rabaey K, Breusegem F, de Mey M, Soetaert W, Boerjan W (2013) Towards a carbon-negative sustainable bio-based economy. Front Plant Sci 4:174
6. Börjesson P, Tufvesson LM (2011) Agricultural crop-based biofuels – resource efficiency and environmental performance including direct land use changes. J Cleaner Prod 19:108–120
7. Börjesson P, Prade T, Lantz M, Björnsson L (2015) Energy-crops-based biogas as vehicle fuel, the impact of crop selection on energy efficiency and greenhouse gas performance. Energies 8(6):6033–6058
8. Alburquerque JA, de la Fuente C, Bernal MP (2012) Chemical properties of anaerobic digestates affecting C and N dynamics in amended soils. Agric Ecosyst Environ 160:15–22
9. Nkoa R (2014) Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: a review. Agron Sustain Dev 34:473–492

10. Batstone DJ, Virdis B (2014) The role of anaerobic digestion in the emerging energy economy. Curr Opin Biotechnol 27:142–149
11. Angelidaki I, Karakashev D, Batstone DJ, Plugge CM, Stams AJM (2011) Chapter sixteen – biomethanation and its potential. In: Amy CR, Stephen WR (eds) Methods in enzymology, vol 494. Academic, New York, pp 327–351. http://www.sciencedirect.com/science/article/pii/B9780123851123000160
12. Lynd LR, Weimer PJ, van Zyl WH, Pretorious IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microb Mol Biol Rev 66:506–577
13. Monalu F, Barakat A, Trabby E, Dumas C, Steyer JP, Carrère H (2013) Lignocellulosic materials into biohydrogen and biomethane: impact of structural features and pre-treatment. Crit Rev Environ Sci Technol 43(3):260–322
14. Azam S, Khadem AF, van Lier JB, Zeeman G, Plugge CM (2015) Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. Crit Rev Env Sci Technol 45:2523–2564
15. Schwartz WH (2001) The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol 56(5):634–649
16. Worm P, Müller N, Plugge CM, Stams AJM, Schink B (2010) Syntrophy in methanogenic degradation. In: Hackstein JHP (ed) (Endo)symbiotic methanogenic archaea, vol. 19. Springer, Berlin, pp 149–173
17. Ramsay IR, Pullammanappallil PC (2001) Protein degradation during anaerobic wastewater treatment: derivation of stoichiometry. Biodegradation 12:247–257
18. Sousa DZ, Smidt H, Alves MM, Stams AJM (2009) Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids. FEMS Microbiol Ecol 68:257–272
19. Drake HL, Küsel K, Matthies C (2002) Ecological consequences of the polygenetic and physiological diversities of acetogens. Antonie Van Leeuwenhoek 81:203–213
20. Drake HL, Gössner AS, Daniel SL (2008) Old acetogens, new light. Ann N Y Acad Sci 1125:100–128
21. Ragsdale SW, Pierce E (2008) Acetogenesis and the Wood–Ljungdahl pathway of CO_2 fixation. Biochim Biophys Acta 1784(12):1873–1898
22. Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61(2):262–280
23. Montag D, Schink S (2016) Biogas process parameters–energetics and kinetics of secondary fermentations in methanogenic biomass degradation. Appl Microbiol Biotechnol 100:1019–1026
24. Felchner-Zwirello M, Winter J, Gallert C (2013) Interspecies distances between propionic acid degraders and methanogens in syntrophic consortia for optimal hydrogen transfer. Appl Microbiol Biotechnol 97(20):9193–9205
25. Morris BEL, Henneberger R, Huber H, Moissl-Eichinger C (2013) Microbial syntrophy: interaction for common good. FEMS Microbiol Rev 37:384–406
26. Shen L, Zhao Q, Wu X, Li X, Li Q, Wang Y (2016) Interspecies electron transfer in syntrophic methanogenic consortia: from cultures to bioreactors. Renewable Sustain Energy Rev 54:1358–1367
27. Regueiro L, Veiga P, Figueroa M, Alonso-Gutierrez J, Stams AJM, Lema JM, Carballa M (2012) Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters. Microbiol Res 167:581–589
28. Costa KC, Leigh JA (2014) Metabolic versatility in methanogens. Curr Opin Biotechnol 29:70–75
29. Ferry JG (2011) Fundamentals of methanogenic pathways that are key to the biomethanation of complex. Curr Opin Biotechnol 22:351–357
30. Cabezas A, de Araujo JC, Callejas C, Galès A, Hamelin J, Marone A, Sousa DZ, Trably E, Etchebehere C (2015) How to use molecular biology tools for the study of the anaerobic digestion process? Rev Environ Sci Biotechnol. doi:10.1007/s11157-015-9380-8
31. Carballa LR, Lema JM (2015) Microbial management of anaerobic digestion: exploiting the microbiome-functionality nexus Marta. Curr Opin Biotechnol 33:103–111

32. Koch C, Müller S, Harms H, Harnisch F (2014) Microbiomes in bioenergy production: from analysis to management. Curr Opin Biotechnol 27:65–72
33. Vanwonterghem I, Jensen PD, Ho DP, Batstone DJ, Tyson GW (2014) Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr Opin Biotechnol 27:55–64
34. Hungate RE (1950) The anaerobic mesophilic cellulolytic bacteria. Bacteriol Rev 14:1–49
35. Li C, Mörtelmaier WJ, Gallert C (2014) Effect of moisture of municipal biowaste on start-up and efficiency of mesophilic and thermophilic dry anaerobic digestion. Bioresour Technol 168:23–32
36. Lu X, Rao S, Shen Z, Lee PKH (2013) Substrate induced emergence of different active bacterial and archaeal assemblages during biomethane production. Bioresour Technol 148:517–524
37. Smith A, Sharma D, Lappin-Scott H, Burton S, Huber D (2014) Microbial community structure of a pilot-scale thermophilic anaerobic digester treating poultry litter. Appl Microbiol Biotechnol 98:2321–2334
38. Zakrzewski M, Goesmann A, Jaenicke S, Jünemann S, Eikmeyer F, Szczepanowski R, Abu Al-Soud W, Sørensen S, Pühler A, Schlüter A (2012) Profiling of the metabolically active community from a production-scale biogas plant by means of high-throughput metatranscriptome sequencing. J Biotechnol 158:248–258
39. Sun L, Pope PB, Eijsink VGH, Schnürer A (2015) Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure. J Microbial Biotechnol 8:815–827
40. Solli L, Håvelsrud OE, Horn SJ, Rike AG (2014) A metagenomic study of the microbial communities in four parallel biogas reactors. Biotechnol Biofuels 7(146):1–15
41. Epstein SS (2013) The phenomenon of microbial uncultivability. Curr Opin Microbiol 16:636–642
42. Schink B (2002) Synergistic interactions in the microbial world. Antonie Van Leeuwenhoek 81:257–261
43. Mori K, Kamagata Y (2014) The challenges of studying the anaerobic microbial world. Microbes Environ 29(4):335–337
44. Nakamura K, Tamaki H, Kang MS, Mochimaru H, Lee S-T, Nakamura K, Kamagata Y (2011) A six-well plate method: less laborious and effective methods for cultivation of obligate anaerobic microorganisms. Microbes Environ 26(4):301–306
45. Dridi B, Fardaeu M-L, Ollivier B, Raoult D, Drancourt M (2012) *Methanomassilicoccus luminyensis gen* nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int J Syst Evol Microbiol 62:1902–1907
46. Narihiro T, Kamagata Y (2013) Cultivating yet-to-be cultivated microbes: the challenge continues. Microbes Environ 28(2):163–165
47. Hori T, Haruta S, Sasaki D, HAnajima D, Ueno Y, Ogata A, Ishii M, Igarashi Y (2015) Reorganization of the bacterial and archaeal populations associated with organic loading conditions in a thermophilic anaerobic digester. J Biosci Bioeng 119(3):337–344
48. Moestedt J, Müller B, Westerholm M, Schnürer A (2015) Ammonia threshold for inhibition of anaerobic digestion of thin stillage and the importance of organic loading rate. J Microbial Biotechnol. doi:10.1111/1751-7915.12330
49. Westerholm M, Müller B, Isaksson I, Schnürer A (2015) Trace element and temperature effects on microbial communities and links to biogas digester performance at high ammonia levels. Biotechnol Biofuels 8:154
50. Bremges A, Maus I, Belman P, Eikmeyser F, Winkler A, Albersmeier A, Pülher A, Schlürer A, Sczyrba A (2015) Deeply sequenced metagenome and metatranscriptome of a biogas-producing microbial community from an agricultural production-scale biogas plant. GigaScience 4(33):1–6
51. Heyer R, Kohrs F, Reichl U, Benndorf D (2015) Metaproteomics of complex communities in biogas plants. J Microbial Biotechnol 8:749–763

52. Lebuhn M, Weiß S, Munk B, Guebitz GM (2015) Microbiology and molecular biology tools for biogas process analysis, diagnosis and control. Biogas Sci Technol Ser Adv Biochem Eng Biotechnol 151:1–40
53. De Vrieze JD, Saunders AM, He Y, Fang J, Nilesen PH, Verstaete W, Boon N (2015) Ammonia and temperature determine the potential clustering in the anaerobic digestion microbiome. Water Res 75:312–323
54. Sundberg C, Al-Soud WA, Larsson M, Alm E, Yekta SS, Svensson BH, Sorensen SJ, Karlsson A (2013) 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol Ecol 85(3):612–626
55. Narihiro T, Sekiguchi Y (2011) Oligonucleotide primers, probes and molecular methods for environmental monitoring of methanogenic archaea. J Microbial Biotechnol 4:585–602
56. Mathai PP, Zitomer DH, Maki JS (2015) Quantitative detection of syntrophic acid degrading bacterial communities in methanogenic environments. Microbiology 161:1189–1197
57. Westerholm M, Dolfing J, Sherry A, Gray ND, Head IM, Schnürer A (2011) Quantification of syntrophic acetate oxidizing microbial communities in biogas processes. Environ Microbiol Rep 3:500–505
58. Ziels RM, Beck DAC, Martí M, Gough HL, Stensel HD, Svensson BH (2015) Monitoring the dynamics of β-oxidising bacteria during anaerobic degradation of oleic acid by quantitative PCR. FEMS Microbiol Ecol 91(4):1–13
59. Lebuhn M, Hanreich A, Klocke M, Schnlüter A, Bauer A, Peréz CM (2014) Towards molecular biomarkers for biogas production from ligno-cellulose-rich substrates. Anaerobe 29:10–21
60. Dziewit L, Pyzik A, Romaniuk K, Sobczak A, Szczesny P, Lipinski L, Bartski D, Drewniak L (2015) Novel molecular markers for the detection of methanogens and phylogenetic analyses of methanogenic communities. Front Microbiol 6(649):1–11
61. Wilkins D, Lu X-Y, Shen Y, Cghen J, Le PKH (2015) Pyrosequencing of mcrA and archeael 16S rRNA genes reveals diversity and substrate preference of methanogenic communities in anaerobic digesters. Appl Environ Microbiol 81(2):604–612
62. Müller B, Sun L, Westerholm M, Schnürer A (2016) Bacterial community composition and FHS profiles of low and high ammonia biogas digesters reveal novel syntrophic acetate-oxidizing bacteria. Accepted for publication in Biotechnology for Biofuels
63. Pereyra LB, Hiibel SR, Prieto Riquelme MV, Reardon KF, Pruden A (2010) Detection and quantification of functional genes of cellulose degrading, fermentative and sulphate reducing bacteria and methanogenic archaea. Appl Environ Microbiol 76:2192–2202
64. Goux X, Calusinska M, Lemaigre S, Marynowska M, Klocke M, Udelhoven T, Benizri E, Delfosse P (2015) Microbial community dynamics sequentially to increasing organic loading rate, acidosis, and process recovery. Biotechnol Biofuels 8:122
65. Lv Z, Leite AF, Harms H, Richnow HH, Liebetrau J, Nikolausz M (2014) Influences of the substrate feeding regime on methanogenic activity in biogas reactors approached by molecular and stable isotope methods. Anaerobe 29:91–99
66. Regueiro L, Lema JM, Carballa M (2015) Key microbial communities steering the functioning of anaerobic digesters during hydraulic and organic overloading shocks. Bioresour Technol 197:208–216
67. Alvarado A, Montañez-Hernández LE, Palacio-Molina SL, Oropeza-Navarro R, Luévanos-Escareño MP, Balagurusamy N (2014) Microbial trophic interactions and *mcr*A gene expression in monitoring of anaerobic digesters. Front Microbiol 5:597
68. Lv W, Zhang W, Yu Z (2013) Evaluation of system performance and microbial communities of a temperature-phased anaerobic digestion system treating dairy manure: thermophilic digester operated at acidic pH. Bioresour Technol 142:625–632
69. Town JR, Links MG, Fonstad AT, Dumonceaux TJ (2015) Molecular characterization of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance. Bioresour Technol 151:249–257
70. Rui J, Li J, Zhang S, Yan X, Wang Y, Li X (2015) The core populations and co-occurrence patterns of prokaryotic communities in household biogas digesters. Biotechnol Biofuels 8 (158):1–15

71. Azman S, Khadem AF, van Lier JB, Zeeman G, Plugge C (2016) Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. Crit Rev Environ Sci Technol 45:2523–2564
72. Li J, Rui J, Yao M, Zhang S, Yan X, Wang Y, Yan Z, Li X (2015) Substrate type and free ammonia determine bacterial community structure in full-scale mesophilic anaerobic digesters treating cattle or swine manure. Front Microbiol 6:1337
73. Li J, Rui J, Pei Z, Sun X, Zhang S, Yan ZY, Wang YP, Liu XP, Zheng T, Li XZ (2014) Straw- and slurry-associated prokaryotic communities differ during co-fermentation of straw and swine manure. Appl Microbiol Biotechnol 98:4771–4780
74. Müller L, Kretzschmar J, Pröter J, Liebetrau J, Nelles M, Scholwin F (2016) Does the addition of proteases affect the biogas yield from organic material in anaerobic digestion? Bioresour Technol 203:267–271
75. Hanrecih A, Schimpf U, Zakrzewski M, Schnluter A, Benndorf D, Heyer R, Rapp E, Puhler A, Reich U, Klocke M (2013) Metagenome and metaproteome analysis of microbial communities in mesophilic biogas-producing batch fermentation indicate concerted plant carbohydrate degradation. Syst Appl Microbiol 36(5):330–338
76. St Pierre B, Wright ADG (2014) Comparative metagenomic analysis of bacterial populations in three full-scale mesophilic anaerobic manure digesters. Appl Microbiol Biotechnol 98 (6):2709–2717
77. Worm P, Koehorst JJ, Visser M, Sedano-Núenz VT, Schaap PJ, Plugge CM, Sousa DZ, Stams AJM (2014) A genomic view on syntrophic versus non-syntrophic life style in anaerobic fatty acid degrading communities. Biochimica et Biophysica Acta 1837:2004–2016
78. Koeck DE, Pechtl A, Zverlov VV, Schwarz WH (2014) Genomics of cellulolytic bacteria. Curr Opin Biotechnol 29:171–183
79. Mba Medie F, Davies GJ, Drancourt M, Nenrissat B (2012) Genome analysis highlight the different biological roles of cellulases. Nature Rev 10:227–234
80. Pelletier E, Kreimeyer A, Bocs S, Rouy Z, Gyapay G, Chouari R et al (2008) 'Candidatus Cloacamonas acidaminovorans': genome sequence reconstruction provides a first glimpse of a new bacterial division. J Bacteriol 190:2572–2579
81. Limam RD, Chouari R, Mazéas L, Wu TD, Li T, Grossin-Debattista J et al (2014) Members of the uncultured bacterial candidate division WWE1 are implicated in anaerobic digestion of cellulose. Microbiol Open 3:157–167
82. Ransom-Jones E, Jones DL, Alan McCarthy AJ, McDonald JE (2012) The fibrobacteres: an important phylum of cellulose-degrading bacteria. Microb Ecol 63:267–281
83. Nobu MK, Narihiro T, Rinke C, Kamagata Y, Tringe SG, Woyke T, Liu W (2015) Microbial dark matter ecogenomics reveals complex synergistic network in a methanogenic bioreactor. ISME J 9(8):1710–1722
84. Welte C, Deppenmeir U (2014) Bioenergetics and anaerobic respiratory chains of aceticlastic methanogens. Biochim Biophys Acta 1837:1130–1147
85. Liu WT, Chan OC, Fang HH (2002) Characterization of microbial community in granular sludge treating brewery wastewater. Water Res 36:1767–1775
86. Campanaro S, Treu L, Kougias PG, De Fransici D, Valle G, Angelidaki I (2016) Metagenomic analysis and functional characterisation of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnol Biofuels 9(26):2–17
87. De Vrieze JD, Hennebel T, Boon N, Verstraete W (2012) Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. Bioresour Technol 112:1–9
88. Miller TL, Wolin MJ (1985) *Methanosphaera stadtmaniaegen*. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. Archiv Microbiol 141:116–122
89. Smith KS, Ingram-Smith C (2007) Methanosaeta, the forgotten methanogen? Trends Microbiol 15(4):150–155
90. Westerholm M, Moesetdt J, Schnürer A (2016) Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance. Appl Energy (Accepted)

91. Carballa M, Regueiro L, Lem JM (2015) Microbial management of anaerobic digestion: exploiting the microbiome-functionality nexus. Curr Opin Biotech 33:103–111
92. Pap B, Györkei Á, Boboescu IZ, Nagy IK, Bíró T, Kondorosi É, Maróti G (2015) Temperature-dependent transformation of biogas-producing microbial communities points to the increased importance of hydrogenotrophic methanogenesis under thermophilic operation. Bioresour Technol 177:375–380
93. Gruninger RJ, Puniya AK, Callaghan TM, Edwards JE, Youssef N, Dagar SD, Fliegerova K, Griffith GW, Forster R, Tsang A, Mcallister T, Elsahed MS (2014) Anaerobic fungi (phylum *Neocallimastigomycota*): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. FEMS Microbiol Ecol 90:1–17
94. Yarlett N, Orpin CG, Munn EA, Yarlett NC, Greenwood CA (1986) Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. J Biochem 236:729–739
95. Khejornsart P, Wanapat M (2010) Diversity of rumen anaerobic fungi and methanogenic archaea in swamp buffalo influenced by various diets. J Anim Vet Adv 9:3062–3069
96. Bauchop T, Mountfort DO (1981) Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Appl Environ Microbiol 42:1103–1110
97. Mountfort DO, Asher RA, Bauchop T (1982) Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. strain RA1 and *Methanosarcina barkeri*. Appl Environ Microbiol 44:128–134
98. Akin DE, Borneman WS (1990) Role of rumen fungi in fibre degradation. J Dairy Sci 73:3023–3032
99. Haitjema CH, Solomon KV, Henske JK, Theodorou MK, O'Malley MA (2014) Anaerobic gut fungi: advances in isolation, culture, and cellulolytic enzyme discovery for biofuel production. Biotechnol Bioeng 111:1471–1482
100. Kazda M, Langer S, Bengelsdorf FR (2014) Fungi open new possibilities for anaerobic fermentation of organic residues. Energy Sustain Soc 4:6
101. Mata-Alvarez J, Dosta J, Romero-Güiza MS, Fonoll X, Peces M, Astals SA (2014) Critical review on anaerobic co-digestion achievements between 2010 and 2013. Renew Sustain Energy Rev 3:412–427
102. Banks CJ, Zhang Y, Jiang Y, Heaven S (2012) Trace element requirements for stable food waste digestion at elevated ammonia concentrations. Bioresour Technol 104:127–135
103. Moestedt J, Nordell E, Shakeri Yekta S, Lundgren J, Marti M, Sundberg C, Ejlertsson J, Svensson BH, Björn A (2016) Effects of trace elements addition on process stability during anaerobic co-digestion of OFMSW and slaughterhouse waste. Waste Manag 47:11–20
104. Moeller L, Görsch K (2015) Foam formation in full-scale biogas plants processing biogenic waste. Energy Sustain Soc 5:1
105. Banks CJ, Heaven S (2013) Optimisation of biogas yields from anaerobic digestion by feedstock type. In: Wellinger A, Murphy J, Baxter D (eds) Woodhead publishing series in energy, Oxford, vol 52. pp 131–165
106. Dandikas V, Heuwinkel H, Lichti F, Drewes JE, Koch K (2014) Correlation between biogas yield and chemical composition of energy crops. Bioresour Technol 174:316–320
107. Li Y, Zhang R, Liu G, Chen C, He Y, Liu X (2013) Comparison of methane production potential, biodegradability, and kinetics of different organic substrates. Bioresour Technol 149:565–569
108. McGenity TJ (eds) (2016) Hydrocarbon and lipid microbiology protocols, Springer protocols handbooks, Springer, Berlin. doi: 10.1007/8623_2016_214
109. Chen JL, Ortiz R, Steele TWJ, Stuckey DC (2014) Toxicants inhibiting anaerobic digestion: a review. Biotechnol Adv 32:1523–1534
110. Rajagopal R, Massé DI, Singh G (2014) A critical review on inhibition of anaerobic digestion process by excess ammonia. Bioresour Technol 143:632–641
111. Esposito G, Frunzo L, Giordano A, Liotta F, Panico A, Pirozzi F (2012) Anaerobic co-digestion of organic wastes. Rev Environ Sci Biotechnol 11:325–341

112. Yekta SS, Svensson BH, Björn A, Skyllberg U (2014) Thermodynamic modeling of iron and trace metal solubility and speciation under sulfidic and ferruginous conditions in full scale continuous stirred tank biogas reactors. Appl Geochem 47:61–73
113. Rasit N, Idris A, Harun R, Ghani WAWAK (2015) Effects of lipid inhibition on biogas production of anaerobic digestion from oily effluents and sludges: an overview. Renew Sustain Energy Rev 45:351–358
114. Ma J, Zhao Q-B, Laurens LLM, Jarvis EE, Nagle NJ, Chen S, Frear CS (2015) Mechanism, kinetics and microbiology of inhibition caused by long-chain fatty acids in anaerobic digestion of algal biomass. Biotechnol Biofuels 8:141
115. Palatsi J, Laureni M, Andrés MV, Flotats X, Nielsen HB, Angelidaki I (2009) Strategies for recovering inhibition caused by long chain fatty acids on anaerobic thermophilic biogas reactors. Bioresour Technol 100:4588–4596
116. Silva SA, Cavaleiro AJ, Pereira MA, Stams AJM, Alves MM, Sousa DZ (2014) Long-term acclimation of anaerobic sludges for high-rate methanogenesis from LCFA. Biomass Bioenergy 67:297–303
117. Cavaleiro AJ, Salvador AF, Alves JI, Alves M (2009) Continuous high rate anaerobic treatment of oleic acid based wastewater is possible after a step feeding start-up. Environ Sci Tech 43:2931–2936
118. Sawatdeenarunat C, Surendra KC, Takara D, Oechsner H, Khanal SK (2015) Anaerobic digestion of lignocellulosic biomass: challenges and opportunities. Bioresour Technol 178:178–186
119. Ariunbaatar J, Panico A, Esposito G, Pirozzi F, Lens PNL (2014) Pretreatment methods to enhance anaerobic digestion of organic solid waste. Appl Energy 123:143–156
120. Behera S, Arora R, Nandhagopal N, Kumar S (2014) Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renew Sustain Energy Rev 36:91–106
121. Cano R, Pérez-Elvira SJ, Fdz-Polanco F (2015) Energy feasibility study of sludge pretreatments: a review. Appl Energy 149:176–185
122. Harris PW, McCabe BK (2015) Review of pre-treatments used in anaerobic digestion and their potential application in high-fat cattle slaughterhouse wastewater. Appl Energy 155:560–575
123. Naegele HJ, Mönch-Tegeder M, Haag NL, Oechsner H (2014) Effect of substrate pretreatment on particle size distribution in a full-scale research biogas plant. Bioresour Technol 172:396–402
124. Bouallagui H, Touhami Y, Ben Cheikh R, Hamdi M (2005) Bioreactor performance in anaerobic digestion of fruit and vegetable wastes. Process Biochem 40:989–995
125. Lindmark J, Thorin E, Fdhila RB, Dahlquist E (2014) Effects of mixing on the result of anaerobic digestion: review. Renew Sustain Energy Rev 40:1030–1047
126. Dhaked RK, Singh P, Singh L (2010) Biomethanation under psychrophilic conditions. Waste Manag 30:2490–2496
127. Mao C, Feng Y, Wang X, Ren G (2015) Review on research achievements of biogas from anaerobic digestion. Renew Sustain Energy Rev 45:540–555
128. Labatut RA, Angenent LT, Scott NR (2014) Conventional mesophilic vs. thermophilic anaerobic digestion: a trade-off between performance and stability? Water Res 53:249–258
129. Sahlström LA (2003) A review of survival of pathogenic bacteria in organic waste used in biogas plants. Bioresour Technol 87:161–166
130. Brambilla M, Romano E, Cutini M, Pari L, Bisaglia C (2013) Rheological properties of manure/biomass mixtures and pumping strategies to improve ingestate formulation: a review. Trans ASABE 56:1905–1920
131. Drosg B (2013) Process monitoring in biogas plants. IEA Bioenergy Task 37, Technical Brochure
132. Risberg K, Sun L, Levén L, Horn SJ, Schnürer A (2013) Biogas production from wheat straw and manure – impact of pretreatment and process operating parameters. Bioresour Technol 149:232–237

133. Ziembinska-Buczynska A, Banach A, Bacza T, Pieczykolan M (2013) Diversity and variability of methanogens during the shift from mesophilic to thermophilic conditions while biogas production. World J Microbiol Biotechnol 30:3047–3053
134. Mauky E, Jacobi HF, Liebetrau J, Nelles M (2015) Flexible biogas production for demand-driven energy supply – feeding strategies and types of substrates. Bioresour Technol 178:262–269
135. De Vrieze J, Verstraete W, Boon N (2013) Repeated pulse feeding induces functional stability in anaerobic digestion. J Microbial Biotechnol 6:414–424
136. Lahav O, Morgan BE (2004) Titration methodologies for monitoring of anaerobic digestion in developing countries - a review. J Chem Technol Biotechnol 79:1331–1341
137. Marchaum U, Krause C (1993) Propionic to acetic acid ratios in overloaded anaerobic digestion. Bioresour Technol 43:195–203
138. Björnsson L, Hörnsten EG, Mattiasson B (2001) Utilization of a palladium–metal oxide semiconductor (Pd-MOS) sensor for on-line monitoring of dissolved hydrogen in anaerobic digestion. Biotechnol Bioeng 73:35–43
139. Lebuhn M, Weiß S, Munk B, Guebitz GM (2015) Microbiology and molecular biology tools for biogas process analysis, diagnosis and control. Biogas Sci Technol Ser Adv Biochem Eng Biotechnol 151:1–40
140. Beale DJ, Karpe AV, McLeod JD, Gondalia SV, Muster TH, Othman MZ, Palombo EA, Joshi D (2016) An 'omics' approach towards the characterisation of laboratory scale anaerobic digesters treating municipal sewage sludge. Water Res 88:346–357
141. Win TT, Ki H, Cho K, Song KG, Park J (2016) Monitoring the microbial community shift throughout the shock changes of hydraulic retention time in an anaerobic moving bed membrane bioreactor. Bioresour Technol 202:125–132
142. Chen S, He Q (2015) Persistence of *Methanosaeta* populations in anaerobic digestion during process instability. J Ind Microbiol Biotechnol 42:1129–1137
143. Leite AF, Janke L, Lv Z, Harms H, Richnow H-H, Nikolausz M (2015) Improved monitoring of semi-continuous anaerobic digestion of sugarcane waste: effects of increasing organic loading rate on methanogenic community dynamics. Int J Mol Sci 16(10):23210–23226
144. Mulat DG, Jacobi HF, Feilberg A, Adamsen APS, Richnow H-H, Nikolausz M (2016) Changing feeding regimes to demonstrate flexible biogas production: effects on process performance, microbial community structure, and methanogenesis pathways. Appl Envion Microbiol 82(2):438–449
145. Polag D, May T, Müller L, König H, Jacobi F, Laukenmann S, Keppler F (2015) Online monitoring of stable carbon isotopes of methane in anaerobic digestion as a new tool for early warning of process instability. Bioresour Technol 197:161–170
146. Romero-Güiza MS, Vila J, Mata-Alvarez M, Chimenos JM, Astal S (2016) The role of additives on anaerobic digestion: a review. Renew Sustain Energy Rev 58:1486–1499
147. Choong YY, Norli I, Abdullah AZ, Yhaya MF (2016) Impacts of trace element supplementation on the performance of anaerobic digestion process: a critical review. Biores Technol 209:369–379
148. Demirel B, Scherer P (2011) Trace element requirements of agricultural biogas digesters during biological conversion of renewable biomass to methane. Biomass Bioenergy 35:992–998
149. Yamada C, Kato S, Ueno Y, Ishii M, Igarashi Y (2015) Conductive iron oxides accelerate thermophilic methanogenesis from acetate and propionate. J Biosci Bioeng 119(6):678–682
150. Kato S (2015) Biotechnological aspects of microbial extracellular electron transfer. Microbes Environ 30(2):133–139
151. Kougias PG, Boe K, Einarsdottir ES, Angelidaki I (2016) Counteracting foaming caused by lipids or proteins in biogas reactors using rapeseed oil or oleic acid as antifoaming agents. Water Res 79:119–127

152. Peng X, Börner RA, Nges IA, Liu J (2014) Impact of bioaugmentation on biochemical methane potential for wheat straw with addition of *Clostridium cellulolyticum*. Bioresour Technol 152:567–571
153. Nkemka VN, Gilroyed B, Yanke J, Gruninger R, Vedres D, McAllister T, Hao X (2015) Bioaugmentation with an anaerobic fungus in a two-stage process for biohydrogen and biogas production using corn silage and cattail. Bioresour Technol 185:79–88
154. Zhang J, Go R-B, Qiu Y-L, Qiao J-T, Yuan X-Z, Shi X-S, Chuan-Shui Wang C-S (2015) Bioaugmentation with an acetate-type fermentation bacterium *Acetobacteroides hydrogenigenes* improves methane production from corn straw. Bioresour Technol 179:306–313
155. Poszytek K, Ciezkowak M, Sklodowska A, Drewniak L (2016) Microbial consortium with high cellulolytic activity (MCHCA) for enhanced biogas production. Front Microbiol 7:324
156. Fotidis IA, Wang H, Fiedel N-R, Luo G, Karakashev DB (2014) Bioaugmentation as a solution to increase methane production from an ammonia-rich substrate. Environ Sci Tech 48:7669–7676
157. Ács N, Bagi Z, Rákhely G, Minárovics J, Nagy K, Kovács KL (2015) Bioaugmentation of biogas production by a hydrogen-producing bacterium. Bioresour Technol 186:286–293
158. Cirne DG, Björnsson L, Alves M, Mattiasson B (2006) Effects of bioaugmentation by an anaerobic lipolytic bacterium on anaerobic digestion of lipid-rich waste. J Chem Technol Biotechnol 81:1745–1752
159. Quinones TS, Plöchl M, Budde J, Heiermann M (2011) Enhanced methane formation through application of enzymes: results from continuous digestion tests. Energy Fuels 2011 (25):5378–5386
160. Kothari R, Pandey KA, Kumar S, Tyagi VV, Tyagi SK (2014) Different aspects of dry anaerobic digestion for bio-energy: an overview. Renew Sustain Energy Rev 39:174–195
161. Sha FA, Mahmood Q, Rashid N, Pervez A, Raja IA (2015) Co-digestion, pretreatment and digester design for enhanced methanogenesis. Renew Sustain Energy Rev 42:627–642
162. van Lier JB, van der Zee FP, Frijters CTMJ, Ersahin ME (2015) Celebrating 40 years of sludge bed rectors for industrial waste water treatment. Rev Environ Biotechnol 14:681–702
163. Apples L, Baeyens J, Degrève J, Dweil R (2008) Principles and potential of the anaerobic digestion of waste – activated sludge. Progess Energy Combust Sci 34:755–718
164. Ozgun H, Dereli RK, Ersahin ME, Kinaci C, Spanjers H, van Lier JB (2013) A review of anaerobic membrane bioreactors for municipal wastewater treatment: integration options, limitations and expectations. Sep Purif Technol 118:89–104
165. Rajagopal R, Cata Saady NM, Torrijos M, Thanikal JV, Hung Y-T (2013) Sustainable agro-food industrial wastewater treatment using high rate anaerobic process. Water 5:292–311
166. Jensen PD, Yap SD, Boyle-Gota A, Janoschka J, Carney C, Pidou M, Batstone DJ (2015) Anaerobic membrane reactors enable high rate treatment of slaughter house wastewater. Biochem Eng J 97:132–141
167. Bustillo-Lecompte CF, Mehrvar M (2015) Slaughterhouse waste water characteristics, treatment, and management in meat processing industry: a review on trends and advances. J Environ Manage 161:287–302
168. Karadag D, Köroglu OE, Ozkaya B, Cakmakci M (2015) A review on anaerobic biofilm reactors for the treatment of dairy industry wastewater. Process Biochem 50:262–271
169. Nasir IM, Ghazi TIM, Omar R (2012) Anaerobic digestion technology in livestock manure treatment for biogas production: a review. Eng Life Sci 12(3):258–269
170. Turkdogan FI, Park J, Evans EA, Ellis TG (2013) Evaluation of pretreatment using UASB and SGBR reactors for pulp and paper plants wastewater treatment. Water Air Soil Pollut 224:1512
171. Lettinga G, van Velsen AFM, Hobma SW, de Zeeuw W, Klapwijk A (1980) Use of the upflow sludge blanket (ASB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol Bioeng 22(4):699–734
172. Verstraete W, de Beer D, Pena M, Lettinga G, Lens P (1996) Anaerobic bioprocessing of organic waste. World J Microbiol Biotechnol 12:221–238

173. Ellis TG, Evars KM (2008) A new high rate anaerobic technology, the static granular bed reactor (SGBR), for renewable energy production from medium strength waste streams. WIT Trans Ecol Environ 109:141–150
174. Lettinga G, Rebac S, Zeeman G (2001) Challenge of psychrophilic anaerobic wastewater treatment. Trends Biotechnol 19(9):363–370
175. Lim JS, Kim T-H (2014) Applicability and trends of anaerobic granular sludge treatment processes. Biomass Bioenergy 60:189–202
176. Hulstoshoff L, Lens P, Castro S, Lettinga G (2004) Anaerobic sludge granulation. Water Res 38:1376–1389
177. Batstone DJ, Keller J (2001) Variation of bulk properties of anaerobic granules with wastewater type. Water Res 35(7):1723–1729
178. McHugh S, Reilly C, Mahony E, Colleran E, Flaherty V (2003) Anaerobic granular sludge technology. Rev Environ Sci Biotechnol 2:225–245
179. Cao X, Sheng Y, Cao H, Zhang Y (2014) Comparison of Mg^{2+} and Ca^{2+}-enhancing anaerobic granulation in an expanded granular sludge-bed reactor. Sci China 57(11):1596–1601
180. Satho H, Tsushoma I, Miura Y, Ito T, Okabe S (2012) Characterization of microbial community structures and their activities in single anaerobic granules by beta imaging, microsensors and fluorescence hybridization. Water Sci Technol 65(12):2125–2131
181. Shrestha PM, Malvankar NS, Werner JJ, Franks AE, Elena-Rotaru A, Shrestha M, Liu F, Nevin KP, Angenent LT, Lovley DR (2014) Correlation between microbial community and granule conductivity in anaerobic bioreactors for brewery wastewater treatment. Biores Technol 174:306–310
182. Dahiya S, Joseph J (2015) High rate biomethanation technology for solid waste management and rapid biogas production: an emphasis on reactor design parameters. Bioresour Technol 188:73–78
183. Divya D, Gopinath LR, Christy M (2015) A review on current aspects and diverse prospects for enhancing biogas production in sustainable means. Renew Sustain Energy Rev 42:690–699
184. Munk B, Bauer A, Gronauer A, Lebuh M (2012) A metabolic quotient for methanogenic archaea. Water Sci Technol 66(11):2311–2317
185. Stukey DC (2012) Recent developments in anaerobic membrane reactors. Bioresour Technol 122:137–148
186. Zhu G, Zou R, Jha AK, Huang X, Liu L, Liu C (2015) Recent developments and future perspectives of anaerobic baffled bioreactor for wastewater treatment and energy recovery. Crit Rev Environ Sci Technol 45:1243–127

Adv Biochem Eng Biotechnol (2016) 156: 235–262
DOI: 10.1007/10_2015_5004

Published online: 24 February 2016

Life in the Anoxic Sub-Seafloor Environment: Linking Microbial Metabolism and Mega Reserves of Methane Hydrate

Varsha Honkalas, Ashwini Dabir, and Prashant K. Dhakephalkar

Abstract Sub-seafloor methane hydrate deposits have attracted attention in recent times as an enormous and yet untapped source of alternate energy. It is interesting to note that methane in sub-seafloor methane hydrate deposits is of biogenic origin. The sub-seafloor environment is mostly anoxic and characterized by high pressure and the presence of complex organic matter. Microorganisms adapted to such extreme sub-seafloor environmental conditions may serve as source of novel taxa and industrially valuable biomolecules. Microbial metabolism is responsible for the degradation of complex organic matter and subsequent formation of methane. Various ecophysiological and nutrient conditions have a significant influence on the rate of methane formation and on the conversion of methane into methane hydrate deposits. Understanding the kinetics of methanogenesis is of utmost importance in predicting the rate and extent of methane hydrate deposits in sub-seafloor environments. This review illustrates the diversity of anaerobes in deep-sea sediments associated with methane hydrates and their metabolism leading to methane generation.

Keywords Metabolism · Methane hydrate · Microbial diversity · Sub-seafloor sediments

Contents

V. Honkalas, A. Dabir, and P.K. Dhakephalkar (✉)
Bioenergy Group, MACS, Agharkar Research Institute, Pune 411004, India
e-mail: pkdhakephalkar@aripune.org; pkdhakephalkar@gmail.com

1 Introduction

Microorganisms, unlike most plants and animals, are not always dependent upon the supply of atmospheric oxygen for their growth. Bacteria and archaea are classified on the basis of their oxygen requirement into the following categories: (1) obligate aerobes which grow only in the presence of oxygen; (2) facultative anaerobes which grow both in the presence and absence of free oxygen (examples of facultative anaerobic bacteria are *Staphylococcus* sp., *Streptococcus* spp., *Escherichia coli*, *Listeria* sp., *Shewanella oneidensis*, etc.) [1]; (3) microaerophilic organisms which can grow best in the presence of low concentrations of molecular oxygen; and (4) obligate anaerobes which can grow only in the absence of oxygen (examples of anaerobic bacteria include *Actinomyces*, *Prevotella*, *Bacteroides*, *Clostridium*, *Fusobacterium*, *Peptostreptococcus*, *Porphyromonas*, *Veillonella*, etc.) [2]. Aerobic bacteria utilize molecular oxygen as a terminal electron acceptor or as an oxidizing agent. Thus, the energy need of aerobes is fulfilled by aerobic respiration. Facultative anaerobes can utilize molecular oxygen as terminal electron acceptor when available. Anaerobic bacteria, however, do not use molecular oxygen to obtain energy – they use fermentation or anaerobic respiration. Some anaerobes can tolerate – but not grow in – the presence of oxygen, whereas the presence of oxygen could be toxic for others such as rumen bacteria and methanogens. Anaerobic bacteria are capable of utilizing other electron oxidants. Thus, in anoxic conditions, microbial communities are sequentially observed which can utilize NO_3^-, Mn^{2+}, Fe^{2+}, SO_4^{2-}, and CO_2. During aerobic respiration, the maximum amount of energy is liberated with a positive O_2 redox potential (820 mV). Redox potential decreases with different electron acceptors and finally, for methanogenesis, it is as low as −240 mV. A negative value indicates high electron activity and intense anaerobic conditions.

The energy-generating metabolism of anaerobes is not as efficient as that of aerobic microorganisms. This is evident because anaerobic fermentation of a glucose molecule yields only 2 molecules of ATP whereas aerobic respiration of a glucose molecule yields 36–38 ATP molecules [3]. However, during metabolism, anaerobes consume less carbon for biomass generation and a bigger carbon pool is directed towards the formation of metabolites of industrial value. Thus, higher product yields make anaerobic metabolism advantageous compared to aerobic metabolism [4]. Anaerobic bacteria have had a long history of industrial applications ranging from the production of biogas, fuels, and chemicals [5, 6] to that of

alcohols and solvents [7]. Substrate flexibility and toxicity tolerance coupled with unique biosynthetic capabilities make anaerobes suitable candidates for industrial applications. However, because of their complex metabolism and often special cultivation requirements, they have been less explored.

Anaerobes thrive in a wide range of environments on planet Earth. Natural systems such as the animal gut, rumen, river sediments, lake sediments, ocean floor, and sub-seafloor deposits are the favored habitats for anaerobic bacteria and archaea. They can also be found in micro-environments where oxygen has been depleted by other aerobic organisms. In the case of submarine sediments, particularly sediments near coastal areas, oxygen is removed rapidly by aerobic respiration. Once oxygen is removed, a microbial population adapted to anaerobic environments predominates. In sediments near coastal areas, anaerobic conditions are generated when oxygen consumption exceeds oxygen supply.

Sub-seafloor sediments associated with methane hydrate deposits form an interesting habitat for anaerobes. Because of its pristine nature, such a habitat forms a fertile source of novel anaerobic taxa. A description of the diversity and potential industrial applications of anaerobes associated with methane hydrate deposits is given below. Further, the role of anaerobic metabolism leading to the formation of methane hydrate deposits is discussed.

2 Methane Hydrates

Methane hydrates are crystalline solid structures formed of a cage of water molecules surrounding natural gas methane under specific conditions of relatively high pressure and low temperature. Methane hydrates are made up of approximately 85 mol% water. Their properties such as physical appearance, refractive index, and density are similar to those of ice, whereas other properties such as mechanical strength, heat capacity, thermal conductivity, etc. differ from those of ice. The structure of methane hydrate (Fig. 1) is stabilized by weak van der Waals interactions between the water and methane molecules [8, 9].

2.1 Occurrence of Methane Hydrates

Large amounts of methane naturally frozen in hydrated form have been discovered in various environments. These are (1) sediment below arctic permafrost, (2) sedimentary deposits along continental margins, (3) deep-water sediments in lakes and seas, and (4) under Antarctic ice. Massive methane hydrate deposits are found in the Siberian arctic region, Kara Sea, Skan Bay in Alaska, Tibetan Plateau, etc. in the Arctic permafrost region, and the Chilean margin, Norwegian margin, Nankai Trough of Japan, Cascadian continental margin, Hikurangi Trough off the East Coast of New Zealand, Gulf of Mexico, Margins of Taiwan, etc. represent methane

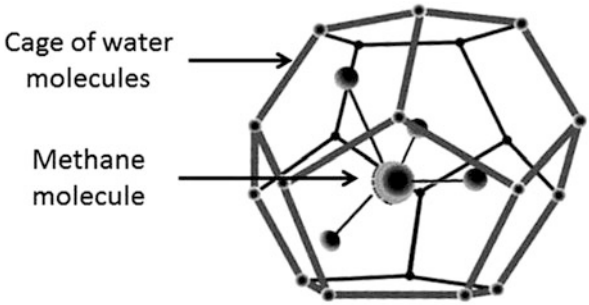


Fig. 1 Schematic presentation of methane hydrate crystal

Table 1 Global methane hydrate deposits inferred, and recovered in the deep submarine sediments (modified after [9])

Site description	Methane hydrates	
	Recovered[a]	Inferred[b]
Arctic Ocean	+	+
Pacific Ocean	+	+
Southern Ocean	+	+
Tibet	+	–
Lake Baikal	+	–
Atlantic Ocean	+	+
Southern Ocean	–	+
Indian Ocean	+	+
Caspian Sea	+	–

–Presence; +Absence
[a]Confirmed by direct sampling
[b]Inferred from the data obtained from Bottom Simulating Reflectors (BSR), seismic profiles, and other geological evidence

hydrate sites along continental margins. Methane hydrate deposits were found in Lake Baikal in Siberia, Krishna Godavari basin of the Bay of Bengal in India, Cascadian margin, Black ridge, Ulleung Basin off the coast of South Korea, Gumsut – Kakap in Malaysia, Shenhu Basin in south China, etc. In Antarctic regions, huge deposits of methane hydrate were found in the Weddell Sea and the edge of glaciers in Antarctica. Table 1 represents the presence of methane hydrates throughout the world.

2.2 Significance of Submarine Methane Hydrate Deposits

Methane content associated with global methane hydrate deposits has been estimated to be around $1–5 \times 10^{15}$ m^3 [10]. This amount is twice the amount of energy contributed by all hydrocarbon reservoirs available worldwide [11]. Approximately 1,900 Trillion Cubic Meters (TCM) of methane gas is accumulated in the form of methane hydrate jus within the Indian subcontinent. This volume of gas is sufficient

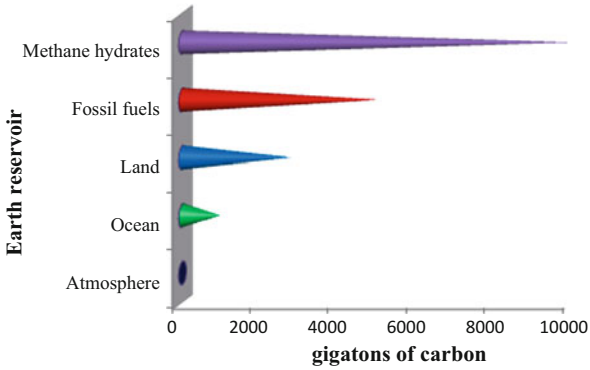


Fig. 2 Schematic representation of energy potential in terms of carbon content of energy reservoirs (modified after [13])

to fulfill India's increasing energy requirement – it is predicted that recovery of even 10% of this huge methane resource can fulfill India's energy requirement for about a century [12]. Hence, methane in the form of hydrate is being considered as an alternate source of energy/fuel for the future. Comparison of energies in conventional hydrocarbons and hydrates in total forms of energy available worldwide is shown in Fig. 2.

The significance of methane hydrate is not limited to its application as an alternate fuel, but is also extended to its potential contribution to global warming. Methane is a greenhouse gas which has been proven to contribute to global warming. Methane associated with methane hydrate deposits can escape into the atmosphere as a result of an earthquake, tectonic disturbances, and leakage during extraction/excavation operations, etc. Methane escaped into the environment could cause enormous holes in the ozone layer, which can significantly contribute to global warming. The potential of methane hydrates as a source of alternate energy and associated environmental concerns have necessitated accurate estimation of global deposits of methane hydrate. Carbon isotopic studies from marine sediments have interpreted the widespread release of carbon from dissociating marine methane hydrates, which would contribute to global warming [14, 15].

2.3 Contribution of Microbes to Methane Formation in the Sub-Seafloor Environment

Stable carbon isotope analysis revealed that most of the methane hydrates and surrounding sediments are of microbial origin [9]. The sub-seafloor production of methane requires the activity of a variety of bacteria and archaea. Organic matter consists of different polymers such as polysaccharides, proteins, nucleic acids, lipids, etc. Organic matter in the uppermost few centimeters of sediments is rich in carbohydrates, both, simple and complex. Methanogens do not possess the ability to utilize carbohydrates as a source of carbon and energy. Hydrolytic bacteria and

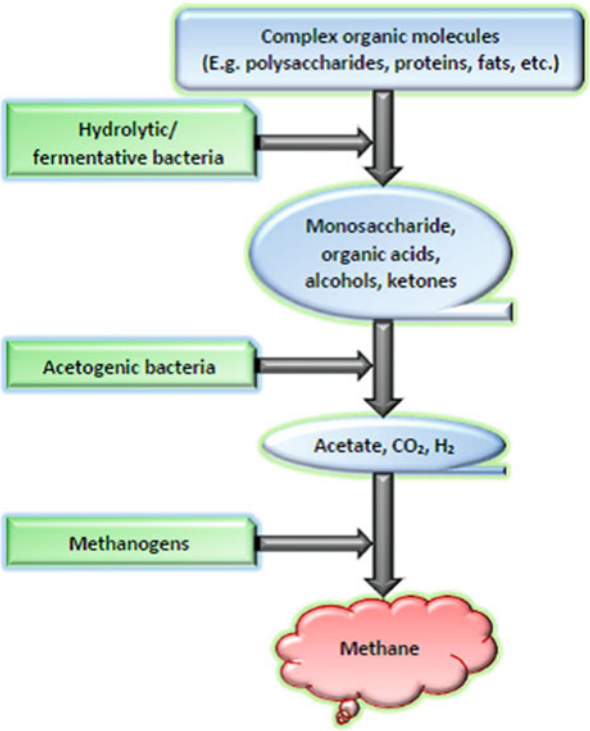


Fig. 3 Schematic representation of conversion of organic matter to methane by bacteria and archaea

fungi can metabolize complex polysaccharides to produce metabolites such as organic acids, volatile fatty acids, and H_2/CO_2. The large, complex polymeric organic molecules are hydrolyzed into smaller molecules (e.g., cellulase catalyzes the hydrolysis of cellulose into glucose and cellobiose; proteases hydrolyze proteins into peptides and amino acids; lipids are hydrolyzed by lipase enzymes to glycerol and fatty acids). Syntrophs and acetogenic bacteria can convert organic acids and some of the volatile fatty acids into acetate, formate, etc. [16]. Acetate, formate, methylamine, methanol, and H_2/CO_2 are utilized by methanogens to produce methane as an end product (Fig. 3). Microbial degradation of organic matter and generation of methane occurs over time scales ranging from minutes to hours for the breakdown of simple biomolecules. Millions of years are required for the mineralization of complex organic compounds in deep submarine sediments. The most rapidly decomposed substances are amino acids, simple sugars, and short-chain carboxylic acids, etc. These substances decompose on time scales of hours to weeks. Complex organic polysaccharides such as cellulose, chitin, pectin, and amino sugars take time scales of years for biodegradation. Several bacteria and archaea are involved in the biodegradation of complex organic matter associated with sediments and further conversion of metabolites into methane. Examples of bacteria involved in biodegradation of organic matter in sub-seafloor sediments include *Oceanirhabdus sediminicola*, *Acetobacterium*, *Marinilactibacillus*,

Clostridium lartetii, *Clostridium bifermentans*, *Clostridium welchii*, *Clostridium sordellii*, *Clostridium botulinum*, *Clostridium celerecrescens*, *Clostridium sulfidigenes*, *Clostridium glycolicum*, etc. [17–20]. Methanogens from deep submarine sediments such as *Methanosarcina mazeii*, *Methanoculleus* sp., *Methanosarcina acetivorans*, *Methanoculleus palmolei*, etc., have been reported for the generation of methane [21–23].

3 Microbial Diversity Associated with Sub-Seafloor Sediments

3.1 Taxonomic Novelty

The sub-seafloor environment is characterized by extreme temperatures (4–50 °C) and pressures (2–1,000 MPa) [9, 11]. Growth of bacteria and methanogens has been observed, even under such extreme conditions. Such a pristine and as yet unexplored environment may serve as a source of taxonomically novel bacteria/archaea, enzymes, and biomolecules of industrial importance. A few novel methanogens have been reported from deep submarine sediments. *Methanoculleus submarinus* was reported as a novel species associated with methane hydrate-bearing deep submarine sediments. Three novel strains of methylotrophic methanogens were isolated. One of the strains, *Methanococcoides alaskense*, was proposed as a novel species, whereas the other two were novel strains within the species *Methanosarcina baltica*. Several bacterial species were reported as novel species from deep submarine sediments. A novel piezotolerant marine lactic acid bacterium *Marinilactibacillus piezotolerans*, barophilic sulfate-reducing bacterium *Desulfovibrio profundus*, and psychrophilic bacterium *Sediminicola arcticus* were reported from different deep-sea sediments. Several taxonomically novel bacterial and methanogen species were reported from deep sub-seafloor sediments. A few of them are listed in Table 2.

Aerobic and anaerobic bacteria have been reported from deep-sea sediments. Anaerobic isolate *Oceanirhabdus sediminicola* was found to be mesophilic, growing in the pH range of 6–8.5 with optimum salinity of 2.5% [17]. Most of the isolates were found to be facultative anaerobes, which could grow in a temperature range of 4–45 °C. *Alcanivorax dieselolei* and *Citreicella marina* were halophilic. *Alcanivorax dieselolei* was industrially important because of its property of alkane degradation. Strict anaerobic metabolism is reported for methanogens which are mesophilic except *Methanococcus aeolicus* which grows at 46 °C. Most methanogens from deep-sea sediments grow at neutral pH [34, 41, 46].

Table 2 Novel species reported from sub-seafloor sediments world over

Novel bacterium	Source of sub-seafloor sediments	Reference
Aestuariivita atlantica	Atlantic Ocean	[24]
Oceanirhabdus sediminicola	South China Sea	[17]
Microbacterium indicum	Chagos Trench, Indian Ocean	[25]
Brevibacterium oceani	Chagos Trench, Indian Ocean	[26]
Streptomyces hoynatensis	Southern Black Sea coast of Turkey	[27]
Celeribacter indicus	Indian Ocean	[28]
Kangiella profundi	Southwest Indian Ocean	[29]
Roseovarius pacificus	Western Pacific Ocean	[30]
Devosia pacifica	South China Sea	[31]
Oceanobacillus profundus	Ulleung Basin of the East Sea, Korea	[32]
Streptomyces indicus	Indian Ocean	[33]
Palaeococcus pacificus	East Pacific Ocean	[34]
Citreicella marina	South-west Indian Ocean Ridge	[35]
Altererythrobacter atlanticus	North Atlantic Rise	[36]
Luteimonas abyssi	South Pacific Gyre	[37]
Roseivivax pacificus	Deep-sea sediments of East Pacific Rise	[38]
Oceanobacillus pacificus	South Pacific Gyre	[39]
Salinimicrobium sediminis	Bay of Bengal at Visakhapatnam, Andhra Pradesh, India	[40]
Alcanivorax dieselolei	East Pacific Ocean	[41]
Methanoculleus sediminis	Upper slope of southwest Taiwan	[42]
Methanobacterium arcticum	Kolyma lowland in the Russian Arctic	[43]
Methanolobus profundi	Natural gas field in Japan	[44]
Methanococcus aeolicus	Nankai Trough	[45]

3.2 Biotechnological Potential

Microorganisms adapted to environmental extremes serve as a source of enzymes that are of biotechnological importance because of their activity at extreme pH, temperature, pressure, etc. Novel bacteria obtained from extreme environments such as sub-seafloor sediments may also serve as a source of valuable biomolecules such as lipids, pigments, antibiotics, enzymes, etc. Most of the microorganisms reported for production of such compounds were found to be facultative anaerobes except *Clostridium* sp. Various cold active enzymes such as amylase, lipase, protease, and pectinase from deep submarine sediments were reported from *Anoxybacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Nocardiopsis* sp., etc. [47, 48]. Cold active enzymes are used in various industrial processes such as synthesis of volatile and heat sensitive compounds (flavors and fragrance), detergents, etc. They are catalytically efficient at low temperature and save both energy

and time [49]. *Streptomyces* sp. isolated from deep-sea sediments yielded antibiotics such as pluramycin which is active against *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium phlei*, and *Shigella dysenteriae* [50]. It also yielded caboxamycin (which is active against Gram-positive bacteria) and anticancer chemical streptokordin [51]. *Bacillus* sp. isolated from deep-sea sediments yielded a phenazine derivative with a novel ring structure which could be used to treat cancer [52]. Polyunsaturated fatty acids present in barophilic and psychrophilic microorganisms from deep-sea sediments were used in the treatment of heart diseases, allergies, rheumatoid arthritis, psychiatric disorders, etc. [53, 54]. *Kocuria* sp., *Pantoea* sp., and *Alcanivorax* sp. from submarine sediments produce biosurfactant which could be used for *n*-alkane degradation and in heavy metal and hydrocarbon bioremediation processes [55–57]. *Clostridium celerecrescence* isolated from deep-sea sediments associated with methane hydrates is known for its hydrogen and organic acid production [19]. Pigments from psychrophiles have potential for use as food coloring agents [47].

3.3 Microbial Diversity Reported from Sub-Seafloor Sediments

3.3.1 Profiling of the Microbial Community by a Culture Independent Approach

A wide variety of microorganisms are associated with the sub-seafloor environment. It is difficult to anticipate the growth and nutritional requirements of these microorganisms. It is important to replicate the growth and nutritional requirements when cultivating the diverse microorganisms in the laboratory. This is especially important in documenting the diversity and to eliminate bias introduced in a culture-based approach. Molecular approaches such as PCR-based DNA fingerprinting techniques, sequencing of cloned SSU rRNA (16S rRNA) gene libraries, and metagenomics facilitate accurate documentation of the diversity of microbial population. Such information can subsequently be used to provide adequate growth and the nutritional environment required for the cultivation of a majority of the constituents of the microbial population. However, several challenges are faced during culture-independent investigation of microbial diversity associated with environmental samples such as sub-seafloor sediments. These challenges include (1) extraction of DNA from marine sediments inhabiting low biomass of extremophiles, (2) PCR bias introduced because of GC rich template inaccessible to primer annealing during PCR, and (3) PCR inhibition in the presence of inhibitors such as humic acids, phenolic substances, etc. which are co-purified with nucleic acids as contaminants.

Extraction of DNA from deep subsurface sediments is difficult considering the complexity of sediments, low biomasses, structures, presence of PCR inhibitors, etc. [58]. The most widely used methods for DNA isolation from deep submarine

sediments is the method described in [59] which was based on the use of bead beating to lyse the microbial cells and release DNA. Other techniques combined freeze thawing and chemical lysis using detergents such as SDS, CTAB, Proteinase K, etc. [23, 59]. Chemical lysis and bead beating methods are the basis for commercially available kits for DNA isolation from soil. These kits are MoBio Ultra Clean Soil DNA kit, Fast DNA SPIN Kit, Geneclean DNA isolation kit, Qiagen soil DNA mini Kit, etc.

Extraction of DNA from sediment samples may result in co-purification of PCR inhibitors such as humic acids and phenolic compounds. These compounds are known to inhibit the activity of Taq polymerase enzyme [60]. Amplification of community DNA from deep-sea sediments was improved by including PCR additives such as bovine serum albumin, Triton X-100, T4 gene 32 proteins, polyethylene glycol 8000, and glycerol [61]. Bovine serum albumin (BSA) increases the thermal stability and half-life of the enzymes in the PCR reactions [62]. Triton X-100 overcomes the inhibitory effects of trace amounts of strong ionic detergents remaining in DNA [63]. Glycerol improves the efficiency of PCR by enhancing hydrophobic interactions between protein domains and lowering the strand-separation temperature [64]. By altering the annealing temperature, Mg^{2+} concentration, and DNA template concentration, the efficiency of amplification of community DNA was enhanced. Following is an illustration of molecular approaches used by researchers to document microbial diversity associated with sub-seafloor methane hydrate deposits.

Bacterial and Archaeal Diversity

Microbial community profiles investigated by a culture-independent approach from various locations (associated with methane hydrate) are described below. Members of phylum *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, *Euryarchaeota*, *and Crenarchaeota* were dominant in most of the sites. *Proteobacteria*, which are found to be most common in the sub-seafloor sediments, perform functions such as degradation of organic matter. They are major contributors in nitrogen and sulfur cycle. Products of their metabolism could be the source of electron acceptors for another group of microorganisms. Members of phylum *Firmicutes* were common members observed in most of the deep submarine sediment. Their presence could be related to their functions at that site. *Firmicutes* are known for survival in extreme conditions. They may be aerobes, facultative anaerobes, or strict anaerobes. Several members of phylum *Firmicutes* are involved in the biodegradation of complex organic matter associated with sediments such as *Clostridium*, *Marinilactibacillus*, etc. They produce gaseous metabolites such as H_2 and CO_2 in large amounts which could be the substrate for methanogens. Phylum *Euryarchaeota* includes methanogens which indicates the presence of the methanogenesis process in a deep sub-seafloor environment. Methanogens of the orders *Methanomicrobiales* and *Methanosarcinales* are detected most commonly. These methanogens are known to utilize acetate, formate, and H_2/CO_2, which are the major bacterial

metabolites. *Methanoculleus* was common in most sites, whereas *Methannosaeta* is found to be unique to the Gulf of Mexico site AT425. All these functions of bacteria and archaea justify their presence in the deep sub-seafloor environment. Bacterial and archaeal diversity from various sites are listed in Table 3.

3.3.2 Culture-Dependent Approach

The microbial community profiled by culture-dependent techniques usually constitutes less than 1% of the diversity existing in situ. This percentage is even lower in the case of extreme habitats such as the deep submarine environment [72]. However, it is important to isolate, identify, and characterize diverse microorganisms to study biochemical processes and physiology of microorganisms associated with sub-seafloor habitats. Several investigators have adopted combinations of special cultivation techniques and parameters such as pressure, temperature, nutrient requirements, etc., to isolate, identify, and characterize microorganisms from the deep sub-seafloor environment. Following is an illustration of cultivation and characterization of diverse microorganisms from sediments associated with methane hydrate deposits in the marine environment.

An adequate supply of nutrients is essential for the cultivation of diverse microbial population. A range of nutrient media was used in different studies to cultivate microorganisms in the laboratory. Generally, basal media comprising organic/inorganic nitrogen source, trace elements, and vitamins was supplemented with complex organic carbon and energy sources. Organic matter in deep-sea sediments consists of complex polysaccharides; hence, the majority of the studies used complex carbohydrates as a source of carbon and energy for bacteria. Marine agar [73] or synthetic anaerobic MM medium [67] were used as basal medium and supplemented with one or a few carbon and energy sources such as Tween 40, carboxymethylcellulose, sodium formate, acetate, methanol, monoethylamine to isolate heterotrophic bacteria, acetogens, sulfate reducers from deep-sea sediments, etc. In another approach, a mixture of glucose, fructose, galactose, lactate, glycolate, glycerol, mannitol, casamino acids, etc., was used with synthetic seawater to isolate bacteria [74]. There are very few reports of isolation of archaea/methanogens from deep submarine sediments. Methanogens cannot utilize complex substrates as bacteria can, and hence they have a very limited substrate range. MSH medium [22] and MJYP medium [75] were used for isolation of methanogens and archaea, respectively, from deep submarine sediments. Acetate, formate, trimethylamine, H_2, and CO_2 were used as carbon/energy sources for isolation of methanogens whereas sulfur was used for isolation of archaea.

Ecophysiological Conditions

The temperature at seafloor level is ca. 4 °C; as sub-seafloor depth increases, there is an increase in temperature. Before isolation of bacteria and archaea from deep submarine sediments, the depth of the samples below seafloor was considered to

Table 3 Bacteria and archaea reported in various locations across the world

Source	Phylum	Species	Reference
Sediments of the Xisha Trough, South China Sea	• *Proteobacteria*	*Stella humosa*	[65]
		Inanidrilus makropetalos	
	• *Firmicutes*	*Moorella* sp.	
	• *Chloroflexi*	*Dehalococcoides* sp.	
	• *Planctomycetes*	*Verrucomicrobia thermoacetica*	
	• *Acidobacteria*		
	• *Actinobacteria*		
	• *Bacteroidetes*		
	• *Crenarchaeota*		
South Hydrate Ridge (SHR) on the Cascadia Margin	• *Actinobacteria*		[66]
	• *Planctomycetes*		
	• *Spirochaetes*		
	• *Chloroflexi*		
	• *Bacteroidetes*		
	• *Proteobacteria*		
	• *Firmicutes*		
	• *Crenarchaeota*		
	• *Euryarchaeota*	*Methanoculleus* sp.	
Deep-sea sediments of Nankai Trough	• *Proteobacteria*	*Ralstonia pickettii*	[67]
		Desulfofrigus fragile	
		Pseudomonas chloritidismutans	
		Acinetobacter lwoffi	
		Klebsiella planticola	
	• *Firmicutes*	*Carnobacterium alterfundicum*	
		Acetobacterium psammolithicum	
		Acetobacterium carbinolicum	
		Marinilactibacillus psychrotolerans	
		Carnobacterium alterfundicum	
		Desemzia incerta	
		Eubacterium limosum	
		Clostridium acetobutylicum	
		Fusibacter paucivorans	
	• *Spirochaetes*	*Spirochaeta* sp.	
		Spirochaeta smaragdinae	

(continued)

Table 3 (continued)

Source	Phylum	Species	Reference
Cascadia Margin in and around Ocean Drilling Program (ODP) site 892B, 889, 890	• *Proteobacteria*	*Thiomicrospira denitrificans*	[23]
		Desulfolobus rhabdoformis	
		Desulfolobus sp.	
		Desulfobacula toluolica	
		Desulfonema magnum	
	• *Crenarchaeota*	*Crenarchaeum symbiosum*	
		Thermoproteus tenax	
		Pyrodictium occultum	
		Thermofilum pendens	
		Solfolobus solfataricus	
	• *Euryarchaeota*	*Methanosarcina mazeii*	
Deeply buried marine sediments of the Pacific Ocean Margins	• *Actinobacteria*		[21]
	• *Bacteroidetes*		
	• *Proteobacteria*	*Desulfobacterium*	
		Desulforhopalus	
		Pelobacter sp.	
		Desulfococcus sp.	
		Desulfosarcina sp.	
	• *Firmicutes*		
	• *Spirochaetes*		
	• *Crenarchaeota*	*Pyrococcus* sp.	
		Thermococcus sp.	
		Archaeoglobu sp.	
	• *Euryarchaeota*	*Methanosarcina acetivorans*	
		Methanoculleus palmolei	
		Methanocaldococcus sp.	
		Methanothermococcus sp.	
Gas hydrate bearing sediments of good weather ridges offshore SW of Taiwan	• *Cloroflexi*		[35]
	• *Planctomycetes*		
	• *Spirochaetes*		
Deep marine sediments in a Forearc Basin	• *Actinobacteria*	*Streptomyces thermodiastaticus*	[68]
	• *Bacteroidetes*	*Prevotella tannerae*	
		Prevotella pallens	
	• *Proteobacteria*	*Kingella denitrificans*	
		Pelobacter acetylenicus	
		Idiomarina loihiensis	
	• *Planctomycetes*	*Pirellula* sp.	

(continued)

Table 3 (continued)

Source	Phylum	Species	Reference
	• *Euryarchaeota*	*Archaeoglobus fulgidus*	
		Thermococcus fumicolans	
		Pyrococcus horikoshii	
		Halobacterium salinarum	
		Thermolasma acidophilum	
	• *Crenarchaeota*	*Staphylothermusmarinus*	
		Thermoproteus nutrophilus	
		Acidianus infernus	
		Thermofilum pendens	
		Desulfurococcus mobilis	
		Sulfolobus sulfataricus	
Hikurangi margin deep-sea sediments, New Zealand	• *Proteobacteria*	*Roseobacter litoralis*	[69]
		Acrobacter halophilus	
		Photobacterium lipolyticum	
		Leucothrix sp.	
	• *Bacteroidetes*	*Flavobacterium granuli*	
	• *Chloroflexi*	*Chloroflexus aggregans*	
	• *Crenararchaeota*		
Shenhu area, South China Sea	• *Proteobacteria*		[70]
	• *Chloroflexi*		
	• *Planctomycetes*		
	• *Crenarchaeota*		
Gulf of Mexico site AT425	• *Proteobacteria*		[71]
	• *Actinobacteria*		
	• *Firmicutes*		
	• *Euryarchaeota*	*Methanosaeta* sp.	

decide ecophysiological conditions during isolation. Isolation of bacteria was carried out at different incubation temperatures. Bacteria isolated from Antarctic deep-sea sediments were enriched at 10 °C. This temperature covered the growth of psychrophilic as well as psychrotolerant bacteria. Bacteria were isolated from Nankai Trough deep-sea sediments by enriching them at 25 °C in dark anaerobic conditions. The pH of the medium in these studies was 6.8–7.8. In a few cases, enrichment was set up at pH 4.5–9 to isolate bacteria [67, 73, 76].

For isolation of methanogens from sediments in Hydrate Ridge, enrichments were set up at neutral pH and incubated at 4 °C and subsequently 15 °C. For

sediments of the Forearc basin of the Nankai Trough, enrichments for methanogens were incubated at 10 °C and 35 °C [22, 45, 77]. Isolation of archaea from deep-sea hydrothermal sediments was carried out at 80 °C.

Special Techniques Used

Various new strategies were used for isolation of different types of bacteria. A few of them are described below.

1. *DeepIsoBUG:* A system was developed for the cultivation of microbes from deep-sea sediments using pressure retaining instruments. DeepIsoBUG is a combination of HYCINTH and PRESS systems. HYCINTH is for pressure retaining and coring of samples and PRESS is for core cutting and processing of samples. In situ pressure (ca. 100 MPA) was applied in this technique for the enrichment of microflora from deep-sea sediments. Bacteria belonging to phylum *Chloroflexi* were isolated from deep-sea sediments associated with methane hydrates using this approach [74].
2. *Continuous flow bioreactor:* The Down flow Hanging Sponge (DHS) continuous flow bioreactor is one of the novel approaches for isolation of microbes from deep-sea sediments [78]. This uses polyurethane sponges to provide a large surface area for microbial colonization [79]. It enriches slow growing anaerobic microbes. Continuous cultivation mode allows the outflow of metabolites which may inhibit microbial growth. Ammonia oxidizing anaerobic bacteria, heterotrophic bacteria, and archaea were enriched from deep-sea sediments of Japan using this approach [80, 81].
3. *High pressure reactor cultivation:* In yet another approach, high pressure and continuous flow bioreactor techniques were combined for the cultivation of microbes of marine origin. An anaerobic environment under high pressure was created using CO_2 (up to 100 MPa). This system enriched methanotrophs and sulfate reducers from deep-sea sediments [82]. Microbial metabolism investigated in this system revealed sulfate-driven anaerobic oxidation of methane wherein methane coupled with sulfate in an anoxic environment to form HCO_3^-, HS^-, and water. This metabolism was mediated by the consortium of methanotrophic archaea and sulfate-reducing bacteria.

For isolation of archaea/methanogens, traditional methods were used. After enrichment, isolation of archaea/methanogens was carried out using the roll tube technique described in [83].

Bacterial and Archaeal Diversity

Most of the bacteria isolated from deep submarine sediments belonged to phylum *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, etc. [67, 74, 84, 85] which are the most dominant members of deep submarine associated environments.

Table 4 Bacterial and archaeal flora reported from deep submarine sediments using various techniques

Phylum	Species	Source	Reference
• *Actinobacteria*	*Brevibacterium iodinum*	Eastern South Atlantic Ocean	[73]
	Micrococcus luteus		
	Nesterenkonia halobia		
• *Firmicutes*	*Paenibacillus glucanolyticus*		
	Brevibacillus parabrevis		
	Bacillus firmus		
	Staphylococcus saprophyticus		
	Oceanobacillus iheyensis		
	Teribacillus saccharophylus		
• *Proteobacteria*	*Pseudoalteromonas tetreodonis*		
	Psychrobacter aquaticus		
	Cobetia marina		
	Marinobacter excellens		
	Halomonas aquamarina		
	Idiomarina loihiensis		
• *Proteobacteria*	*Desulfofrigus fragile*	Nankai Trough, Leg 190	[67]
	Pseudomonas chloritidismutans		
• *Firmicutes*	*Carnobacterium alterfundicum*		
	Acetobacterium psammolithicum		
• *Spirochaetes*	*Spirochaeta* sp.		
• *Actinobacteria*	*Rhodococcus*, *Microbacterium*	Shimokita Peninsula	[76]
• *Proteobacteria*	*Paracoccus* sp.		
	Halomonas sp.		
	Pseudoalteromonas sp.		
	Pseudomonas sp.		
• *Euryarchaeota*	*Methanoculleus submarinus*	Nankai Trough	[22]
• *Euryarchaeota*	*Methanococcoides alaskense*	Skan Bay, Alaska	[77]
	Methanosarcina baltica		
• *Crenarchaeota*	*Paleococcus pacificus*	East Pacific Ocean	[34]

Important sub-seafloor phylotypes such as *Chloroflexi*, candidate division JS1, etc., were also enriched from sea sediments [86]. The presence of these bacteria was confirmed using molecular approaches [21, 68]. Bacterial flora from deep submarine sediments across the world are detailed in Table 4.

4 Microbial Metabolism in Sub-Seafloor Sediments Leading to Methanogenesis

Sediment surface at the sea bottom represents an oxic environment, whereas sub-seafloor layers or the buried sediment represents an anoxic environment. Both aerobic and anaerobic biodegradation of organic matter in marine sediments has been reported. However, whether the metabolism is faster under aerobic or anoxic conditions is a contentious issue. Organic matter is usually deposited and partially degraded in an oxic environment. The degradation process proceeds/completes when anaerobic decomposers attack organic matter. Biodegradability of organic matter has been reported to decrease with the depth [87]. This observation emphasizes that direct comparison of the biodegradation rates under aerobic and anaerobic environment is deceptive and does not reveal the significance of the metabolism occurring in an anaerobic environment. Complex organic matter is aerobically metabolized by heterotrophic microorganisms to form CO_2 and build biomass. The simpler organic matter is usually metabolized via an aerobic route, whereas decomposition of complex organic compounds is effected under anaerobic conditions. Anaerobic metabolism involves hydrolysis and fermentation of complex organic compounds to simpler metabolites such as volatile fatty acids, organic acids, etc. These metabolites are mineralized to CO_2 by microorganisms in an anoxic environment using inorganic compounds such as nitrates and sulfates as electron acceptors. Thus, anaerobic decomposition is effected by a consortium of organisms in two steps: (1) hydrolysis of macromolecules such as polysaccharides, proteins, lipids, etc., and (2) mineralization of metabolites/intermediates into CO_2. Comparison of microbial metabolism of organic compounds in oxic and anoxic conditions at the sediment surface and in sub-seafloor sediments reveals two important aspects. Aerobic metabolism almost always occurs at a higher rate. This observation could be attributed to the dual role played by oxygen in oxic metabolism. Oxygen acts as a terminal electron acceptor, a role that can be shared by inorganic electron acceptors such as nitrates and sulfates in anoxic microbial metabolism. Oxygen also acts as a potent reactant in the oxygenase-catalyzed initial attack on organic substrates. No such equivalent to oxygen as a reactant in the primary transformation of organic compounds is known in anaerobic metabolism. These observations emphasize the role played by facultative microorganisms in the metabolism of the organic matter a in sub-seafloor environment [88].

Organic matter in sub-seafloor sediments is mainly contributed by dead and decaying material of plant and animal origin. Over the period, this organic matter gets buried under fresh sediments. Analysis of sediments revealed that sediments below seafloor level seem to be deposited with different sedimentation rates which vary from hours to years [89, 90]. Organic matter consists of complex carbohydrates, proteins, lipids, etc. [91–93]. Carbohydrates are among the most abundant and reactive components which consist of pectin, lignin, cellulose, hemicellulose, starch, xylan, chitin, etc. [94]. Different microalgae contribute different carbohydrates such as carrageenans and alginates in marine sediments. Carrageenan and

carrageenin are a family of linear sulfated polysaccharides, synthesized by red seaweeds. Alginates, a linear hetero-polyuronic acid consisting of 1,4 linked a-L-guluronic acid (G) and its C5 epimer b-D-mannuronic acid (M) are synthesized by brown seaweeds. These carbohydrates are utilized by marine organisms. Glucose (>70% of total carbohydrate) is the most abundant monosaccharide observed in deep-sea sediments. The abundance of other simple sugars such as fructose, rhamnose, ribose, arabinose, and galactose in marine sediments decreases as depth increases [95]. The polysaccharide, chitin, which is a structural polymer of *N*-acetyl-D-glucosamine, is produced through degradation of different marine organisms, mainly crustaceans, some molluscs, coelenterate, protozoa, lower fungi, copepoda, and filamentous yeast [96]. Another complex sugar, pectin, is a major cell wall component of plants made up of polymers of methoxylated galacturonic acid. Proteins are the major source of nitrogen for growth of microorganisms. Protein concentration in deep-sea sediments ranged from 507 to 4,098 μg g^{-1}. Cell membranes of plants and animals contain lipids and hence the remains of them could act as a source of fats for microbes. Lipids from deep-sea sediments were found to be present in the range of 23–518 μg g^{-1} [97]. Most of the organic matter buried in marine sediments subjected to elevated temperatures and pressures gets transformed into a complex compound, namely kerogen, a mixture of polycyclic aromatic hydrocarbons [98].

Extracellular enzyme activity is needed for the degradation of complex carbohydrates, proteins, and lipids. Chitin, the most abundant complex carbohydrate, is degraded by hydrolytic bacteria (*Pseudomonas*, *Aeromonas*, *Xanthomonas*, *Serratia*, *Cytophaga*, *Arthrobacter*, *and Bacillus*) through the action of chitinolytic enzymes [99]. About 10% of marine bacterial population could be supported by chitin [100]. Hemicellulose and lignocellulose, part of plant cell wall, becomes degraded into simple sugars and organic acids by cellulolytic bacteria such as *Clostridium thermocellum*, *Bacteroides cellulosolvens*, and *Clostridium cellulolyticum* using cellulase enzyme [101]. Acetogenic bacteria convert these metabolites into acetate. Another major polysaccharide, pectin, is degraded by pectinases including pectin esterases and depolymerases. These enzymes are produced by various Gram-positive and Gram-negative bacteria. Some of them are *Pseudoalteromonas haloplanktis*, *Georgenia muralis*, *Bacillus subtilis*, etc. [102–104]. Decomposition of pectin results in the formation of H_2/CO_2, methanol, and acetate, which are the catabolic substrates for methanogens [105]. *Pseudomonas*, *Cytophaga*, *Alteromonas atlantica*, *Alteromonas carrageenovora*, and some unidentified strains have been found to possess the carrageenan-degrading enzymes. Alginate lyases, which are alginate-degrading enzymes, have been isolated from various marine fungi and bacteria [106].

Proteins present in organic matter are degraded into amino acids by bacteria such as *Planococcus* and *Psychrobacter* using proteases. Lipids are degraded into fatty acids and glycerol by bacteria such as *Halomonas*, *Methylarcula*, *Micrococcus*, and *Psychrobacter* using lipase [107–109]. Bacteria such as *Cycloclasticus*, *Alteromonas*, *Marinobacter*, *Neptunomonas*, etc. degrade polyaromatic hydrocarbons [110].

Metabolites produced from the degradation of carbohydrates, proteins, and lipids are hydrogen, carbon dioxide, formate, acetate, methanol, etc. These metabolites are the ultimate source of carbon/energy for methanogens [111, 112]. Methylated amines produced by degradation of choline, creatine, betaine, trimethylamine oxide, etc., which are common excretory products of marine organisms, also act as substrates for methanogens. *Methanosarcina barkeri* and *Methanococcus mazeii* utilize methanol and methylated amines [113].

Large amounts of H_2/CO_2 occur in the upper region of deep submarine sediments; hence, hydrogenotrophic methanogens are dominant in this region (*Methanoculleus*, *Methanothermobacter*). Acetoclastic methanogenesis was found to be dominant at deeper sediment sites. In some cases, both hydrogenotrophic and acetoclastic methanogenesis were taking place simultaneously in deep-sea sediments [114–116]. Sulfate reduction activity is detected at depths greater than 20 m below seafloor. Here, SO_4^{2-}, which is a major electron acceptor, is contributed by organic matter. Sulfate-reducing bacteria make use of sulfate as electron acceptor. Hydrogen, which acts as an electron donor in this process, is also required for methanogens. Where competition between methanogens and sulfate-reducing bacteria occurs for acetate and hydrogen, methanol and methylated amines act as an important resource for methane production. Different processes occurring below sub-seafloor are represented in Fig. 4.

Organic matter degradation is a complex process and involves the interplay of several biotic and abiotic factors [118–120]. One of the crucial factors is age of the organic matter in sediments. Microbial degradation decreases the amount of available substrates with an extended period of microbial activity. Subsequently, bulk organic matter breaks down at an increasingly lower rate as it degrades [121, 122]. Temperature is an important controlling factor in biodegradation. Generally, reaction rate increases with increase in temperature. However, for sediments, multiple factors such as microbial physiology, the reaction pathway, the timescale of interest, the intensity and duration of temperature perturbation, and the climatic zone play important role in the temperature response [123, 124]. Another important factor affecting degradation is the physical protection of organic matter. It is aided by the formation of mineral matrix which controls degradation on a micro scale [125, 126]. Presence of metal ions also affects the growth of microorganisms in deep-sea sediments. Metal ions such as zinc, copper, cadmium, lead, and nickel have also been reported in sub-seafloor sediments. Most of these trace metals are required for growth of bacteria and archaea. These metal ions act as cofactors for several enzymatic reactions in microorganisms [23].

Thus, methane generation in deep sediments below seafloor level is a cascade of activities of different groups of microorganisms. Huge deposits of such biogenic methane in deep-sea sediments are found throughout the world which could be used as an alternate, clean energy source in the future. Considering global warming produced by methane, better understanding of the exact estimation of methane hydrates is a must. Determining accurate rates for microbial activities in deep submarine environments associated with methane hydrates is difficult. The direct measurement of in situ activities by using methods such as radiotracer-labeled

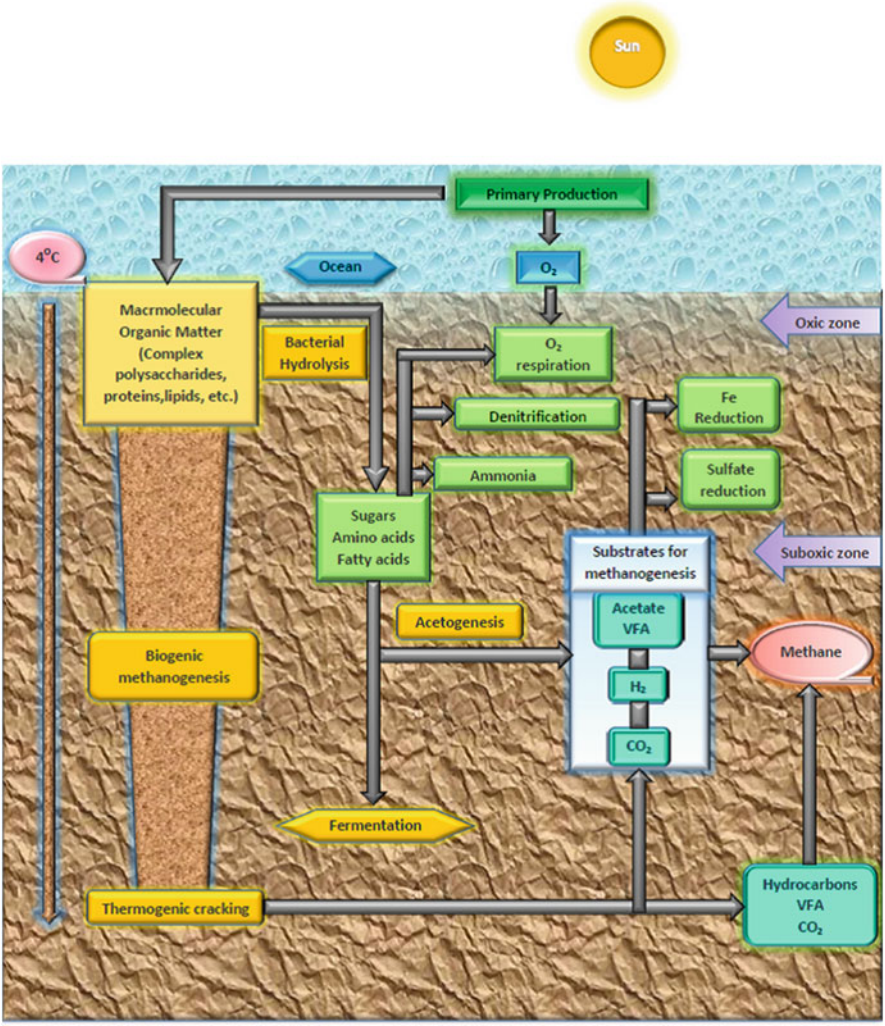


Fig. 4 Schematic representation of processes occurring below sub-seafloor (modified after [117])

substrate turnover is often used to assess microbial activity, although it could overestimate the rate of microbial metabolism compared to geochemical modeling [127]. One approach used biomass recycle reactors (BRRs) or retentostats which were developed to measure the metabolic rates of starved cells from deep-sea sediments associated with methane hydrates. This estimated the in situ methanogenesis rates in Hydrate Ridge (HR) sediments by coupling experimentally derived minimal rates of methanogenesis to methanogen biomass. When starved in a biomass recycle reactor, *Methanoculleus submarinus* produced ca. 0.017 fmol methane/cell/day [128].

Current estimates of methane hydrates are based on extrapolation of field data and geochemical transport reaction modeling. Even then, predictions made by different researchers vary over three orders of magnitude. Different models and observations have helped in the determination of critical parameters for methane hydrate formation. These include accumulation of particulate organic carbon at the sea floor, kinetics of organic matter degradation and methane generation, thickness of the gas hydrate stability zone, solubility of methane in pore fluids, sediment compaction, and transport of methane gas and pore fluid from sediment [129].

Model-based estimates of global methane hydrates were made for the first time by Buffet and Archer. Particulate organic carbon rain rate was considered as the major external driving force for stimulation of hydrate formation. It was calculated as a function of water depth. Another important factor considered was the rate of upward fluid flow. The model was calibrated using data obtained at Black Ridge and the Cascadia margin. A value of 700–900 Gt C has been predicted based on this model [130]. In another study on the determination of global methane hydrate deposits, it was assumed that the entire pool of particulate organic carbon is completely degradable. This model has predicted the presence of 55–700 Gt C [131, 132]. The first simulation of gas hydrate formation under quaternary boundary conditions was performed [133], predicting 995 Gt C in marine sediments. Evaluation of methane hydrate resource potential in the entire outer continental margin, including Alaskan, Atlantic, Gulf of Mexico, and Pacific margins, has been carried out by the Minerals Management Service. It has conducted extensive research for comprehensive assessment of undiscovered methane hydrates across the U.S. The model was derived from a Monte Carlo mass balance progression which utilized a combination of spatially resolved geologic inputs and empirically defined probability distributions. The assessment model works on various modules including a charge module, a container module, a concentration module, and an integration module. A mean in place volume of 606.87 trillion m^3 has been predicted for Gulf of Mexico OCS [134].

Acknowledgement We gratefully acknowledge financial support from Swedish Research Council and Keshava Deva Malaviya Institute of Petroleum Exploration, ONGC, India.

References

1. Ryan KJ (2004) Staphylococci; Streptococci and Enterococci; Corynebacterium, Listeria, and Bacillus. In: Ryan KJ, Ray CG (eds) Sherris medical microbiology, 4th edn. McGraw Hill, New York, pp 261–308
2. Prescott LM, Harley JP, Klein DA (1996) Microbiology, 3rd edn. Brown Publishers, Dubuque, pp 130–131
3. Madigan MT, Martinko JM (2006) Brock biology of microorganisms, 11th edn. Pearson-Prentice Hall, Upper Saddle River
4. Weusthuis RA, Lamot I, van der Oost J, Sanders JP (2011) Microbial production of bulk chemicals: development of anaerobic processes. Trends Biotechnol 29:153–158

5. Goldstein EJ (1995) Anaerobes under assault: from cottage industry to industrialization of medicine and microbiology. Clin Infect Dis 20:S112–S116
6. Wolfe RS (1999) Anaerobic life - a centennial view. J Bacteriol 181:3317–3320
7. Kumar M, Gayen K (2011) Developments in biobutanol production: new insights. Appl Energy 88:1999–2012
8. Sloan ED, Koh CA (2008) Clathrate hydrates of natural gases, 3rd edn. CRC Press, Boca Raton, p 721
9. Kvenvolden KA (1995) A review of the geochemistry of methane in natural gas hydrate. Org Geochem 23:997–1008
10. Milkov AV (2004) Global estimates of hydrate-bound gas in marine sediments: how much is really out there? Earth Sci Rev 66:183–197
11. Sloan ED Jr, Koh C (2007) Clathrate hydrates of natural gases, 3rd edn. CRC press, Boca Raton
12. Sain K, Gupta H (2012) Gas hydrates in India: potential and development. Gondwana Res 22:645–657
13. Boswell R (2009) Is gas hydrate energy within reach? Science 325:957–958
14. Dickens GR, Castillo MM, Walker JC (1997) A blast of gas in the latest paleocene: simulating first-order effects of massive dissociation of oceanic methane hydrate. Geology 25:259–262
15. Zachos JC, Dickens GR, Zeebe RE (2008) An early Cenozoic perspective on greenhouse warming and carbon-cycle dynamics. Nature 451:279–283
16. Marty D (1981) Distribution of different anaerobic bacteria in Arabian Sea sediments. Mar Biol 63:277–281
17. Pi RX, Zhang WW, Fang MX, Zhang YZ, Li TT, Wu M, Zhu XF (2013) *Oceanirhabdus sediminicola* gen. nov., sp. nov., an anaerobic bacterium isolated from sea sediment. Int J Syst Evol Micr 63:4277–4283
18. Toffin L, Zink K, Kato C, Pignet P, Bidault A, Bienvenu N, Prieur D (2005) *Marinilactibacillus piezotolerans* sp. nov., a novel marine lactic acid bacterium isolated from deep sub-seafloor sediment of the Nankai Trough. Int J Syst Evol Micr 55:345–351
19. Honkalas VS, Dabir AP, Arora P, Ranade DR, Dhakephalkar PK (2015) Draft genome sequence of *Clostridium celerecrescens* 152B isolated from sub-seafloor methane hydrate deposits. Mar Gen 21:23–24
20. Kusel K, Karnholz A, Trinkwalter T, Devereux R, Acker G, Drake HL (2001) Physiological ecology of *Clostridium glycolicum* RD-1, an aerotolerant acetogen isolated from sea grass roots. Appl Environ Microb 67:4734–4741
21. Inagaki F, Nunoura T, Nakagawa S, Teske A, Lever M, Lauer A, Jorgensen BB (2006) Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean margin. Proc Natl Acad Sci U S A 103:2815–2820
22. Mikucki JA, Liu Y, Delwiche M, Colwell FS, Boone DR (2003) Isolation of a methanogen from deep marine sediments that contain methane hydrates, and description of *Methanoculleus submarinus* sp. nov. Appl Environ Microb 69:3311–3316
23. Bidle K, Kastner M, Bartlett D (1999) A phylogenetic analysis of microbial communities associated with methane hydrate containing marine fluids and sediments in the Cascadia margin (ODP site 892B). Fems Microbiol Lett 177:101–108
24. Li XG, Xiao X, Xu J (2015) *Kangiella profundi* sp. nov., isolated from a deep-sea sediment. Int J Syst Evol Micr. 10.1099/ijs.0.000257
25. Shivaji S, Bhadra B, Rao RS, Chaturvedi P, Pindi PK, Raghukumar C (2007) *Microbacterium indicum* sp. nov., isolated from a deep-sea sediment sample from the Chagos Trench, Indian Ocean. Int J Syst Evol Micr 57:1819–1822
26. Bhadra B, Raghukumar C, Pindi PK, Shivaji S (2008) *Brevibacterium oceani* sp. nov., isolated from deep-sea sediment of the Chagos Trench, Indian Ocean. Int J Syst Evol Micr 58:57–60

27. Veyisoglu A, Sahin N (2014) *Streptomyces hoynatensis* sp. nov., isolated from deep marine sediment. Int J Syst Evol Micr 64:819–826
28. Lai Q, Cao J, Yuan J, Li F, Shao Z (2014) *Celeribacter indicus* sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium from deep-sea sediment and reclassification of *Huaishuia halophila* as *Celeribacter halophilus* comb. nov. Int J Syst Evol Micr 64:4160–4167
29. Li G, Lai Q, Du Y, Liu X, Sun F, Shao Z (2015) *Aestuariivita atlantica* sp. nov., isolated from deep sea sediment of the Atlantic Ocean. Int J Syst Evol Micr. 10.1099/ijsem.0.000406
30. Wang B, Tan T, Shao Z (2009) *Roseovarius pacificus* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Micr 59:1116–1121
31. Jia YY, Sun C, Pan J, Zhang WY, Zhang XQ, Huo YY, Wu M (2014) *Devosia pacifica* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Micr 64:2637–2641
32. Kim YG, Choi DH, Hyun S, Cho BC (2007) *Oceanobacillus profundus* sp. nov., isolated from a deep-sea sediment core. Int J Syst Evol Micr 57:409–413
33. Luo Y, Xiao J, Wang Y, Xu J, Xie S, Xu J (2011) *Streptomyces indicus* sp. nov., an actinomycete isolated from deep-sea sediment. Int J Syst Evol Micr 61:2712–2716
34. Zeng X, Zhang X, Jiang L, Alain K, Jebbar M, Shao Z (2013) *Palaeococcus pacificus* sp. nov., an archaeon from deep-sea hydrothermal sediment. Int J Syst Evol Micr 63:2155–2159
35. Lai Q, Fu Y, Wang J, Chen S, Zhong H, Sun F, Shao Z (2011) *Citreicella marina* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Micr 61:728–731
36. Wu YH, Xu L, Meng FX, Zhang DS, Wang CS, Oren A, Xu XW (2014) *Altererythrobacter atlanticus* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Micr 64:116–121
37. Fan X, Yu T, Li Z, Zhang XH (2014) *Luteimonas abyssi* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Micr 64:668–674
38. Wu YH, Meng FX, Xu L, Zhang XQ, Wang CS, Oren A, Xu XW (2013) *Roseivivax pacificus* sp. nov., isolated from deep-sea sediment. Int J Syst Evol Micr 63:4574–4579
39. Yu C, Yu S, Zhang Z, Li Z, Zhang XH (2014) *Oceanobacillus pacificus* sp. nov., isolated from a deep-sea sediment. Int J Syst Evol Micr 64:1278–1283
40. Subhash Y, Sasikala C, Ramana CV (2014) *Salinimicrobium sediminis* sp. nov., isolated from a deep-sea sediment. Int J Syst Evol Micr 64:984–988
41. Liu C, Shao Z (2005) *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. Int J Syst Evol Micr 55:1181–1186
42. Chen SC, Chen MF, Lai MC, Weng CY, Wu SY, Lin S, Chen PC (2015) *Methanoculleus sediminis* sp. nov., a methanogen from sediments near submarine mud volcano Southwestern Taiwan. Int J Syst Evol Micr. 10.1099/ijs.0.000233
43. Shcherbakova V, Rivkina E, Pecheritsyna S, Laurinavichius K, Suzina N, Gilichinsky D (2011) *Methanobacterium arcticum* sp. nov., a methanogenic archaeon from Holocene Arctic permafrost. Int J Syst Evol Micr 61:144–147
44. Mochimaru H, Tamaki H, Hanada S, Imachi H, Nakamura K, Sakata S, Kamagata Y (2009) *Methanolobus profundi* sp. nov., a methylotrophic methanogen isolated from deep subsurface sediments in a natural gas field. Int J Syst Evol Micr 59:714–718
45. Kendall MM, Liu Y, Sieprawska-Lupa M, Stetter KO, Whitman WB, Boone DR (2006) *Methanococcus aeolicus* sp. nov., a mesophilic, methanogenic archaeon from shallow and deep marine sediments. Int J Syst Evol Micr 56:1525–1529
46. Lai MC, Hung CC, Ding JY, Wu SY, Lai SJ, Wu CY (2011) Prokaryotic community structure of potential gas hydrate bearing sediments from the good weather ridge. A proceeding of the 7th international conference on gas hydrates (ICGH 2011), SW Taiwan
47. Ohgiya S, Hoshino T, Okuyama H, Tanaka S, Ishizaki K (1999) Biotechnology of enzymes from cold-adapted microorganisms. Biotechnological applications of cold-adapted organisms. Springer, Berlin/Heidelberg, pp 17–34
48. Pote S, Chaudhary Y, Upadhayay S, Tale V, Walujkar S, Shouche Y, Bhadekar R (2014) Identification and biotechnological potential of psychrotrophic marine isolates. Eurasia. J Biosci 8:51–60

49. Margesin R, Feller G, Gerday C, Russell NJ (2002) Cold-adapted microorganisms: adaptation strategies and biotechnological potential. Encyclopedia of Environmental Microbiology. doi:10.1002/0471263397.env150
50. Takeuchi T, Hikiji T, Nitta K, Umezawa H (1957) Effect of pluramycin A on Ehrlich carcinoma of mice studies on antitumor substances produced by *actinomycetes*. XIII. J Antibiotics (Japan) Ser A 10:143
51. Hohmann C, Schneider K, Bruntner C, Irran E, Nicholson G, Bull AT, Jones AL, Brown R, Stach JEM, Goodfellow M et al (2009) Caboxamycin, a new antibiotic of the benzoxazole family produced by the deep-sea strain *Streptomyces* sp. NTK 937. J Antibiot 62:99–104
52. Davidson BS, Schumacher RW (1993) Isolation and synthesis of caprolactins A and B, new caprolactams from a marine bacterium. Tetrahedron 49:6569–6574
53. Lee S, Gura KM, Kim S, Arsenault DA, Bistrian BR, Puder M (2006) Current clinical applications of omega-6 and omega- 3 fatty acids. Nutr Clin Pract 21:323–341
54. Anandan C, Nurmatov U, Sheikh A (2009) Omega 3 and 6 oils for primary prevention of allergic disease: systematic review and meta-analysis. Allergy 64:840–848
55. Pacwa-Płociniczak M, Płaza GA, Piotrowska-Seget Z, Cameotra SS (2011) Environmental applications of biosurfactants: recent advances. Int J Mol Sci 12:633–654
56. Tonkova VE, Gesheva V (2007) Biosurfactant production by Antarctic facultative anaerobe *Pantoea* sp. during growth on hydrocarbons. Curr Microbiol 54:136–141
57. Yakimov MM, Giuliano L, Bruni V, Scarfi S, Golyshin PN (1999) Characterization of Antarctic hydrocarbon-degrading bacteria capable of producing bioemulsifiers. New Microbiol 22:249–256
58. Webster G, Newberry CJ, Fry JC, Weightman AJ (2003) Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16S rDNA-based techniques: a cautionary tale. J Microbiol Methods 55:155
59. Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microb 62:316–322
60. Lloyd KG, MacGregor BJ, Teske A (2010) Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition. FEMS Microbiol Ecol 72:143–151
61. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64:795–799
62. Kreader CA (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol 62:1102–1106
63. Gelfand DH, White TJ (1990) Thermostable DNA polymerases. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 129–141
64. Nagai M, Yoshida A, Sato N (1998) Additive effects of bovine serum albumin, dithiothreitol, and glycerol on PCR. Biochem Mol Biol Int 44:157–163
65. Tao L, Peng W, Pinxian W (2008) Microbial diversity in surface sediments of the Xisha Trough, the South China Sea. Acta Ecol Sin 28:1166–1173
66. Nunoura T, Inagaki F, Delwiche ME, Colwell FS, Takai K (2008) Sub-seafloor microbial communities in methane hydrate-bearing sediment at two distinct locations (ODP Leg204) in the Cascadia Margin. Microbes Environ 23:317–325
67. Toffin L, Webster G, Weightman AJ, Fry JC, Prieur D (2004) Molecular monitoring of culturable bacteria from deep-sea sediment of the Nankai Trough, Leg 190 ocean drilling program. FEMS Microbiol Ecol 48:357–367
68. Reed DW, Fujita Y, Delwiche ME, Blackwelder DB, Sheridan PP, Uchida T, Colwell FS (2002) Microbial communities from methane hydrate-bearing deep marine sediments in a forearc basin. Appl Environ Microbiol 68:3759–3770

69. Ruff SE, Arnds J, Knittel K, Amann R, Wegener G, Ramette A, Boetius A (2013) Microbial communities of deep-sea methane seeps at Hikurangi continental margin (New Zealand). PLoS One 8, e72627
70. Jiao L, Su X, Wang Y, Jiang H, Zhang Y, Chen F (2014) Microbial diversity in the hydrate-containing and-free surface sediments in the Shenhu area, South China Sea. Geosci Front 6:627–633
71. Lanoil BD, Sassen R, La Duc MT, Sweet ST, Nealson KH (2001) Bacteria and archaea physically associated with Gulf of Mexico gas hydrates. Appl Environ Microbiol 67:5143–5153
72. Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169
73. Silva D, Castro MA, Cavalett A, Spinner A, Rosa DC, Jasper RB, Quecine MC, Bonatelli ML, Pizzirani-Kleiner A, Corcao G, Lima AO (2013) Phylogenetic identification of marine bacteria isolated from deep-sea sediments of the eastern South Atlantic Ocean. SpringerPlus 2:127
74. Parkes RJ, Sellek G, Webster G, Martin D, Anders E, Weightman AJ, Sass H (2009) Culturable prokaryotic diversity of deep, gas hydrate sediments: first use of a continuous high-pressure, anaerobic, enrichment and isolation system for sub-seafloor sediments (DeepIsoBUG). Environ Microbiol 11:3140–3153
75. Takai K, Sugai A, Itoh T, Horikoshi K (2000) *Palaeococcus ferrophilus* gen. nov., sp. nov., a barophilic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney. Int J Syst Evol Micr 50:489–500
76. Kobayashi T, Koide O, Mori K, Shimamura S, Matsuura T, Miura T, Horikoshi K (2008) Phylogenetic and enzymatic diversity of deep sub-seafloor aerobic microorganisms in organics-and methane-rich sediments off Shimokita Peninsula. Extremophiles 12:519–527
77. Singh N, Kendall MM, Liu Y, Boone DR (2005) Isolation and characterization of methylotrophic methanogens from anoxic marine sediments in Skan Bay, Alaska: description of *Methanococcoides alaskense* sp. nov., and emended description of *Methanosarcina baltica*. Int J Syst Evol Micr 55:2531–2538
78. Zhang Y, Arends JBA, Van de Wiele T, Boon N (2011) Bioreactor technology in marine microbiology: from design to future application. Biotechnol Adv 29:312–321
79. Imachi H, Aoi K, Tasumi E, Saito Y, Yamanaka Y, Saito Y, Takai K (2011) Cultivation of methanogenic community from sub-seafloor sediments using a continuous-flow bioreactor. ISME J 5:1913–1925
80. Haroon MF, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan Z, Tyson GW (2013) Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature 500:567–570
81. Raghoebarsing AA, Pol A, van de Pas-Schoonen KT, Smolders AJP, Ettwig KF, Rijpstra WIC, Schouten S, Damste JSS, Op den Camp HJM, Jetten MSM, Strous M (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. Nature 440:918–921
82. Ohtomo Y, Ijiri A, Ikegawa Y, Tsutsumi M, Imachi H, Uramoto GI, Inagaki F (2013) Biological CO_2 conversion to acetate in subsurface coal-sand formation using a high-pressure reactor system. Front Microbiol 4:361. doi:10.3389/fmicb.2013.00361
83. Hungate RE (1969) A roll tube method for cultivation of strict anaerobes. Method Microbiol 3B:117–132
84. Bale SJ, Goodman K, Rochelle PA, Marchesi JR, Fry JC, Weightman AJ, Parkes RJ (1997) *Desulfovibrio profundus* sp. nov., a novel barophilic sulfate reducing bacterium from deep sediment layers in the Japan Sea. Int J Syst Bacteriol 47:515–521
85. Barnes SP, Bradbrook SD, Cragg BA, Marchesi JR, Weightman AJ, Fry JC, Parkes RJ (1998) Isolation of sulfate-reducing bacteria from deep sediment layers of the Pacific Ocean. Geomicrobiol J 15:67–83

86. Gordon W, Sass H, Cragg BA, Gorra R, Knab NJ, Green CJ, Mathes F, Fry JC, Weightman AJ, Parkes RJ (2011) Enrichment and cultivation of prokaryotes associated with the sulfate–methane transition zone of diffusion-controlled sediments of Aarhus Bay, Denmark, under heterotrophic conditions. FEMS Microbiol Ecol 77:248–263
87. Kristensen E (1993) Seasonal variations in benthic community metabolism and nitrogen dynamics in a shallow, organic-poor Danish lagoon. Estuar Coast Shelf Sci 36:565–586
88. Kristensen E, Ahmed SI, Devol AH (1995) Aerobic and anaerobic decomposition of organic matter in marine sediment: which is fastest? Limnol Oceanogr 40:1430–1437
89. Boswell R, Collett TS, Frye M, Shedd W, McConnell DR, Shelander D (2012) Subsurface gas hydrates in the northern gulf of Mexico. Mar Petrol Geol 34:4–30
90. Mazumdar A, Dewangan P, Joao HM, Peketi A, Khosla VR, Kocherla M, Avanzinelli R (2009) Evidence of paleo–cold seep activity from the Bay of Bengal, offshore India. Geochem Geophys Geosyst 10(6):1–15. doi:10.1029/2008GC002337
91. Fichez R (1991) Composition and fate of organic-matter in submarine cave sediments - implications for the biogeochemical cycle of organic-carbon. Oceanol Acta 14:369–377
92. Danovaro R, Fabiano M, Della Croce N (1993) Labile organic matter and microbial biomasses in deep-sea sediments (Eastern Mediterranean Sea). Deep Sea Res Pt I 40:953–965
93. Fabiano M, Danovaro R, Fraschetti S (1995) A three-year time series of elemental and biochemical composition of organic matter in subtidal sandy sediments of the Ligurian Sea (northwestern Mediterranean). Cont Shelf Res 15:1453–1469
94. Pakulski JD, Benner R (1994) Abundance and distribution of carbohydrates in the ocean. Limnol Oceanogr 39:930–940
95. Tanoue E, Handa N (1987) Monosaccharide composition of marine particles and sediments from the Bering Sea and northern North Pacific. Oceanol Acta 10:91–99
96. Campbell LL, Williams OB (1951) A study of chitin-decomposing microorganisms of marine origin. J Gen Microbiol 5:894–905
97. Fabiano M, Danovaro R (1998) Enzymatic activity, bacterial distribution, and organic matter composition in sediments of the Ross Sea (Antarctica). Appl Environ Microbiol 64:3838–3845
98. Canfield DE, Kristensen E, Thamdrup B (2005) Aquatic geomicrobiology, vol 48, Advances in marine biology. Elsevier, London, p 640
99. Gartner A, Blumel M, Wiese J, Imhoff JF (2011) Isolation and characterisation of bacteria from the Eastern Mediterranean deep sea. Anton Leeuw 100:421–435
100. Kirchman DL, White J (1999) Hydrolysis and mineralization of chitin in the Delaware estuary. Aquat Microb Ecol 18:187–196
101. Leschine SB (1995) Cellulose degradation in anaerobic environments. Annu Rev Microbiol 49:399–426
102. Tuyen H, Helmke E, Schweder T (2001) Cloning of two pectate lyase genes from the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* strain ANT/505 and characterization of the enzymes. Extremophiles 5:35–44
103. Sasaki M, Koide O, Kobayashi T, Usami R, Horikoshi K (2015) A pectate lyase from a deep sub-seafloor *Georgenia muralis* with unusual molecular characteristics. Extremophiles 19:119–125
104. Nasser W, Chalet F, Robert Baudouy J (1990) Purification and characterization of extracellular pectate lyase from *Bacillus subtilis*. Biochimie 72:689–695
105. Schink B, Ward JC, Zeikus JG (1981) Microbiology of wetwood: importance of pectin degradation and *Clostridium* species in living trees. Appl Environ Microbiol 42:526–532
106. Wong TY, Preston LA, Schiller NL (2000) Alginate lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. Annu Rev Microbiol 54:289–340
107. Dang H, Zhu H, Wang J, Li T (2009) Extracellular hydrolytic enzyme screening of culturable heterotrophic bacteria from deep-sea sediments of the Southern Okinawa Trough. World J Microbiol Biot 25:71–79

108. Biddle JF, Lipp JS, Lever MA, Lloyd KG, Sorensen KB, Anderson R, Hinrichs KU (2006) Heterotrophic archaea dominate sedimentary subsurface ecosystems off Peru. Proc Natl Acad Sci U S A 103:3846–3851
109. Lomstein BA, Langerhuus AT, D'Hondt S, Jorgensen BB, Spivack AJ (2012) Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. Nature 484:101–104
110. Dong C, Bai X, Sheng H, Jiao L, Zhou H, Shao Z (2015) Distribution of PAHs and the PAH-degrading bacteria in the deep-sea sediments of the high-latitude Arctic Ocean. Biogeosciences 12:2163–2177
111. Rowe GT, Deming JW (1985) The role of bacteria in the turnover of organic carbon in deep-sea sediments. J Mar Res 43:925–950
112. Orcutt BN, Sylvan JB, Knab NJ, Edwards KJ (2011) Microbial ecology of the dark ocean above, at, and below the seafloor. Microbiol Mol Biol Rev 75:361–422
113. Oremland RS, Marsh LM, Polcin S (1982) Methane production and simultaneous sulphate reduction in anoxic, salt marsh sediments. Nature 296:143–145
114. Newberry CJ, Webster G, Cragg BA, Parkes RJ, Weightman AJ, Fry JC (2004) Diversity of prokaryotes and methanogenesis in deep subsurface sediments from the Nankai Trough, ocean drilling program Leg 190. Environ Microbiol 6:274–287
115. Whiticar MJ (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chem Geol 161:291–314
116. Parkes RJ, Webster G, Cragg BA, Weightman AJ, Newberry CJ, Ferdelman TG, Kallmeyer J, Jorgensen BB, Aiello IW, Fry JC (2005) Deep sub-seafloor prokaryotes stimulated at interfaces over geological time. Nature 436:390–394
117. Parkes RJ, Cragg B, Roussel E, Webster G, Weightman A, Sass H (2014) A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere: geosphere interactions. Mar Geol 352:409–425
118. Muller PJ, Suess E (1979) Productivity, sedimentation rate, and sedimentary organic matter in the oceans—I. Organic carbon preservation. Deep Sea Res Pt I 26:1347–1362
119. Calvert SE, Pedersen TF (1992) Productivity accumulation and preservation of organic matter in recent and ancient sediments. Columbia University Press, New York, pp 231–263
120. Tromp TK, Van Cappellen P, Key RM (1995) A global model for the early diagenesis of organic carbon and organic phosphorus in marine sediments. Geochim Cosmochim Acta 59:1259–1284
121. Parkes RJ, Cragg BA, Bale SJ, Getlifff JM, Goodman K, Rochelle PA, Harvey SM (1994) Deep bacterial biosphere in Pacific Ocean sediments. Nature 371:410–413
122. Wellsbury P, Goodman K, Barth T, Cragg BA, Barnes SP, Parkes RJ (1997) Deep marine biosphere fuelled by increasing organic matter availability during burial and heating. Nature 388:573–576
123. Arnosti C, Jorgensen BB, Sagemann J, Thamdrup B (1998) Temperature dependence of microbial degradation of organic matter in marine sediments: polysaccharide hydrolysis, oxygen consumption, and sulfate reduction. Mar Ecol Prog Ser 165:59–70
124. Thamdrup B, Fleischer S (1998) Temperature dependence of oxygen respiration, nitrogen mineralization, and nitrification in aquatic sediments. Aquat Microb Ecol 15:191–199
125. Mayer LM, Rahaim PT, Guerin W, Macko SA, Watling L, Anderson FE (1985) Biological and granulometric controls on sedimentary organic matter of an intertidal mudflat. Estuar Coast Shelf Sci 20:491–503
126. Keil RG, Kirchman DL (1994) Abiotic transformation of labile protein to refractory protein in sea water. Mar Chem 45:187–196
127. Phelps TJ, Murphy EM, Pfiffner SM, White DC (1994) Comparison between geochemical and biological estimates of subsurface microbial activities. Microb Ecol 28:335–349
128. Colwell FS, Boyd S, Delwiche ME, Reed DW, Phelps TJ, Newby DT (2008) Estimates of biogenic methane production rates in deep marine sediments at Hydrate Ridge, Cascadia Margin. Appl Environ Microbiol 74:3444–3452

129. Wallmann K, Pinero E, Burwicz E, Haeckel M, Hensen C, Dale A, Ruepke L (2012) The global inventory of methane hydrate in marine sediments: a theoretical approach. Energies 5:2449–2498
130. Buffett B, Archer D (2004) Global inventory of methane clathrate: sensitivity to changes in the deep ocean. Earth Planet Sci Lett 227:185–199
131. Klauda JB, Sandler SI (2005) Global distribution of methane hydrate in ocean sediment. Energy Fuel 19:459–470
132. Davie MK, Buffett BA (2003) A steady state model for marine hydrate formation: constraints on methane supply from pore water sulfate profiles. J Geophys Res Sol Ea 1978–2012:108 (B10)
133. Burwicz EB, Rupke LH, Wallmann K (2011) Estimation of the global amount of submarine gas hydrates formed via microbial methane formation based on numerical reaction-transport modeling and a novel parameterization of Holocene sedimentation. Geochim Cosmochim Acta 75:4562–4576
134. Frye M, Herndon VA, Schuenemeyer J, Cortez CO, Shedd W, Piper K, Herman B (2011, July) Gas hydrate resource assessment on the United States outer continental shelf: a mass balance model. Paper in proceedings of the 7th international conference on gas hydrates (ICGH 2011), Edinburgh, Scotland, United Kingdom

Adv Biochem Eng Biotechnol (2016) 156: 263–292
DOI: 10.1007/10_2015_5001

Published online: 24 February 2016

Anaerobes in Bioelectrochemical Systems

Marika E. Kokko, Annukka E. Mäkinen, and Jaakko A. Puhakka

Abstract In bioelectrochemical systems (BES), the catalytic activity of anaerobic microorganisms generates electrons at the anode which can be used, for example, for the production of electricity or chemical compounds. BES can be used for various purposes, including wastewater treatment, production of electricity, fuels and chemicals, biosensors, bioremediation, and desalination. Electrochemically active microorganisms are widely present in the environment and they can be found, in sediment, soil, compost, wastewaters and their treatment plants. Exoelectrogens are microorganisms capable of donating electrons to anode electrode or accepting electrons from cathode electrode and are mainly responsible for current generation or use in BES. However, current generation from fermentable substrates often requires the presence of electrochemically inactive microorganisms that break down complex substrates into metabolites which can be further utilized by exoelectrogens. The growth and electron transfer efficiency of anaerobes depend on several parameters, such as system architecture, electrode material and porosity, electrode potential and external resistance, pH, temperature, substrate concentration, organic loading rate, and ionic strength. In this chapter, the principles and microbiology of bioelectrochemical systems as well as selective factors for exoelectrogens are reviewed. The anaerobic microorganisms and their electron transfer mechanisms at the anode and cathode are described and future aspects are briefly discussed.

Keywords Anaerobe · Bioelectrochemical system · Exoelectrogen

M.E. Kokko (✉), A.E. Mäkinen, and J.A. Puhakka
Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland
e-mail: marika.kokko@tut.fi

Contents

Abbreviations

BES	Bioelectrochemical system
BOD	Biological oxygen demand
CE	Coulombic efficiency
MDC	Microbial desalination cell
MEC	Microbial electrolysis cell
MES	Microbial electrosynthesis
MFC	Microbial fuel cell
OLR	Organic loading rate
VFA	Volatile fatty acid

1 Introduction

Bioelectrochemical systems (BES) have received increasing attention in the past decade. They can be used for various purposes, including production of electricity, fuels and chemicals, wastewater treatment, biosensors, bioremediation, and desalination. In BES, the catalytic activity of anaerobic microorganisms is used at the anode to generate current. At the cathode, electrons can be accepted by anaerobic microorganisms that utilize them, e.g., for the reduction of carbon dioxide to acetate, or utilised for abiotic reduction reactions. Aerobic microorganisms [1, 2] and enzymes [3, 4] can also be used as biocatalysts at the cathode but are

not within the scope of this chapter and are not further discussed. There are two main types of BES – microbial fuel cells (MFC) in which the anaerobic oxidation of organic matter is used for the production of electricity and microbial electrolysis cells (MEC) where applied electricity is required to overcome thermodynamically unfavorable biotic or abiotic reactions at the cathode.

Electrochemically active microorganisms are widely present in the environment and they can be found, for example, in sediment, soil, compost, wastewaters, and their treatment plants. The most studied electrochemically active pure cultures are *Geobacter* and *Shewanella* species. Microorganisms capable of transferring electrons outside of the cell to the anode electrode are called exoelectrogens [5] and are mainly responsible for current generation in BES. These anaerobic bacteria can use the anode electrode as electron acceptor either through direct contact via *c*-type cytochromes or nanowires or via electron shuttling compounds called mediators. However, the current generation from fermentable substrates, such as glucose or wastewaters, often requires the presence of electrochemically inactive microorganisms that break down the complex substrates into organic acids or alcohols which can be more readily utilized for current production by exoelectrogens. Anaerobes have also been shown to be capable of accepting electrons from the cathode electrode. Although the electron-accepting mechanisms at the cathode are still fairly unknown, it has been shown that enzymes such as *c*-type cytochromes and hydrogenases are involved in the process. The growth of anaerobes and their electron transfer efficiencies depend on several parameters, including system architecture, electrode material and porosity, electrode potential and external resistance, pH, temperature, substrate concentration, organic loading rate, and ionic strength.

Bioelectrochemical systems are an attractive approach to capture the chemical energy stored in waste streams containing easily degradable organics and to convert this energy into valuable products. BESs have many advantages over traditional wastewater treatment systems, including energy savings due to lack of aeration, simultaneous production of electricity, and less sludge production. In addition, MECs can be used for the production of valuable chemicals, bioremediation, or CO_2 fixation. Current densities of laboratory-scale BESs approach values that would be suitable for practical implementation for wastewater treatment. However, more studies with real wastewaters are required to develop strategies for improving the degradation of complex substrates, controlling the microbial reactions, and optimizing the performance of full-scale BES [6].

In this chapter, the principles and microbiology of bioelectrochemical systems as well as selective factors for exoelectrogens are reviewed. The anaerobic microorganisms and their electron transfer mechanisms at the anode and cathode are described, and future aspects are briefly discussed.

2 Bioelectrochemical Systems

2.1 Principles of Bioelectrochemical Systems

There are various applications of bioelectrochemical systems where anaerobes are used as biocatalysts for the production of electrons from biodegradable materials at the anode and/or for the utilization of electrons at the cathode (Table 1). BESs traditionally consists of anode and cathode chambers separated by a selective membrane or separator. At the anode, microorganisms anaerobically oxidize organic or inorganic materials producing electrons that generate current when transferred from anode to cathode electrode through an external load. Simultaneously, protons are transferred through the separator to the cathode. At the cathode, electrons and protons react with electron acceptor either abiotically or biologically. The generated current can be directly utilized in the form of electricity, in which case the bioelectrochemical systems are called microbial fuel cells. Although MFCs often have abiotic cathodes where oxygen reduction completes the electron transfer, biological anaerobic cathodes can also be utilized (Fig. 1a). One example of an anaerobic biocathode is the denitrification of nitrate into nitrite [19] or directly to nitrogen [8].

Instead of producing electricity, current can be applied to the system to produce different compounds at the cathode. By adding current, thermodynamic limitations are overcome and the otherwise unfavorable biological reactions are supported energetically [20]. In microbial electrolysis cells (MEC, Fig. 1b), protons combine at the cathode with electrons or CO_2 to produce hydrogen or methane, respectively. The reactions can be abiotic [21] or biotic [6, 22]. One form of MEC are microbial electrosynthesis cells (MES; [23]), where CO_2 or other carbon sources are reduced to, e.g., acetate or ethanol [13, 14, 24].

Table 1 Different applications of bioelectrochemical systems with anaerobic microorganisms at the anode and/or at the cathode

BES	Function/purpose	Reference
Microbial fuel cell (MFC)	Electricity production	[7]
	Electricity production and denitrification at the cathode	[8, 9]
	Biological oxygen demand (BOD) sensor	[10]
Microbial desalination cell (MDC)	NaCl removal from saline waters with simultaneous electricity production	[11]
Microbial electrolysis cell (MEC)	H_2 or CH_4 production at the cathode with applied voltage	[6, 12]
Microbial electrosynthesis (MES)	Production of organics at the cathode with applied voltage	[13, 14]
Pollutants removal	Bioremediation of organic/inorganic compounds with or without applied voltage	[15, 16]
Resource recovery	Recovery of metals at abiotic cathode with or without applied voltage	[17, 18]

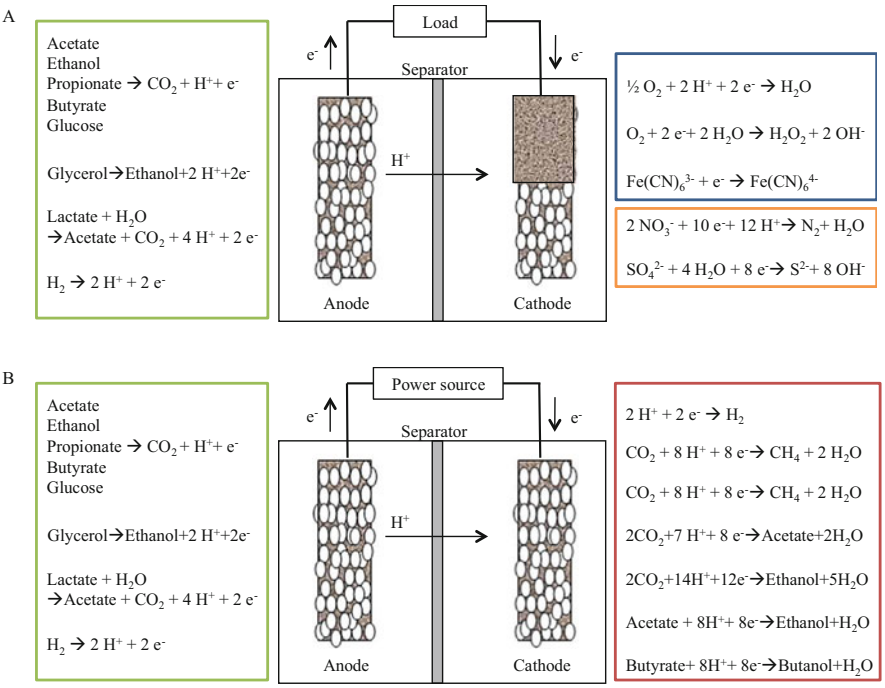


Fig. 1 Schematic diagrams of (**a**) two-chamber microbial fuel cell with abiotic (*blue*) or biotic (*orange*) cathode, and (**b**) production and synthesis of compounds in microbial electrochemical cells or through microbial electrosynthesis at the cathode, which requires additional voltage

2.2 Bioelectrochemical Calculations

In bioelectrochemical systems, oxidation-reduction reactions and their biological standard potentials (Fig. 2) at the anode and cathode determine whether the whole cell potential is positive, i.e., electricity is produced, or negative when applied voltage is required to drive the reaction. Gibbs free energy of reaction in standard conditions ($\Delta G^{0'}_{r}$) (available in [25]) can be used to calculate the electrode potentials at standard conditions by using the Nernst equation (1), where $E^{0}_{an/cat}$ is the standard reduction potential (2), R the universal gas constant (8.31447 J/mol K), T the temperature (K), n the number of electrons per reaction mol, F the Faraday's constant (96 485 C/mol), and [P] and [S] the concentrations of products and substrates, respectively.

The whole cell voltage (E_{eq}) is determined by the difference between the anodic (E_{an}) and cathodic (E_{cat}) redox potentials – see (3). Thus, the higher the cathodic redox potential and the lower the anodic redox potential, the higher the whole cell voltage. If the redox potential at the cathode is lower than at the anode, voltage has to be applied to the system. The performance of the BES is often interpreted as current (I) flowing through the system. This can be further converted into current density calculated based on the area of the anode electrode (I_{an}) or volume (I_{v}).

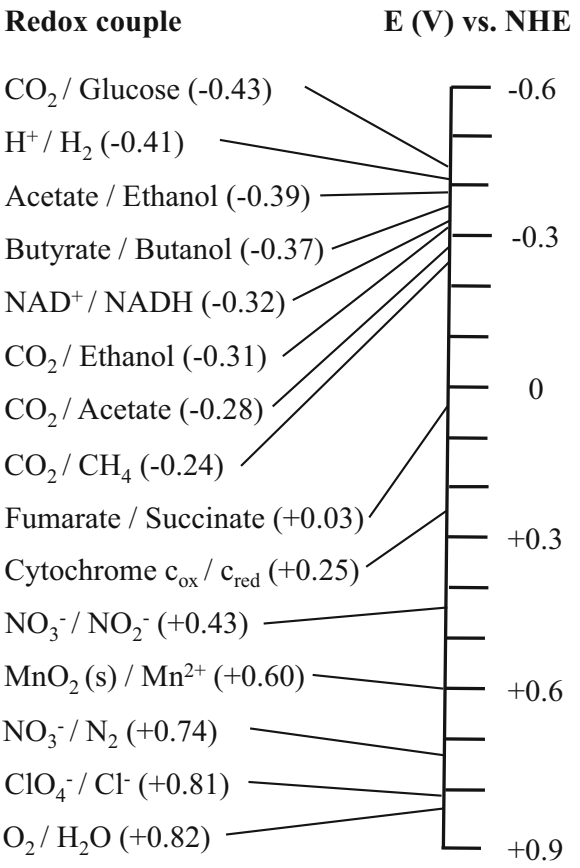


Fig. 2 Biological redox tower of electron donors and acceptors at pH 7

Another way of analyzing the performance of BES is to calculate Coulombic efficiency – see (4) – that gives the ratio of total electrons derived from the oxidized substrate for current production to maximum electrons present in the added substrate. In (4), C_p is calculated by integrating the current over time ($\int I\,dt$) and C_t according to $C_t = n{\cdot}F{\cdot}c{\cdot}V$, where c is the concentration of substrate (mol/L) and V the liquid volume at the anode (L).

$$E = E^0_{an/cat} - \frac{RT}{nF}\ln\left(\frac{[P]^x}{[S]^y}\right), \tag{1}$$

$$E^o_{an/cat} = \frac{-\Delta G^{o'}_r}{nF}, \tag{2}$$

$$E_{eq} = E_{cat} - E_{an}, \tag{3}$$

$$CE = \frac{C_p}{C_t}\cdot 100\%. \tag{4}$$

Theoretically, all the biochemical energy in the substrate can be converted into electricity. In practice, however, losses occur due to microbial growth and BES configuration. Electrons can be lost because of activation, ohmic, and mass transport losses. Activation losses occur due to the activation barrier present in the substrate or electron acceptor [26]. These losses can be decreased by enhancing the biofilm thickness [27] or by increasing the electrode surface area, temperature, or substrate concentration [28], which enhances the electron transfer between anaerobes and the electrode [29]. Ohmic losses are associated with the electron and proton flows through the electrodes, electrolytes, and interconnections (such as separators) [28, 30]. Ohmic losses can be minimized by selecting highly conductive electrodes, improving contacts, decreasing the distance between anode and cathode electrodes, or by increasing solution conductivity [21, 26, 31]. Substrate diffusion or product removal close to the electrodes causes mass transport losses [30]. For example, a thick biofilm may prevent diffusion at the electrode [32]. Mass transport losses can be decreased by optimizing the operating conditions, geometry of BES or electrode materials [28].

3 Anaerobic Microorganisms at the Anode

The current at the anode of bioelectrochemical systems is produced by anaerobic bacteria called exoelectrogens that are able to transfer electrons outside the cell to an insoluble electron acceptor, i.e. anode electrode. Exoelectrogens have been shown to convert, e.g., H_2 [33], acetate [34], lactate [35], ethanol [34], and glucose [36, 37] directly to current. However, direct conversion of more complex substrates, such as wastewater, into current is not possible, and even the oxidation of glucose or lactate to current often requires syntrophic interaction of different bacterial species [38–40]. The fermentable substrates are first oxidized into soluble metabolites, volatile fatty acids (VFAs) and alcohols, which are further converted to electrons, protons, and CO_2 by exoelectrogens. In addition to producing current, the utilization of metabolites by exoelectrogens decreases feedback inhibition to fermentative bacteria [41]. To optimize current production, competing anaerobic biological reactions have to be avoided. These include methanogens, homoacetogens, fermentative bacteria not leading to products amenable to exoelectrogens, nitrate reducers, aerobic microorganisms, and sulfate reducers that do not directly compete with current production but require carbon for their growth [42].

When bacteria oxidize organic or inorganic materials (Table 2), they have to dispose the produced electrons. In aerobic conditions, electrons are donated to oxygen, which has the highest redox potential of electron acceptors (Fig. 2). In anaerobic conditions, possible electron acceptors include nitrate, sulfate, carbon dioxide, ferric iron, fumarate, as well as the anode electrode. In BES, the competing electron acceptors are avoided so that the anode electrode is the sole means for bacteria to complete respiration. The electron transfer mechanisms of

Table 2 Potential electron donors at the bioanode

Electron donor	Reaction	Reference
Hydrogen	$H_2 \rightarrow 2\ H+ + 2\ e-$	[33]
Acetic acid	$CH_3COOH + 4\ H_2O \rightarrow 2\ HCO_3^- + 10\ H^+ + 8\ e^-$	[34]
Lactic acid	$C_2H_5OCOOH + 6\ H_2O \rightarrow 3\ HCO_3^- + 15\ H^+ + 12\ e^-$	[43]
Butyric acid	$C_3H_7COOH + 10\ H_2O \rightarrow 4\ HCO_3^- + 24\ H^+ + 20\ e^-$	[21]
Propionic acid	$C_2H_5COOH + 7\ H_2O \rightarrow 3\ HCO_3^- + 17\ H^+ + 14\ e^-$	[44]
Xylose	$C_5H_{10}O_5 + 10\ H_2O \rightarrow 5\ HCO_3^- + 25\ H^+ + 20\ e^-$	[45]
Glucose	$C_6H_{12}O_6 + 12\ H_2O \rightarrow 6\ HCO_3^- + 30\ H^+ + 24\ e^-$	[7]
Sulfur compounds	$H_2S \rightarrow S^0 + 2\ H^+ + 2\ e^-$	[46]
	$HS^- \rightarrow S^0 + 2\ H^+ + 2\ e^-$	

exoelectrogens originate from nature where, e.g., solid iron or manganese oxides can be used as electron acceptors by metal-reducing bacteria [47, 48]. For example, metal-reducing bacteria *Geobacter sulfurreducens* [33] and *Shewanella putrefaciens* [49] have been shown to donate electrons directly to the anode electrode.

The selection of efficient exoelectrogenic communities is crucial because the anaerobic metabolism and the rate and nature of electron transfer determine the anode performance [50]. Thus, the anaerobic culture affects the biofilm formation on the electrode, internal resistance of the BES, and the overall current generation [51, 52]. Both pure and mixed cultures can be used for current production in BES. Exoelectrogenic pure cultures are usually capable of utilizing only certain substrates [53]. Mixed cultures are often preferred over pure cultures because they (1) are more suitable for wastewater treatment, (2) allow wider substrate versatility due to the presence of both acidophilic and exoelectrogenic microorganisms, (3) have higher resistance to process disturbances, (4) often give higher current outputs, and (5) obligate aerobes present minimize the effects of oxygen diffusion through the separator [54–56]. However, pure culture studies are required to understand in detail electron transfer mechanisms and metabolism of microorganisms in BES and to evaluate how dominant strains evolve in mixed cultures to optimize BES performance [57].

3.1 Pure Cultures

Direct electron transfer by bacteria attached to the anode electrode was first reported in the late 1990s by Kim et al. [49] with a pure culture of *Shewanella putrefaciens*. Exoelectrogens are found from many bacterial groups including metal-reducing bacteria, such as *G. sulfurreducens* [33] and *S. putrefaciens* [49], sulfate-reducing bacteria, such as *Desulfobulbus propionicus* [58], and denitrifying bacteria, e.g., *Orchobactrum anthropic* [34] and *Comamonas denitrificans* [59]. Pure exoelectrogenic cultures and their currently known electron transfer mechanisms and substrates used for current generation are listed in Table 3.

Table 3 Pure cultures of exoelectrogenic bacteria their substrate versatility, and proposed electron transfer mechanisms (without added external mediators)

Bacterium	Substrate(s)	Electron transfer mechanism	Reference
Aeromonas hydrophila	Yeast extract	*c*-Type cytochromes[a]	[60]
Bacillus selenitireducens	Lactate	Nr	[39]
Clostridium butyricum	Glucose	Nr	[61]
Comamonas denitrificans	Acetate	Nr	[59]
Deltasulfuromonas acetoxidans	Acetate	Nr	[62]
Desulfobulbus propionicus	Lactate, propionate, pyruvate, H_2	Direct	[58]
Enterobacter cloacea	Sucrose, glycerol, glucose, cellulose	Nr	[63]
Geobacter sulfurreducens	H_2, Acetate	*c*-Type cytochromes, nanowires	[33, 64, 65]
Geothrix fermentas	Acetate, propionate, malate, lactate, succinate	Excreted electron shuttle	[66]
Geopsychrobacter electrodiphilus	Acetate, malate, fumarate, citrate	*c*-Type cytochromes	[67]
Haloferax volcanii	Yeast extract + peptone	Nr	[68]
Klebsiella pneumoniae	Starch, glucose	Direct[a]	[69]
Lactococcus lactis	Glucose	Excreted electron shuttle (soluble quinone)	[70]
Natrialba magadii	Yeast extract	Nr	[68]
Ochrobactrum anthropic	Acetate, lactate, propionate, butyrate, glucose, sucrose, cellobiose, glycerol, ethanol	Nr	[34]
Pseudomonas sp.	Tryptone and yeast extract	Excreted electron shuttle (phenazine-1-carboxamide)	[71]
Rhodoferax ferrireducens	Glucose	Nr	[72]
Rhodopseudomonas palustris	Acetate, lactate, ethanol, yeast extract, valerate, fumarate, glycerol, butyrate, propionate, thiosulfate	Direct[a]	[73]
Shewanella japoinica	Sucrose	Excreted electron shuttles	[74]
Shewanella marisflavi	Lactate	Nr	[75]
Shewanella oneidensis	Lactate	Nanowire	[35, 76]

(continued)

Table 3 (continued)

Bacterium	Substrate(s)	Electron transfer mechanism	Reference
Shewanella putrefaciens	Lactate	Outer membrane cytochromes	[43, 49]
Thermincola sp.	Acetate	Direct[a]	[77]
Thermincola ferriacetica	Acetate	Direct[a]	[78]

[a]Suggested, *Nr* not reported

Most known exoelectrogens are Gram-negative bacteria but a few electrochemically active Gram-positive bacteria have also been recognized. The first evidence of direct electron transfer by Gram-positive bacteria *Thermincola* sp. and *Thermincola ferriacetica* was reported by Wrighton et al. [77] and Marshall and May [78], respectively. Direct metabolism of carbohydrates into solely current is rare [63, 79]. For example, in addition to current, *Lactococcus lactis* produced lactate and smaller amounts of acetate and pyruvate from glucose [70]. Current production from cellulose by *Enterobacter cloacae* resulted in accumulation of many VFAs and alcohols with acetate as the main by-product [63].

3.2 Mixed Cultures

Current-producing microbial communities can be enriched and isolated from different natural and industrial environments, including anaerobic sludge from wastewater treatment plants [80, 81] and reactors treating brewery waste [82], domestic wastewater [51, 83, 84], paper recycling wastewater [85], compost [45, 86, 87], cow rumen, [88], soil [1, 89], sediment [75, 90], and river water [91]. During the enrichment of exoelectrogenic cultures, the inhibition of methanogens is crucial because they compete for the same organic substrate with exoelectrogens and are the most critical cause of decreasing Coulombic efficiency in BES [12]. Methanogens can be inhibited, e.g., by initial selection of pH and buffer concentrations [92], periodic aeration [38], and controlled substrate loading [93]. However, Rismani-Yazdi et al. [94] showed that methanogenesis in MFCs stopped over time and performance of MFC improved without any need for methanogenic inhibition.

The microbial communities in MFCs usually contain species from phyla *Proteobacteria* and *Firmicutes* [88, 95]. The bacterial composition depends on the original culture and substrate used for enrichment. With fermentable substrates, more diverse cultures are enriched than with non-fermentable substrates, which enhances the fermentation of sugars and more complex substrates [88, 96]. These diverse cultures have been shown to contain fermentative bacteria, such as *Clostridium* [97], *Rhodopseudomonas* [40] and *Escherichia* and *Bacteroides* [45],

when fed with cellulose, glucose, and xylose, respectively. Gram-negative bacteria, such as *G. sulfurreducens* [41], often dominate the exoelectrogenic communities [55] and generally result in higher current production than Gram-positive bacteria [42]. Although Gram-negative bacteria are most often associated with current generation, Gram-positive bacteria have also been shown to transfer electrons to the anode electrode [77].

3.3 Electron Transfer Mechanisms

For current to be produced in BES, electrons have to be transferred from the inside of the cell membrane to its outside and, further, to the anode electrode. The intercellular electron transfer can occur through physical transfer with reduced compounds or via electron hopping across the cell membrane using membrane-bound redox enzymes [50]. Figure 3 shows examples of proposed intercellular electron transfer mechanisms that start from NADH derived from substrate

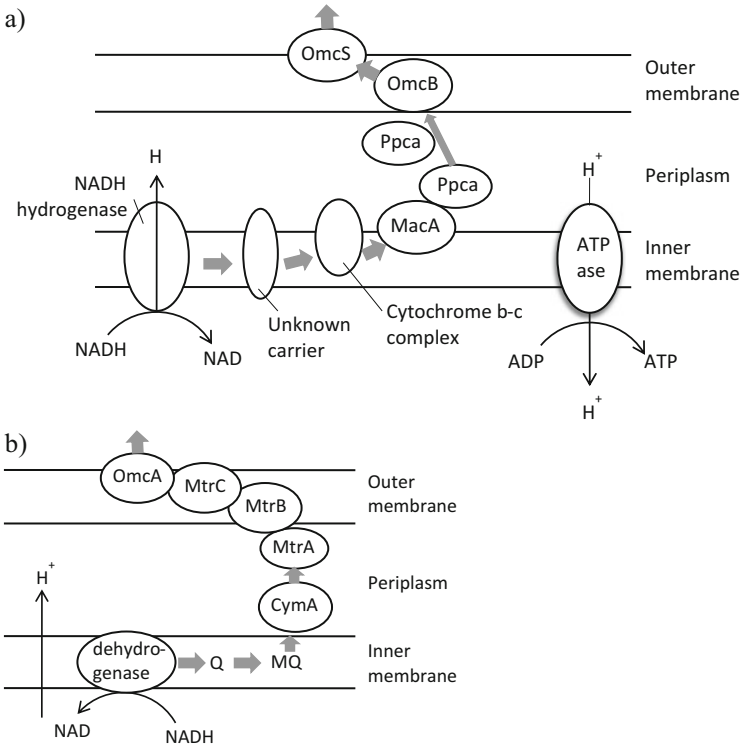


Fig. 3 Proposed intercellular electron transport system in (**a**) *Geobacter sulfurreducens* [98] and (**b**) *Shewanella oneidensis* [99]

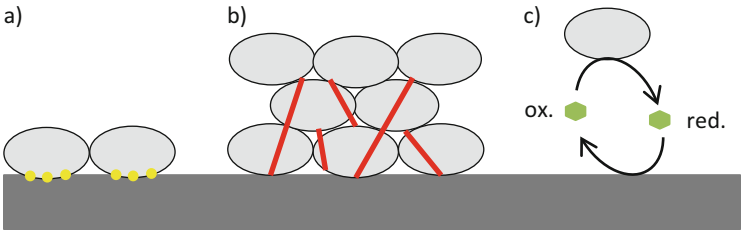


Fig. 4 Electron transfer mechanisms in BES anode: direct electron transfer with (**a**) outer membrane cytochromes (*yellow circles*) or (**b**) nanowires (*red sticks*), and (**c**) mediated electron transfer with electron shuttling compound (*green cycle*). *Ox* oxidized, *red* reduced

oxidation. The disposal of electrons by *G. sulfurreducens* is proposed to occur via different cytochromes (OmcS, OmcB, Ppca, MacA) [98]. In *Shewanella oneidensis*, outer surface cytochromes (OmcA, MtrC) and other proteins are involved in intercellular electron transfer [99]. Electron transfer to the anode electrode occurs only if other electron acceptors, e.g., oxygen, sulfate, nitrate, or fumarate, are not present.

Several electron transfer mechanisms from bacterial cell to the electrode have been proposed (Fig. 4). In direct electron transfer, exoelectrogens have to be in close contact with the electrode and thus form a monolayered or multilayered biofilm on the anode. Direct electron transfer requires either the utilization of electrically active membrane-bound enzymes, such as *c*-type cytochromes [64, 100], or nanowires that can transfer electrons from longer distances [65]. In addition to current generation, nanowires also play a structural role in biofilm formation [101]. Electrons from planktonic microorganisms as well as inside biofilm can also be transferred to the anode with endogenous or exogenous electron shuttles called mediators [102–104]. Exogenous, i.e., added mediators include humic acids, thionine, viologens, methylene blue, and sulfur species [55, 105]. However, synthetic mediators are often expensive and even toxic, which limits their use in BES [106]. Some bacteria can secrete electron shuttles (i.e., endogenous mediators). For example, *Shewanella* sp. can produce riboflavins [107], *L. lactis* quinones [70], and *Pseudomonas* sp. phenazines [71]. However, production of electron shuttles can be thermodynamically unfavorable [108], although they have also been observed in continuous flow MFCs [109].

The electron-transfer mechanisms of pure cultures of *G. sulfurreducens* and *S. odeinensis* have been widely studied. *G. sulfurreducens* has been reported to transfer electrons in direct contact with the electrode via *c*-type cytochromes [33, 64] or through nanowires [107]. The electron-transfer mechanisms of *S. oneidensis* are are more diverse. *S. oneidensis* has been reported to use direct electron transfer mechanism through both outer membrane cytochromes [110, 111] and nanowires [76]. Furthermore, *S. oneidensis* can excrete flavins to mediate electron transfer [112].

4 Anaerobic Microorganisms at the Cathode

Anaerobic microbes can be used at biological cathodes of MFCs and MECs for wastewater treatment (e.g., denitrification), production of compounds, CO_2 fixation, and bioremediation (Table 4). In biocathodes, electrons for the reduction reactions are provided by the oxidation reactions at the anode. An external power source is used in MECs to overcome cathodic reaction overpotentials [2] and thermodynamic limitations [20]. The use of anaerobes at the cathode has many advantages [1]. They replace the expensive catalysts otherwise required at the cathode electrodes, which decreases construction and operation costs. Further, the use of anaerobic cathodes eliminates the diffusion of oxygen to the anode, which could result in aerobic respiration by facultative exoelectrogens or by other bacteria [5]. In addition, a life cycle assessment showed that MECs producing hydrogen resulted in larger environmental benefits when compared to electricity generation in MFCs [115]. Aerobic biocathodes can also be used, e.g., for the reduction of oxygen, Fe^{2+}, or Mn^{2+} (e.g., [1]), but are not within the scope of this chapter.

Electrochemically active anaerobic microorganisms used at the cathode include pure cultures, such as *G. sulfurreducens* [116], *Geobacter metallireducens* [117], and *Methanobacterium palustre* [22], as well as mixed cultures. Anaerobes can form electrochemically active biofilms on the cathode electrodes, although the extracellular electron-transfer mechanisms at the cathodes are still poorly known [42]. In nature, some bacteria are known to accept electrons from solid electron donors. For example, chemolithotrophic iron and sulfur oxidizers can accept electrons from Fe^{2+},

Table 4 Potential cathodic reactions without (MFC) or with (MEC) applied voltage

Purpose (MFC/MEC)	Reaction	Reference
Nitrate reduction (MFC)	$NO_3^{2-} + 2\ H^+ + 2\ e^- \rightarrow NO_2^- + H_2O$ $2\ NO_3^{2-} + 12\ H^+ + 10\ e^- \rightarrow N_2 + 6\ H_2O$	[8, 9]
Sulfate reduction (MFC)	$2\ H_2O + 2\ e^- \rightarrow H_2 + 2\ OH^-$ $SO_4^{2-} + 4\ H_2 \rightarrow S^{2-} + 4\ H_2O$ $SO_4^{2-} + 4\ H_2O + 8\ e^- \rightarrow S^{2-} + 8\ OH^-$	[113]
Hydrogen production (MEC)	$2\ H^+ + 2\ e^- \rightarrow H_2$	[6]
Methane production (MEC)	$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2\ H_2O$	[12]
Acetate synthesis from CO_2 (MEC/MES)	$CO_2 + 7\ H^+ + 8e^- \rightarrow$ Acetate $+ 2\ H_2O$	[13]
Acetate synthesis to ethanol (MEC/MES)	Acetate $+ 5\ H^+ + 4\ e^- \rightarrow$ Ethanol $+ H_2O$	[14]
Fumarate reduction to succinate (MEC)	Fumarate $+ 2\ H^+ + 2\ e^- \rightarrow$ Succinate	[114]
Trichloroethane (TCE) reduction to ethane or ethene (MEC)	TCE $\rightarrow$ Ethane	[15]
Perchlorate reduction to chloride (MEC)	$ClO_4^- \rightarrow Cl^-$	[16]

S^0, or S^{2-} in oxic/anoxic interfaces where oxygen is used as electron acceptor [2]. In BES biocathodes, the electrode serves as the only electron donor for the microorganisms, whereas for the carbon source a small amount of CO_2 or other carbon has to be added.

4.1 Pure Cultures

Pure electrochemically active cultures have been shown to accept electrons at the cathode for various different purposes, including denitrification and reduction of protons, CO_2, and environmental contaminants (Table 5). Some bacteria have

Table 5 Anaerobic pure and mixed exoelectrogenic cultures detected in biological cathodes (with or without mediators)

Culture	Reduction reaction	Electron transfer mechanism	Reference
Pure culture			
Actinobacillus succinogenes	Fumarate/succinate	Exogenous NR mediator	[118]
Azospira suillum	ClO_4^-/Cl^-	Exogenous AQDS mediator	[119]
Dechloromonas agitata	ClO_4^-/Cl^-	Exogenous AQDS mediator	[119]
Desulfovibrio vulgaris	H^+/H_2	Exogenous MV mediator	[120]
Geobacter lovleyi	PCE/*cis*-DCE	Direct[a]	[121]
Geobacter metallireducens	NO_3^-/NO_2^-	Direct[a]	[117]
Geobacter sulfurreducens	Fumarate/succinate	Direct[a]	[117]
Geobacter sulfurreducens	Fumarate/succinate	Direct[a]	[116]
Geobacter sulfurreducens	U(VI)/U(IV)	Nr (mediatorless)	[122]
Methanobacterium palustre	CO_2/CH_4	Direct[a]	[22]
Sporomusa ovate	CO_2/acetate	Direct[a]	[13]
Mixed culture			
Anaerobic sludge	NO_3^-/N_2	Nr	[123]
Anaerobic sludge	NO_3^-/N_2	Nr	[8]
Hydrogenophilic mixed culture	H^+/H_2	Nr	[6]
Hydrogenophilic mixed culture	H^+/H_2	Nr	[124]
Hydrogenophilic methanogenic culture	H^+/H_2, CO_2/CH_4	Exogenous MV mediator/ Direct[a]	[125]
Anaerobic sludge	Acetate/ethanol	Exogenous MV mediator	[14]
Sulfate-reducing bacteria	Acetate/ethanol Butyrate/butanol	Direct[a]	[24]
Hydrogenophilic dechlorinating culture	TCE/*cis*-DCE (VC/ethane)	Endogenous mediator	[126]
Anaerobic digester effluent	Cr(VI)/Cr(III)	Nr	[127]

[a]Suggested, *AQDS* anthraquinone-2,6-disulfonate, *MV* methyl viologen, *NR* neutral red, *Nr* not reported

been reported both to donate electrons to anode electrode and to accept electrons from cathode electrode. For example, *G. sulfurreducens* can act as biocatalyst both at the anode to oxidize acetate [65] and at the cathode to reduce fumarate [116] or uranium [122].

4.2 Mixed Cultures

Mixed cultures used at biocathodes are listed in Table 5. Not many microbial communities from biocathodes have been characterized. Croese et al. [128] produced hydrogen at a biocathode of an MEC and reported that *Proteobacteria* dominated. *Methanobacterium* spp. was a dominant anaerobe at an MEC biocathode producing methane [22]. Similar to bioanodes, the growth of methanogens at MEC biocathodes producing hydrogen should be prevented. Methanogens disturb the process by decreasing CE, utilizing produced H_2, and reducing the purity of the produced gas [129]. For example, Wang et al. [130] reported that hydrogenotrophic methanogens (producing methane from H_2 and CO_2) were responsible for methane production in one-chamber MEC. The easiest way to decrease methane production is to use two-chamber MEC, where methanogens may occur at the anode but are separated from the produced H_2 by a separator. Using higher applied voltages of >0.6 V have also been reported to reduce methane production in one-chamber MECs [130].

4.3 Electron Transfer Mechanisms

At the cathode, anaerobes have been suggested to accept electrons directly or with mediators. However, the precise electron-transfer mechanisms of direct electron transfer are not yet known. The electrode has been reported to serve as a direct electron source, for example, for the following cultures: *Sporomusa ovata* for CO_2 reduction to acetate [13], *Geobacter metallireducens* to reduce nitrate to nitrite, *Geobacter sulfurreducens* to reduce fumarate to succinate [117], and for methanogens [30]. Rosenbaum et al. [2] suggested that *c*-type cytochromes and hydrogenases would play a role in cathodic electron transfer. Strychartz et al. [131] and Rosenbaum et al. [2] reported that the electron transfer mechanisms between the anode and cathode differed significantly, despite the similar gene expression. This was due to the different redox potentials of the electron transfer components [2]. Geelhoed et al. [99] suggested a mechanism for biological hydrogen production at the cathode of an MEC (Fig. 5).

Exogenous mediators used for cathodic reduction reactions include methyl viologen, anthraquinone-2,6-disulfonate (AQDS), and neutral red. Methyl viologen has been used as mediator, e.g., for the reduction of protons to hydrogen [120, 125], acetate to ethanol [14], or for the reduction of trichloroethane (TCE) to ethane or

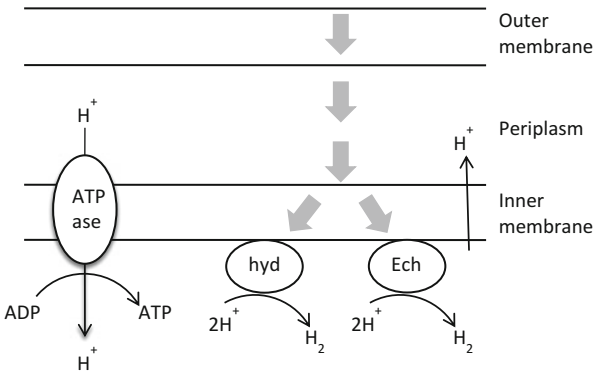


Fig. 5 Hydrogen production mechanism coupled to proton transport at biocathode as suggested by Geelhoed et al. [99]. Hydrogen is produced either with hydrogenase (hyd) or energy-conserving hydrogenase (Ech)

ethene [15]. Thrash et al. [119] reported perchlorate reduction with an exogenous AQDS mediator. However, perchlorate was also reduced in a mediatorless BES with a novel strain isolated from a natural culture [119]. Neutral red was used for fumarate reduction by Park and Zeikus [118]. Aulenta et al. [126] reported that a mixed hydrogenophilic dechlorinating culture produced unknown endogenous mediators when reducing TCE at an anaerobic biocathode.

5 Factors Affecting the Growth of Electroactive Anaerobic Bacteria

In addition to the type of inoculum, operational parameters affect the growth of anaerobic electroactive communities. The structure and activity of the exoelectrogenic cultures are affected by various physical and chemical parameters, including pH, temperature, substrate concentration and loading rate, conductivity, shear stress, external resistance, electrode potential, and materials for electrodes and separators. This chapter presents a short introduction to these different parameters. Due to the low number of studies on the effects of operational parameters on biocathodes, most of the chapters deal mainly with bioanodes.

5.1 Temperature

Current production in BES is affected by temperature changes because anaerobes are sensitive to the operating temperature. Most BES studies are conducted with mesophilic bacteria, but few studies have investigated the BES performance at higher temperatures (above 50°C) [79, 86, 90]. Elevated temperatures make bioprocesses less sensitive to contamination, favor the kinetics and stoichiometry of chemical, electrochemical, and enzymatic reactions, and increase conductivity

according to the Arrhenius laws [132, 133]. Increasing temperature up to a certain point enhances microbial growth which helps microbial attachment to an electrode [134]. For example, Patil et al. [135] reported that increasing the temperature from 15 to 35°C increased the current densities and reduced lag times. The main drawbacks of thermophilic conditions are lower cell densities, complex nutrient requirements, and energy required for process heating [132, 136]. However, high temperature waste streams may enable the use of higher temperatures.

5.2 *pH*

Neutral anodic pH has been used in several BES studies [33, 88, 137]. In BES, cathodic pH tends to rise and anodic pH to decrease due to poor proton transfer through the separator. The resulting pH difference between the anode and cathode leads to increased internal resistance and reduces the whole cell voltage by 0.06 V per pH unit [129]. Further, low pH can decrease bacterial activity at the anode [138] and may set limitations to materials and chemicals used. There are few studies utilizing lower pH at the anode and/or the cathode. For example, Borole et al. [139] oxidized glucose at pH below 4 and Sulonen et al. [140] oxidized tetrathionate at pH below 2.5. Substrate oxidation or reduction at the biofilms can also lead to pH gradients across the biofilm and result, e.g., in lower pH values close to the anode electrode surface. The local pH changes reduce the performance of microorganisms and introduce a higher stress level to the anaerobes [141, 142].

5.3 *Anodic Substrate, Substrate Concentration, and Organic Loading Rate*

The BES performance is greatly affected by the type, concentration, and feeding rate of substrate [56]. Electricity production from many different substrates has been investigated, varying from simple organic acids, such as acetate [34, 137] and butyrate [21], to more complex substrates, including sugars [7, 53], cellulose [143], and real waste materials. Real wastewaters used for current production include domestic [83], brewery [144], paper recycling [85], and food processing [80] wastewaters. In addition, biological sulfide oxidation to sulfate with simultaneous current production was reported by Sun et al. [52].

Substrate influences the bacterial community composition, CE, and current density of the BES. The more complex the substrate, the more diverse microbial community develops due to the syntrophic bacterial interactions required for substrate degradation and electricity generation [44, 145, 146]. Using fermentable

instead of non-fermentable substrates often results in decreased CEs, because a proportion of the electrons are directed to the production of soluble metabolites instead of current [85, 147]. Wastewaters may also contain inorganic or non-biodegradable compounds that interfere with current production and decrease current densities and CEs [148]. Wastewaters from food-processing industries, breweries, and animal confinements that contain high levels of easily degradable organic material and have high water content are especially suitable for BES [54].

Substrate concentration and organic loading rate (OLR) also affect the current generation in BES. Substrate concentration controls the current production according to the Monod relationship ((5); [149]), where j is current density (A/m^2), $j_{max,app}$ the maximum current density, S substrate concentration (e.g., g COD/m^3), and $K_{S,app}$ the half-maximum concentration (g COD/m^3). Increased substrate concentrations and OLR increase the current [150] but only up to a certain limit [151]. High substrate concentrations in MFCs may lead to enhanced formation of fermentation products that decrease anodic pH, lowering bacterial activity [152]. At higher substrate concentrations more substrate is used for bacterial growth or alternative reactions, such as methanogenesis, lowering the CE [152]. In general, substrate removal efficiency decreases at high substrate concentrations [153]. Substrate concentrations may form gradients across the biofilm, which decreases the activity and performance of electroactive anaerobes close to the anode electrode [154].

$$j = j_{\max,\mathrm{app}}\left(\frac{S}{K_{\mathrm{S,app}} + S}\right) \tag{5}$$

OLR has an effect on current density and substrate degradation [155]. It has been reported that with small external resistance increase in OLR results in enhanced current generation [151]. However, Martin et al. [156] reported that increased portion of substrate was used for methane production at increasing OLR.

5.4 Ionic Strength

Ionic strength of an electrolyte in BES increases the solution conductivity and current production [85] and decreases the internal resistance. However, there are only few microbial strains that can produce electricity at a very high ionic strength [75]. Liu et al. [157] reported that power production was enhanced from 720 to 1,330 mW/m^2 by increasing ionic strength from 0.1 to 0.4 M, respectively. Furthermore, halophilic bacteria *Shewanella marisflavi* and halophilic archaea *Haloferax volcanii* and *Natrialba magadii* have been reported to produce electricity

at very high ionic strengths of 1.1 M (9.6 mW/m^2), 2.7 M (119 mW/m^2), and 3.6 M (46 mW/m^2), respectively [68, 75]. Thus, BES can also be effective for treatment of saline industrial wastewaters [62].

5.5 External Resistance and Anode Potential

External resistance regulates the anode availability as electron acceptor and electron flux through the circuit [158]. External resistance (R_{ext}) controls the ratio between the current (I) generation and cell voltage (U) according to Ohm's law (6). In general, the lower the external resistance, the higher the current and coulombic efficiency [158–160]. Up to certain point, lower external resistance may select exoelectrogens that can meet their metabolic energy requirements with a small potential gradient between the redox potential of their electron donor and the anode [158]. Thus, external resistance can be used in the enrichment of exoelectrogens because low external resistance facilitates electron transfer and favors the enrichment of exoelectrogens [161].

$$U = \mathrm{IR}_{\mathrm{ext}}. \tag{6}$$

Anode potential, on the other hand, regulates the activity of a bacterial community in BES. Theoretically, microbes gain more energy by reducing a terminal electron acceptor with a more positive potential [50] according to Gibb's free energy ($\Delta G^{0'}$; (7)), where n is the number of electrons transferred, F is Faradays constant (96 485 C/mol), and $\Delta E^{0'}$ the difference in the potentials between the electron donor and electron acceptor, e.g., outer membrane cytochrome and anode electrode. More positive anode potential should increase the growth rate of bacteria, resulting in higher biocatalyst density, respiration rates, faster start-up of electricity production, and higher current generation [159, 162]. However, microbes must have metabolic pathways capable of capturing the available energy and maximizing their energy gain for a given anode potential [163, 164]; e.g., *Geobacter* sp. that use only a small portion of their net electron flow to ATP production dominated microbial communities at low anode potentials [162].

$$\Delta G^{0'} = -nF\Delta E^{0'} \tag{7}$$

Although more positive anode potentials theoretically result in higher energy gain for bacteria, Wagner et al. [164] proposed that it is primarily the potential of the terminal respiratory proteins used by exoelectrogenic bacteria, rather than the anode potential, which determines the optimal growth conditions in the reactor. This is supported by the studies of Finkelstein et al. [163] and Wei et al. [165], who reported the anode potential selected for exoelectrogens whose terminal respiratory proteins had redox potentials just negative of the anode potential. Theoretically, to maximize current flow in BES, anode potential should be as negative as possible

– see (3). However, experimental results on the effects of anode potentials on current production remain contradictory. For example, Torres et al. [166] reported increased current production at lower anode potentials, whereas Wei et al. [165] and Sun et al. [167] obtained higher current densities at higher anode potentials.

5.6 Electrode and Separator Materials

Several electrode materials are applicable in BES and their main requirements include conductivity, biocompatibility, high surface area, chemical stability, high mechanical strength, and low cost [26, 168]. The electrode material affects the growth and electrochemical activity of the anaerobic culture [151, 169]. Electrode materials used in BES include graphite plates and rods, carbon cloths and papers, graphite fiber brushes, activated carbon, carbon mesh, graphite foam, carbon nanotubes, tungsten, and stainless steel [170–173]. High surface area minimizes activation and ohmic losses and provides more space for the growth of anaerobes [174]. For example, Liu et al. [169] reported 40% higher current densities with electrodes having higher surface area (carbon fiber or carbon paper) than graphite rod. Graphite fiber brushes, activated carbon cloth electrodes, and carbon nanotube-based materials have high surface areas.

Separators are used in two-chamber BES and often in one-chamber BES. A separator is used to separate physically anode and cathode chambers, to reduce oxygen diffusion to the anode, to increase CE, and to allow closer electrode spacing. Further, in MECs the use of a separator reduces H_2 losses caused by methanogenesis and increases the purity of gases [129]. Separators used include salt bridges, proton exchange membranes, cation exchange membranes, anion exchange membranes, bipolar membranes, porous fabrics, and glass fibers [173]. Although the use of separator is often compulsory, its use has many problems. It increases the BES construction costs and the internal resistance, and may result in a pH gradient across the membrane [129]. Further, the membrane surface can meet fouling, which affects the performance of the separator [175].

6 Future Directions

Anaerobes are used in various BES applications at both the anode and the cathode chambers. Further studies using different electrochemically active pure cultures are required to understand better the electron transfer mechanisms to and from the electrode. Oxidation of simple synthetic compounds has produced a fundamental mechanistic understanding during the past 15 years. However, more research is required on the oxidation of real wastewaters in the anode chamber and the possible inhibitory effects of wastewaters on exoelectrogens and current generation.

The utilization of electrochemically active anaerobes at the cathode is a rather new area of research. In recent years it has been reported that many pure and mixed cultures accept electrons from the cathode for the reduction of various different compounds. Of these processes, the biological production of H_2 and CH_4 are the most widely studied. More knowledge is required on the anaerobic cultures catalyzing reduction reactions at the cathode electrodes and their electron transfer mechanisms, reaction routes, and the effects of operational parameters on the reduction reactions.

In the future, BES may not be applicable solely for electricity production and/or wastewater treatment [6]. Bioelectrochemical systems are more likely to become viable sooner when combined with other valuable processes, such as bioremediation, denitrification, or hydrogen production at the cathode [176, 177]. Prior to commercialization, BESs have to be scaled up. A few studies on the up-scaling of MFCs [178] and MECs [179] have reported various problems that require further attention. Challenges that need to be solved include the development of lower cost and more efficient electrode and separator materials, scaling-up by maintaining the current densities obtained at laboratory scale, and minimizing the losses in BES.

References

1. He Z, Angenent LT (2006) Application of bacterial biocathodes in microbial fuel cells. Electroanalysis 18:2009–2015
2. Rosenbaum M, Aulenta F, Villano M, Angenent LT (2011) Cathodes as electron donors for microbial metabolism: which extracellular electron transfer mechanisms are involved? Bioresour Technol 102:324–333
3. Lapinsonniére L, Picot M, Barriére F (2012) Enzymatic versus microbial bio-catalyzed electrodes in bio-electrochemical systems. ChemSusChem 5:995–1005
4. Rubenwolf S, Kerzenmacher S, Zengerle R, von Stetten F (2011) Strategies to extend the lifetime of bioelectrochemical enzyme electrodes for biosensing and biofuel cell applications. Appl Microbiol Biotechnol 89:1315–1322
5. Logan BE, Regan JM (2006) Electricity-producing bacterial communities in microbial fuel cells. Trends Microbiol 14:512–518
6. Rozendal RA, Hamelers HVM, Rabaey K, Keller J, Buisman CJN (2008) Towards practical implementation of bioelectrochemical wastewater treatment. Trends Biotechnol 26:450–459
7. Rabaey K, Lissens G, Siciliano SD, Verstraete W (2003) A microbial fuel cell capable of converting glucose to electricity at high rate and efficiency. Biotechnol Lett 25:1531–1535
8. Clauwaert P, Rabaey K, Aelterman P, de Schamphelaire L, Pham TH, Boeckx P, Boon N, Verstraete W (2007) Biological denitrification in microbial fuel cells. Environ Sci Technol 41:3354–3360
9. Lefebvre O, Al-Mamun A, Ng HY (2008) A microbial fuel cell equipped with a biocathode for organic removal and denitrification. Water Sci Technol 58:881–885
10. Chang IS, Jang JK, Gil GC, Kim M, Kim HJ, Cho BW, Kim BH (2004) Continuous determination of biochemical oxygen demand using microbial fuel cell type biosensor. Biosens Bioelectron 19:607–613
11. Cao X, Huang X, Liang P, Xiao K, Zhou Y, Zhang X, Logan BE (2009) A new method for water desalination using microbial desalination cells. Environ Sci Technol 43:7148–7152

12. Chae KJ, Choi MF, Kim KY, Ajayi FF, Park W, Kim CH, Kim IS (2010) Methanogenesis control by employing various environmental stress conditions in two-chambered microbial fuel cells. Bioresour Technol 101:5350–5357
13. Nevin KP, Woodard TL, Franks AE, Summers ZM, Lovley DR (2010) Microbial electrosynthesis: feeding microbes electricity to convert carbon dioxide and water to multicarbon extracellular organic compounds. mBio 1:e00103-10
14. Steinbusch KJ, Hamelers HV, Schaap JD, Kampman C, Buisman CJ (2010) Bioelectrochemical ethanol production through mediated acetate reduction by mixed cultures. Environ Sci Technol 44:513–517
15. Aulenta F, Catervi A, Majone M, Panero S, Reale P, Rossetti S (2007) Electron transfer from a solid-state electrode assisted by methyl viologen sustains efficient microbial reductive dechlorination of TCE. Environ Sci Technol 41:2554–2559
16. Butler CS, Clauwaert P, Green SJ, Verstraete W, Nerenberg R (2010) Bioelectrochemical perchlorate reduction in microbial fuel cell. Environ Sci Technol 44:4685–4691
17. ter Hejne A, Liu F, van der Weijden R, Weijma J, Buisman CJN, Hamelers HVM (2010) Copper recovery combined with electricity production in a microbial fuel cell. Environ Sci Technol 44:4376–4381
18. Modin O, Wang X, Wu X, Rauch S, Fedje KK (2012) Bioelectrochemical recovery of Cu, Pb, Cd, and Zn from dilute solutions. J Hazard Mater 235:291–297
19. Park HI, Kim DK, Choi YJ, Pak D (2005) Nitrate reduction using an electrode as direct electron donor in a biofilm-electrode reactor. Process Biochem 40:3383–3388
20. Zhang Y, Angelidaki I (2014) Microbial electrolysis cells turning to be versatile technology: recent advances and future challenges. Water Res 56:11–25
21. Liu H, Grot S, Logan BE (2005) Electrochemically assisted microbial production of hydrogen from acetate. Environm Sci Technol 39:4317–4320
22. Cheng S, Xing D, Call DF, Logan BE (2009) Direct biological conversion of electrical current into methane by electromethanogenesis. Environ Sci Technol 43:3953–3958
23. Rabaey K, Girguis P, Nielsen LK (2011) Metabolic and practical considerations on microbial electrosynthesis. Curr Opin Biotechnol 22:371–377
24. Sharma M, Aryal N, Sarma PM, Vanbroekhoven K, Lal B, Benetton XD, Pang D (2013) Bioelectrocalatyzed reduction of acetic and butyric acids via direct electron transfer using a mixed culture of sulfate-reducers drives electrosynthesis of alcohols and acetone. Chem Comm 49:6495–6497
25. Heijnen JJ (1999) Bioenergetics of microbial growth. In: Flickinger MC, Drew SD (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. Wiley, New York, pp 267–291
26. Logan BE, Hamelers B, Rozendal R, Schröder U, Keller J, Fregula S, Aelterman P, Verstraete W, Rabaey K (2006) Microbial fuel cells: methodology and technology. Environ Sci Technol 40:5181–5192
27. Rabaey K, Rodríguez J, Blackall LL, Keller J, Gross P, Batstone D, Verstraete W, Nealson KH (2007) Microbial ecology meets electrochemistry: electricity-driven and driving communities. Int Soc Microb Ecol J 1:9–18
28. Rismani-Yazdi H, Carver SM, Christy AD, Tuovinen OH (2008) Cathodic limitations in microbial fuel cells: an overview. J Power Sources 180:683–694
29. Pham TH, Aelterman P, Verstraete W (2009) Bioanode performance in bioelectrochemical systems: recent improvements and prospects. Trends Biotechnol 27:168–178
30. Clauwaert P, Aelterman P, Pham TH, de Schamphelaire L, Carballa M, Rabaey K, Verstraete W (2008) Minimizing losses in bio-electrochemical systems: the road to applications. Appl Microbiol Biotechnol 79:901–913
31. Clauwaert P, van der Ha D, Verstraete W (2008) Energy recovery from energy rich vegetable products with microbial fuel cells. Biotechnol Lett 30:1947–1951

32. Behera M, Jana PS, More TT, Ghangrekar MM (2010) Rice mill wastewater treatment in microbial fuel cells fabricated using proton exchange membrane and earthen pot at different pH. Bioelectrochem 79:228–233
33. Bond DR, Lovley DR (2003) Electricity production by *Geobacter sulfurreducens* attached to electrodes. Appl Environ Microbiol 69:1548–1555
34. Zuo Y, Xing D, Regan JM, Logan BE (2008) Isolation of the exoelectrogenic bacterium *Ochobactrum anthropic* YZ-1 by using a U-tube microbial fuel cell. Appl Environ Microbiol 74:3130–3137
35. Ringeisen BR, Henderson E, Wu PK, Pietron J, Ray R, Little B, Biffinger JC, Jones-Meean JM (2006) High power density from a miniature microbial fuel cell using *Shewanella oneidensis* DSP10. Environ Sci Technol 40:2629–2634
36. Chaudhuri SK, Lovley DR (2003) Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. Nat Biotechnol 21:1229–1232
37. Chung K, Okabe S (2009) Characterization of electrochemical activity of a strain ISO2-3 phylogenetically related to *Aeromonas* sp. isolated from a glucose-fed microbial fuel cell. Biotechnol Bioeng 104:901–910
38. Freguia S, Rabaey K, Yuan Z, Keller J (2008) Syntrophic processes drive the conversion of glucose in microbial fuel cell anodes. Environ Sci Technol 42:7937–7943
39. Miller LG, Oremland RS (2008) Electricity generation by anaerobic bacteria and anoxic sediments from hypersaline soda lakes. Extremophiles 12:837–848
40. Xing D, Cheng S, Regan JM, Logan BE (2009) Change in microbial communities in acetate- and glucose-fed microbial fuel cells in the presence of light. Biosens Bioelectron 25:105–111
41. Kiely PD, Cusick R, Call DF, Selembo PA, Regan JM, Logan BE (2011) Anode microbial communities produced by changing from microbial fuel cell to microbial electrolysis cell operation using two different wastewaters. Bioresour Technol 102:388–394
42. Borole AP, Reguera G, Ringeisen B, Wang ZW, Feng Y, Kim BH (2011) Electroactive biofilms: current status and future research needs. EnergyEnviron Sci 4:4813–4834
43. Kim HJ, Park HS, Hyun MS, Chang IS, Kim M, Kim BH (2002) A mediator-less microbial fuel cell using a metal reducing bacterium, *Shewanella putrefaciens*. Enzyme Microb Technol 30:145–152
44. Chae KJ, Choi MJ, Lee JW, Kim KY, Kim IS (2009) Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells. Bioresour Technol 100:3518–3525
45. Mäkinen AE, Lay CH, Nissilä ME, Puhakka JA (2013) Bioelectricity production on xylose with a compost enrichment culture. Int J Hydrogen Energy 38:15606–15612
46. Zhao F, Rahunen N, Varcoe JR, Roberts AJ, Avignone-Rossa C, Thumser AE, Slade RCT (2009) Factors affecting the performance of microbial fuel cells for sulfur pollutants removal. Biosens Bioelectron 24:1931–1936
47. El-Naggar MY, Gorby YA, Xia W, Nealson KH (2008) The molecular density of states in bacterial nanowires. Biophys J 95:L10–L12
48. Lovley DR (2011) Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. Energy Environ Sci 4:4896–4906
49. Kim BH, Kim HJ, Hyun MS, Park DH (1999) Direct electrode reaction of Fe(III)-reducing bacterium, *Shewanella putrefaciens*. J Microbiol Biotechnol 9:127–131
50. Schröder U (2007) Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. Phys Chem Chem Phys 9:2619–2629
51. Jiang D, Li B, Jia W, Lei Y (2010) Effect of inoculum types on bacterial adhesion and power production in microbial fuel cells. Appl Biochem Biotechnol 160:182–196
52. Sun M, Mu ZX, Chen YP, Cheng GP, Liu XW, Chen YZ, Zhao Y, Wang HL, Yu HQ, Wei L, Ma F (2009) Microbe-assisted sulfide oxidation in the anode of a microbial fuel cell. Environ Sci Technol 43:3372–3377
53. Catal T, Li K, Bermek H, Liu H (2008) Electricity production from twelve monosaccharides using microbial fuel cells. JPower Sources 175:196–200

54. Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domíguez-Espinosa R (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 22:477–485
55. Chang IS, Moon H, Bretschger O, Jang JK, Park HI, Nealson KH, Kim BH (2006) Electrochemically active bacteria (EAB) and mediator-less microbial fuel cells. J Microbiol Biotechnol 16:163–177
56. Du Z, Li H, Gu T (2007) A state of the art review on microbial fuel cells: a promising technology for wastewater treatment and bioenergy. Biotechnol Adv 25:464–482
57. Han JL, Wang CT, Hu YC, Liu Y, Chen WM, Chang CT, Xu HZ, Chen BY (2010) Exploring power generation of single-chamber microbial fuel cell using mixed and pure cultures. J Taiwan Inst Chem Eng 41:606–611
58. Holmes DE, Bond DR, Lovley DR (2004) Electron transfer by *Desulfobulbus propionicus* to Fe(III) and graphite electrodes. Appl Environ Microbiol 70:1234–1237
59. Xing D, Cheng S, Logan BE, Regan JM (2010) Isolation of the exoelectrogenic denitrifying bacterium *Comamonas denitrificans* based on dilution to extinction. Appl Microbiol Biotechnol 85:1575–1587
60. Pham TH, Rabaey K, Aelterman P, Cauwaert P, de Schamphelaire L, Boon N, Verstraete W (2006) Microbial fuel cells in relation to conventional anaerobic digestion technology. Eng Life Sci 6:285–292
61. Park HS, Kim BH, Kim HS, Kim HJ, Kim TG, Kim M, Chang IS, Park YK, Chang HI (2001) A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Clostridium butyricum* isolated from a microbial fuel cell. Anaerobe 7:297–306
62. Bond DR, Holmes DE, Tender LM, Lovley DR (2002) Electrode-reducing microorganisms that harvest energy from marine sediments. Science 295:483–485
63. Rezaei F, Xing D, Wagner R, Regan JM, Richard TL, Logan BE (2009) Simultaneous cellulose degradation and electricity production by *Enterobacter cloacae* in a microbial fuel cell. Appl Environ Microbiol 75:3673–3678
64. Holmes DE, Chaudhuri SK, Nevin KP, Mehta T, Methé BA, Liu A, Ward JE, Woodard TL, Webster J, Lovley DR (2006) Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. Environ Microbiol 8:1805–1815
65. Reguera G, McCarthy KD, Mehta T, Nicoll JS, Tuominen MT, Lovley DR (2005) Extracellular electron transfer via microbial nanowires. Nature 435:1098–1101
66. Bond DR, Lovley DR (2005) Evidence for involvement of an electron shuttle in electricity generation by *Geothrix fermentans*. Appl Environ Microbiol 71:2186–2189
67. Holmes DE, Nicoll JS, Bond DR, Lovley DR (2004) Potential role of a novel psychrotolerant member of the family *Geobacteraceae*, *Geopsychrobacter electrodiphilus* gen. nov., sp. nov., in electricity production by a marine sediment fuel cell. Appl Environ Microbiol 70:6023–6030
68. Abrevaya XC, Sacco N, Mauas PJD, Cortón E (2011) Archaea-based microbial fuel cell operating at high ionic strength conditions. Extremophiles 15:633–642
69. Zhang L, Zhou S, Zhuang L, Li W, Zhang J, Lu N, Deng L (2008) Microbial fuel cell based on *Klebsiella pneuoniae* biofilm. Electrochem Comm 10:1641–1643
70. Freguia S, Masuda M, Tsujimura S, Kano K (2009) *Lactococcus lactis* catalyses electricity generation at microbial fuel cell anodes via excretion of a soluble quinone. Bioelectrochemistry 76:14–18
71. Pham TH, Boon N, Aelterman P, Clauwaert P, de Schamphelaire L, Vanhaecke L, de Maeyer K, Höfte M, Verstraete W, Rabaey K (2008) Metabolites produced by *Pseudomonas* sp. enable a Gram-positive bacterium to achieve extracellular electron transfer. Appl Microbiol Biotechnol 77:1119–1129
72. Liu ZD, Li HR (2007) Effects of bio- and abio-factors on electricity production in a mediatorless microbial fuel cell. Biochem Eng J 36:209–214
73. Xing D, Zuo Y, Cheng S, Regan JM, Logan BE (2008) Electricity generation by *Rhodopseudomonas palustris* DX-1. Environ Sci Technol 42:4146–4151

74. Biffinger JC, Fitzgerald LA, Ray R, Little BJ, Lizewski SE, Petersen ER, Ringeisen BR, Sanders WC, Sheehan PE, Pietron JJ, Baldwin JW, Nadeau LJ, Johnson GR, Ribbens M, Finkel SE, Nealson KH (2011) The utility of *Shewanella Japonica* for microbial fuel cells. Bioresour Technol 102:290–297
75. Huang J, Sun B, Zhang X (2010) Electricity generation at high ionic strength in microbial fuel cell by a newly isolated *Shewanella marisflavi* EP1. Appl Microbiol Biotechnol 85:1141–1149
76. Gorby YA, Yanina S, McLeanJS RKM, Moyles D, Dohnalkova A, Beveridge TJ, Chang IS, Kim BH, Kim KS, Culley DE, Reed SB, Romine MF, Saffarini DA, Hill EA, Shi L, Elias DA, Kennedy DW, Pinchuk G, Watanabe K, Ishii S, Logan B, Nealson KH, Fredrickson JK (2006) Electrically conductive bacterial nanowire by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proc Natl Acad Sci U S A 103:11358–11363
77. Wrighton KC, Agbo P, Warnecke F, Weber KA, Brodie EL, DeSantis TZ, Hugenholtz P, Andersen GL, Coates JD (2008) A novel ecological role of the *Firmicutes* identified in thermophilic microbial fuel cell. ISME J 2:1146–1156
78. Marshall CW, May HD (2009) Electrochemical evidence of direct electrode reduction by a thermophilic Gram-positive bacterium, *Thermincola ferriacetica*. Energy Environ Sci 2:699–705
79. Choi Y, Jung E, Park H, Paik SR, Jung S, Kim S (2004) Construction of microbial fuel cells using thermophilic microorganisms, *Bacillus licheniformis* and *Bacillus thermoglucosidasius*. Bull Korean Chem Soc 25:813–818
80. Oh SE, Logan BE (2005) Hydrogen and electricity production from a food processing wastewater using fermentation and microbial fuel cell technologies. Water Res 39:4673–4682
81. You S, Zhao Q, Zhang J, Jiang J, Zhao S (2006) A microbial fuel cell using permanganate as the cathodic electron acceptor. J Power Sources 162:1409–1415
82. Jong BC, Kim BH, Chang IS, Liew PWY, Choo YF, Kang GS (2006) Enrichment performance, and microbial diversity of a thermophilic mediatorless microbial fuel cell. Environ Sci Technol 40:6449–6454
83. Liu H, Logan BE (2004) Electricity generation using an air-cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. Environ Sci Technol 38:4040–4046
84. Zhang Y, Min B, Huang L, Angelidaki I (2011) Electricity generation and microbial community response to substrate changes in microbial fuel cell. Bioresour Technol 102:1166–1173
85. Huang L, Logan BE (2008) Electricity generation and treatment of paper recycling wastewater using a microbial fuel cell. Appl Microbiol Biotechnol 80:349–355
86. Carver SM, Vuoriranta P, Tuovinen OH (2011) A thermophilic microbial fuel cell design. J Power Sources 196:3757–3370
87. Nercessian O, Parot S, Délia ML, Bergel A, Achouak W (2012) Harvesting electricity with *Geobacter bremensis* isolated from compost. PLoS One 7:1–8
88. Rismani-Yazdi H, Christy AD, Dehority BA, Morrison M, Yu Z, Tuovinen OH (2007) Electricity generation from cellulose by rumen microorganisms in microbial fuel cells. Biotechnol Bioeng 97:1398–1407
89. Ishii S, Shimoyama T, Hotta Y, Watanabe K (2008) Characterization of a filamentous biofilm community established in a cellulose-fed microbial fuel cell. BMC Microbiol 8:6
90. Mathis BJ, Marshall CW, Milliken CE, Makkar RS, Creager SE, May HD (2008) Electricity generation by thermophilic microorganisms from marine sediment. Appl Microbiol Biotechnol 78:147–155
91. Phung NT, Lee J, Kang KH, Chang IS, Gadd GM, Kim BH (2004) Analysis of microbial diversity in oligotrophic microbial fuel cells using 16S rRNA sequences. FEMS Microbiol Lett 223:77–82

92. Zhu H, Béland M (2006) Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. Int J Hydrogen Energy 31:1980–1988
93. He Z, Shelley D, Minteer SD, Angenent LT (2005) Electricity generation from artificial wastewater using an upflow microbial fuel cell. Environ Sci Technol 39:5262–5267
94. Rismani-Yazdi H, Carver SM, Christya AD, Yu Z, Bibby K, Peccia J, Tuovinen OH (2013) Suppression of methanogenesis in cellulose-fed microbial fuel cells in relation to performance, metabolite formation, and microbial population. Bioresour Technol 129:281–288
95. Chung K, Okabe S (2009) Continuous power generation and microbial community structure of the anode biofilms in a three-stage microbial fuel cell system. Appl Microbiol Biotechnol 83:965–977
96. Jung S, Regan JM (2007) Comparison of anode bacterial communities and performance in microbial fuel cells with different electron donors. Appl Microbiol Biotechnol 77:393–402
97. Cheng S, Kiely P, Logan BE (2011) Pre-acclimation of a wastewater inoculum to cellulose in an aqueous-cathode MEC improves power generation in air-cathode MFCs. Bioresour Technol 102:367–371
98. Lovley DR (2008) The microbe electric: conversion of organic matter to electricity. Curr Opin Biotechnol 19:564–571
99. Geelhoed JS, Hamelers HVM, Stams AJM (2010) Electricity-mediated biological hydrogen production. Curr Opin Microbiol 13:307–315
100. Lies DP, Hernandez ME, Kappler A, Mielke RE, Gralnick JA, Newman DK (2005) *Shewanella oneidensis* MR-1 uses overlapping pathways for iron reduction at a distance and by direct contact under conditions relevant for biofilms. Appl Environ Microbiol 71:4414–4426
101. Reguera G, Pollina RB, Nicoll JS, Lovley DR (2007) Possible nonconductive role of *Geobacter sulfurreducens* pilus nanowires in biofilm formation. J Bacteriol 189:2125–2127
102. Marsili E, Baron DB, Shikhare ID, Coursolle D, Gralnick JA, Bond DR (2008) *Shewanella* secretes flavins that mediate extracellular electron transfer. Proc Natl Acad Sci U S A 105:3968–3973
103. Marsili E, Sun J, Bond DR (2010) Voltammetry and growth physiology of *Geobacter sulfurreducens* biofilms as a function of growth stage and imposed electrode potential. Electroanalysis 22:865–874
104. Srikanth S, Marsili E, Flickinger MC, Bond DR (2008) Electrochemical characterization of *Geobacter sulfurreducens* cells immobilized on graphite paper electrodes. Biotechnol Bioeng 99:1065–1073
105. Stams AJ, de Bok FA, Plugge CM, van Eekert MH, Dolfing J, Schraa G (2006) Exocellular electron transfer in anaerobic microbial communities. Environ Microbiol 8:371–382
106. Gil GC, Chang IS, Kim BH, Kim M, Jang JK, Park HS, Kim HJ (2003) Operational parameters affecting the performance of a mediator-less microbial fuel cell. Biosens Bioelectr 18:327–334
107. Reguera G, Nevin KP, Nicoll JS, Covalla SF, Woodard TL, Lovley DR (2006) Biofilm and nanowire production leads to increased current in *Geobacter sulfurreducens* fuel cells. Appl Environ Microbiol 72:7345–7348
108. Childers SE, Ciufo S, Lovley DR (2002) *Geobacter metallireducens* accesses insoluble Fe (III) oxide by chemotaxis. Nature 416:767–769
109. Aelterman P, Rabaey K, Pham HT, Boom N, Verstraete W (2006) Continuous electricity generation at high voltages and currents using stacked microbial fuel cells. Environ Sci Technol 40:3388–3394
110. Bretschger O, Obraztsove A, Sturm CA, Chang IS, Gorby YA, Reed SB, Culley DE, Reardon CL, Barua S, Romine MF, Zhou J, Beliaev AS, Bouhenni R, Saffarini D, Mansfeld F, Kim BH, Fredrickson JK, Nealson KH (2007) Current production and metal oxide reduction by *Shewanella oneidensis* MR-1 wild type and mutants. Appl Environ Microbiol 73:7003–7012

111. Meitl LA, Eggleston CM, Colberg PJS, Khare N, Reardon CL, Shi L (2009) Electrochemical interaction of *Shewanella oneidensis* MR-1 and its outer membrane cytochromes OmcA and MtrC with hematite electrodes. Geochim Cosmochim Acta 73:5292–5307
112. Coursolle D, Baron DB, Bond DR, Gralnick JA (2010) The Mtr respiratory pathway is essential for reducing flavins and electrodes in *Shewanella oneidensis*. J Bacteriol 192:467–474
113. Coma M, Puig S, Pous N, Balaguer MD, Colprim J (2013) Biocatalysis sulphate removal in a BES cathode. Bioresour Technol 130:218–223
114. Park DH, Laivenieks M, Guettler MV, Jain MK, Zeikus JG (1999) Microbial utilization of electrically reduced neutral red as the sole electron donor for growth and metabolite production. Appl Environ Sci 65:2912–2917
115. Foley JM, Rozendal RA, Hertle CK, Lant PA, Rabaey K (2010) Life cycle assessment of high-rate anaerobic treatment, microbial fuel cells, and microbial electrolysis cells. Environ Sci Technol 44:3629–3637
116. Dumas C, Basseguy R, Bergel A (2008) Microbial electrocatalysis with *Geobacter sulfurreducens* biofilm on stainless stell cathodes. Electrochim Acta 53:2494–2500
117. Gregory KB, Bond DR, Lovley DR (2004) Graphite electrode as electron donors for anaerobic respiration. Environ Microbiol 6:596–604
118. Park DH, Zeikus JG (1999) Utilization of electrically reduced neutral red by *Actinobacillus succinogenes*: physiological function of neutral red in membrane driven fumarate reduction and energy conservation. J Bacteriol 181:2403–2410
119. Thrash JC, van Trump JI, Wever KA, Miller E, Achenbach LA, Coates JD (2007) Electrochemical stimulation of microbial perchlorate reduction. Environ Sci Technol 41:1740–1746
120. Lojou E, Durand MC, Dolla A, Bianco P (2002) Hydrogenase activity control at *Desulfovibrio vulgaris* cell-coated carbon electrodes: biochemical and chemical factors influencing the mediated bioelectrocatalysis. Electroanalysis 14:913–922
121. Strycharz SM, Woodard TL, Johnson JP, Nevin KP, Sanford RA, Loffler FE, Lovley DR (2008) Graphite electrode as a sole electron donor for reductive dechlorination of tetrachloroethene by *Geobacter lovleyi*. Appl Environ Microbiol 74:5943–5947
122. Gregory KB, Lovley DR (2005) Remediation and recovery of uranium from contaminated subsurface environments with electrodes. Environ Sci Technol 39:8943–8947
123. Zhang LH, Jia JP, Ying DW, Zhu NW, Zhu YC (2005) Electrochemical effect on denitrification in different microenvironments around anodes and cathodes. Res Microbiol 156:88–92
124. Jeremiasse AW, Hamelers HVM, Buisman CJN (2010) Microbial electrolysis cell with a microbial biocathode. Bioelectrochemistry 78:39–43
125. Villano M, Aulenta F, Ciucci C, Ferri T, Giuliano A, Majone M (2010) Bioelectrochemical reduction of CO_2 to CH_4 via direct and indirect extracellular electron transfer by a hydrogenophilic methanogenic culture. Bioresour Technol 101:3085–3090
126. Aulenta F, Reale P, Canosa A, Rossetti S, Panero S, Majone M (2010) Characterization of an electro-active biocathode capable of dechlorinating trichloroethene and cis-dichloroethene to ethane. Biosens Bioelectron 25:1796–1802
127. Tandukar M, Huber SJ, Onodera T, Pavlostathis SG (2009) Biological chromium (VI) reduction in the cathode of a microbial fuel cell. Environ Sci Technol 43:8159–8165
128. Croese E, Pereira MA, Euverink GJW, Stams AJM, Geeldhoed JS (2011) Analysis of the microbial community of the biocathode of a hydrogen-producing microbial electrolysis cell. Appl MicrobiolBiotechnol 92:1083–1093
129. Logan BE, Call D, Cheng S, Hamelers HVM, Sleitels THJA, Jeremiasse AW, Rozendal RA (2008) Microbial electrolysis cells for high yield hydrogen gas production from organic matter. Environ Sci Technol 42:8630–8640
130. Wang A, Liu W, Cheng S, Xing D, Zhou J, Logan BE (2009) Source of methane and methods to control its formation in single chamber microbial electrolysis cells. Int J Hydrogen Energy 34:3653–3658

131. Strychartz SM, Glaven R, Coppi M, Gannon S, Perpetua L, Liu A, Nevin K, Lovley DR (2011) Gene expression and deletion analysis of mechanisms for electron transfer from electrodes to *Geobacter sulfurreducens*. Bioelectrochemicstry 80:142–150
132. van Groenestijn JW, Hazewinkel JHO, Nienoord M, Bussmann BJT (2002) Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Int J Hydrogen Energy 27:1141–1147
133. Zumdahl SS (1998) Chemical principles, 3rd edn. Hourson Mifflin Company, Boston, 1040 pp
134. Min B, Román ÓB, Angelidaki I (2008) Importance of temperature and anodic medium composition on microbial fuel cell (MFC) performance. Biotechnol Lett 30:1213–1218
135. Patil SA, Harnisch F, Kapadnis B, Schröder U (2010) Electroactive mixed culture biofilms in microbial bioelectrochemical systems: the role of temperature for biofilm formation and performance. Biosens Bioelectron 26:803–808
136. Hallenbeck PC (2005) Fundamentals of fermentative production of hydrogen. Water Sci Technol 52:21–29
137. Borole AP, Hamilton CY, Vishnivetskaya T, Leak D, Andras C (2009) Improving power production in acetate-fed microbial fuel cells via enrichment of exoelectrogenic organisms in flow-through systems. Biochem Eng J 48:71–80
138. Biffinger JC, Pietron J, Bretschger O, Nadeau LJ, Johnson GR, Williams CC, Nealson KH, Ringeisen BR (2008) The influence of acidity on microbial fuel cells containing *Shewanella oneidensis*. Biosens Bioelectron 24:900–905
139. Borole AP, O'Neill H, Tsouris C, Cesar S (2008) A microbial fuel cell operating at low pH using the acidophile *Acidiphilium cryptum*. Biotechnol Lett 30:1367–1372
140. Sulonen ML, Kokko ME, Lakaniemi AM, Puhakka JA (2015) Electricity generation from tetrathionate in microbial fuel cells by acidophiles. J Hazard Mater 284:182–189
141. Franks AE, Nevin KP, Jia H, Izallalen M, Woodard TL, Lovley DR (2009) Novel strategy for three-dimensional real-time imaging of microbial fuel cell communities: monitoring the inhibitory effects of proton accumulation within the anode biofilm. Energy Environ Sci 2:113–119
142. Torres CI, Marcus AK, Rittmann BE (2008) Proton transport inside the biofilm limits electrical current generation by anode-respiring bacteria. Biotechnol Bioeng 100:872–881
143. Ren Z, Ward TE, Regan JM (2007) Electricity production from cellulose in a microbial fuel cell using a defined binary culture. Environ Sci Technol 41:4781–4786
144. Wang X, Feng YJ, Lee H (2008) Electricity production from beer brewery wastewater using single chamber microbial fuel cell. Water Sci Technol 57:1117–1121
145. Rodrigo MA, Cañizares P, Carcía H, Linares JJ, Lobato J (2009) Study of the acclimation stage and of the effect of the biodegradability on the performance of a microbial fuel cell. Bioresour Technol 100:4704–4710
146. Velasquez-Orta SB, Yu E, Katuri KP, Head IM, Curtis TP, Scott K (2011) Evaluation of hydrolysis and fermentation rates in microbial fuel cells. Appl Microbiol Biotechnol 90:789–798
147. Lee HS, Parameswaran P, Kato-Marcus A, Torres CI, Rittmann BE (2008) Evaluation of energy-conversion efficiencies in microbial fuel cells (MFCs) utilizing fermentable and non-fermentable substrates. Water Res 42:1501–1510
148. Nam JY, Kim HW, Lim KH, Shin HS (2010) Effects of organic loading rates on the continuous electricity generation from fermented wastewater using a single-chamber microbial fuel cell. Bioresour Technol 101:S33–S37
149. Torres CI, Marcus AK, Rittmann BE (2007) Kinetics of consumption of fermentation products by anode-respiring bacteria. Appl Microbiol Biotechnol 77:689–697
150. Behera M, Ghangrekar MM (2009) Performance of microbial fuel cell in response to change in sludge loading rate at different anodic feed pH. Bioresour Technol 100:5114–5121

151. Aelterman P, Versichele M, Marzorati M, Boon V, Verstraete W (2008) Loading rate and external resistance control the electricity generation in microbial fuel cells with different three-dimensional anodes. Bioresour Technol 99:8895–8902
152. Sharma Y, Li B (2010) The variation of power generation with organic substrates in single-chamber microbial fuel cells (SCMFCs). Bioresour Technol 101:1844–1850
153. Sleutels THJA, Hamelers HVM, Buisman CJN (2011) Effect of mass and charge transport speed and direction in porous anodes on microbial electrolysis cell performance. Bioresour Technol 102:399–403
154. Lee HS, Torres CI, Rittmann BE (2009) Effects of substrate diffusion and anode potential on kinetic parameters for anode-respiring bacteria. Environ Sci Technol 43:7571–7577
155. Mohan SV, Raghavulu SV, Srikanth S, Sarma PN (2007) Bioelectricity production by mediatorless microbial fuel cell under acidophilic condition using wastewater as substrate: influence of substrate loading rate. Curr Sci 92:1720–1726
156. Martin E, Savadogo O, Guiot SR, Tartakovsky B (2010) The influence of operational conditions on the performance of a microbial fuel cell seeded with mesophilic anaerobic sludge. Biochem Eng J 51:132–139
157. Liu H, Cheng S, Logan BE (2005) Production of electricity from acetate or butyrate using a single-chamber microbial fuel cell. Environ Sci Technol 39:658–662
158. Jung S, Regan JM (2011) Influence of external resistance on electrogenesis, methanogenesis, and anode prokaryotic communities in microbial fuel cells. Appl Environ Microbiol 77:564–571
159. Aelterman P, Freguia S, Keller J, Verstraete W, Rabaey K (2008) The anode potential regulates bacterial activity in microbial fuel cells. Appl Microbiol Technol 78:409–418
160. Rismani-Yazdi H, Christy AD, Carver SM, Yu Z, Dehority BA, Tuovinen OH (2011) Effect of external resistance on bacterial diversity and metabolism in cellulose-fed microbial fuel cells. Bioresour Technol 102:278–283
161. Lefebvre O, Shen Y, Tan Z, Uzabiaga A, Chang IS, Ng HY (2011) A comparison of membranes and enrichment strategies for microbial fuel cells. Bioresour Technol 102:6291–6294
162. Bond DR (2010) Electrodes as electron acceptors, and the bacteria who love them. In: Barton LL, Mandl M, Loy A (eds) Geomicrobiology: molecular and environmental perspective. Springer, Netherlands, pp 385–399
163. Finkelstein DA, Tender LM, Zeikus JG (2006) Effect of electrode potential on electrode-reducing microbiota. Environ Sci Technol 40:6990–6995
164. Wagner RC, Call DF, Logan BE (2010) Optimal set anode potentials vary in bioelectrochemical systems. Environ Sci Technol 44:6036–6041
165. Wei J, Liang P, Cao X, Huang W (2010) A new insight into potential regulation on growth and power generation of *Geobacter sulfurreducens* in microbial fuel cells based on energy viewpoint. Environ Sci Technol 44:3187–3191
166. Torres CI, Krajmalnik-Brown R, Parameswaran P, Kato Marcus A, Wanger G, Gorby YA, Rittmann BE (2009) Selecting anode-respiring bacteria based on anode potential: phylogenetic, electrochemical and microscopic characterization. Environ Sci Technol 43:9519–9524
167. Sun D, Call DF, Kiely PD, Wang A, Logan BE (2012) Syntrophic interactions improve power production in formic acid fed MFCs operated with set anode potentials or fixed resistances. Biotechnol Bioeng 109:405–414
168. Li F, Sharma Y, Lei Y, Li B, Zhou Q (2010) Microbial fuel cells: the effects of configurations, electrolyte solutions, and electrode materials on power generation. Appl Biochem Biotechnol 160:168–181
169. Liu Y, Harnisch F, Fricke K, Schröder U, Climent V, Feliu JM (2010) The study of electrochemically active microbial biofilms on different carbon-based anode materials in microbial fuel cells. Biosens Bioelectron 25:2167–2171
170. Logan BE (2010) Scaling up microbial fuel cells and other bioelectrochemical systems. Appl Microbiol Biotechnol 85:1665–1671

171. Logan B, Cheng S, Watson V, Estadt G (2007) Graphite fiber brush anodes for increased power production in air-cathode microbial fuel cells. Environ Sci Technol 41:3341–3346
172. Mohanakrishna G, Mohan SK, Mohan SV (2012) Carbon based nanotubes and nanopowder as impregnated electrode structures for enhanced power generation: evaluation with real field wastewater. Appl Energy 95:31–37
173. Wei J, Liang P, Huang X (2011) Recent progress in electrodes for microbial fuel cells. Bioresour Technol 102:9335–9344
174. Gnana Kumar G, Sathiya Sarathi VG, Nahm KS (2013) Recent advances and challenges in the anode architecture and their modification for the applications of microbial fuel cells. Biosens Bioelectron 43:461–475
175. Zhang X, Cheng S, Wang X, Huang X, Logan BE (2009) Separator characteristics for increasing performance of microbial fuel cells. Environ Sci Technol 43:8456–8461
176. Jia YH, Tran HT, Kim DH, Oh SJ, Park DH, Zhang RH, Ahn DH (2008) Simultaneous organics removal and bio-electrochemical denitrification in microbial fuel cells. Bioprocess Biosyst Eng 31:315–321
177. Lovley DR, Nevin KP (2011) A shift in the current: new applications and concepts for microbe-electrode electron exchange. Curr Opin Biotechnol 22:441–448
178. Jiang D, Curtis M, Troop E, Scheible K, McGrath J, Hu B, Suib S, Raymond D, Li B (2011) A pilot-scale study on utilizing multi-anode/cathode microbial fuel cells (MAC MFCs) to enhance the power production in wastewater treatment. Int J Hydrogen Energy 36:876–884
179. Cusick RD, Bryan B, Parker DS, Merrill MD, Mehanna M, Kiely PD, Liu G, Logan BE (2011) Performance of a pilot-scale continuous flow microbial electrolysis cell. Appl Microbiol Biotechnol 89:2053–2063

Adv Biochem Eng Biotechnol (2016) 156: 293–322
DOI: 10.1007/10_2015_5005

Published online: 9 March 2016

Low-Carbon Fuel and Chemical Production by Anaerobic Gas Fermentation

James Daniell, Shilpa Nagaraju, Freya Burton, Michael Köpke, and Séan Dennis Simpson

Abstract World energy demand is expected to increase by up to 40% by 2035. Over this period, the global population is also expected to increase by a billion people. A challenge facing the global community is not only to increase the supply of fuel, but also to minimize fossil carbon emissions to safeguard the environment, at the same time as ensuring that food production and supply is not detrimentally impacted. Gas fermentation is a rapidly maturing technology which allows low carbon fuel and commodity chemical synthesis. Unlike traditional biofuel technologies, gas fermentation avoids the use of sugars, relying instead on gas streams rich in carbon monoxide and/or hydrogen and carbon dioxide as sources of carbon and energy for product synthesis by specialized bacteria collectively known as acetogens. Thus, gas fermentation enables access to a diverse array of novel, large volume, and globally available feedstocks including industrial waste gases and syngas produced, for example, via the gasification of municipal waste and biomass. Through the efforts of academic labs and early stage ventures, process scale-up challenges have been surmounted through the development of specialized bioreactors. Furthermore, tools for the genetic improvement of the acetogenic bacteria have been reported, paving the way for the production of a spectrum of ever-more valuable products via this process. As a result of these developments, interest in gas fermentation among both researchers and legislators has grown significantly in the past 5 years to the point that this approach is now considered amongst the mainstream of emerging technology solutions for near-term low-carbon fuel and chemical synthesis.

J. Daniell
LanzaTech Inc., 8045 Lamon Ave, Suite 400, Skokie, IL 60077, USA

School of Biological Sciences, University of Auckland, Auckland, New Zealand

S. Nagaraju, F. Burton, M. Köpke, and S.D. Simpson (✉)
LanzaTech Inc., 8045 Lamon Ave, Suite 400, Skokie, IL 60077, USA
e-mail: sean@lanzatech.com

Keywords Acetogens · Biofuels · Clostridia · Gas fermentation · Synthesis gas · Wood–Ljungdahl pathway

Contents

Abbreviations

ABE	Acetone-butanol-ethanol fermentation
ACS	Acetyl-CoA synthase
AOR	Acetaldehyde: ferredoxin oxidoreductase
BDO	Butanediol
BOF	Basic oxygen furnace
CO	Carbon monoxide
CO_2	Carbon dioxide
CODH	Carbon monoxide dehydrogenase
CSTR	Continuous Stirred Tank Reactor
GHG	Greenhouse gas
H_2	Hydrogen
LCA	Life-cycle analysis
MSW	Municipal solid waste
RED	Renewable Energy Directive
SLP	Substrate-level phosphorylation
THF	Tetrahydrofolate
WL	Wood–Ljungdahl

1 Introduction

Concerns about climate change and energy supply are driving the production of more sustainable fuels and chemicals [1]. Renewable fuels such as bioethanol are a promising alternative to fossil-based transport fuels, and extensive research and development has been carried out to deploy technologies for their commercial

production [2]. In addition, similar approaches to make commodity chemicals from non-petrochemical feedstocks are under development [3].

The production of most renewable fuels and chemicals involves microbial fermentation of farmed sugars. Examples include yeast-based fermentation to produce bioethanol, and *Escherichia coli* fermentation to produce renewable 1,4-butanediol [4, 5] or 1,3-propanediol [6]. Although these technologies have successfully entered the market, the crops used as feedstocks compete for the use of arable land. Consequently, advanced technologies for the production of low-carbon fuels and commodity chemicals are being developed to access additional carbon sources [2]. Advanced technologies such as gas fermentation use microbial platforms which offer key advantages over traditional yeast and *E. coli*. Gas fermentation uses anaerobic acetogenic bacteria to ferment carbon-rich gas generated from a range of sources, including forestry residues, municipal solid waste (MSW), and industrial waste gases to produce a spectrum of fuel and chemical products. Although feedstock-specific legislative hurdles exist in some geographies, gas fermentation technology provides numerous benefits over established technologies and is nearing the commercial scale for the production of low-carbon fuels and chemicals [7].

2 Alternative Carbon-Containing Feedstocks

The fermentation of sugar to bioethanol is the dominant microbial-based renewable fuel technology. Typical substrates include corn, sugar cane, and molasses [2]. Although these technologies are mature, new approaches which can utilize alternative, more abundant feedstocks are required to displace adequately petroleum-derived fuels and chemicals and meet legislated biofuel demands and climate targets [8, 9]. The requirement for water and arable land may limit the availability of sugar-based feedstocks and can put their production in direct competition with the production of food [10]. Consequently, reports highlight an inherent limit on the level of first-generation biofuel production before biodiversity and food security are negatively impacted [11]. There are also considerable emissions associated with making new land available for crop production [12]. To encourage the development of advanced biofuels, mandates such as the United States Energy Independence and Security Act of 2007 limit the incentives for biofuel production from traditional feedstocks [13].

In response to the limitations of available technologies, new approaches to low-carbon fuel and chemical production are under development which allow the utilization of abundant, alternative carbon-containing feedstocks. Low-carbon fuels and chemicals are those which deliver material greenhouse gas savings [14]. Examples include lignocellulosic hydrolysis and fermentation, biomass gasification and fermentation, and microalgae fermentation [2, 14]. The production of low-carbon fuels from a diverse range of biomass sources allows greater potential displacement of traditional fossil fuels. These biomass sources include lignocellulosic energy

crops such as wheat straw and willow, and waste sources such as MSW or carbon-containing industrial waste gases. Furthermore, many of these advanced technologies allow access to non-commodity feedstocks, thus enabling the paradigm of producing commodities such as fuels and chemicals from commodity feedstocks to be broken. Although the price of sugar-based crops fluctuates significantly, the price of feedstocks such as carbon-containing industrial waste is more stable as they only participate in the energy market. Furthermore, many of these advanced technologies have flexible feedstock requirements, preventing their reliance on the economics of a single input. This is important because feedstock price makes the largest contribution to the cost of biofuel production.

Gas fermentation uses synthesis gas, composed of carbon dioxide and/or hydrogen, and carbon monoxide [15]. This can be generated from the gasification of carbon-containing feedstocks such as industrial and municipal solid waste and lignocellulosic forestry residues. Gasification is an efficient process to convert feedstocks to synthesis gas thermochemically using oxygen, steam, or air as a medium [16, 17]. In addition, carbon dioxide and carbon monoxide-rich gas streams are also available as a by-product of different industrial processes such as steel production and oil refining, and through the reforming of methane (biogas or natural gas), which can also be utilized as feedstock for the gas-fermentation process.

3 Advancement of Non-traditional Host Organisms

E. coli and yeast have become model microorganisms over the past 50 years as they are easy to cultivate, genetically accessible, and well-studied, with over half a million published research articles. Bioengineering and synthetic biology capabilities for these model organisms have advanced recently, benefiting from rapid DNA design [18] and automated high-throughput strain engineering platforms which allow rapid prototyping [19]. The high-throughput bioengineering capabilities established in these systems have set the technological benchmark for other bioproduction organisms.

Despite these advancements, only a handful of examples of successful commercialization of *E. coli* and yeast processes for fuels and commodity chemical (such as 1,3-propanediol or 1,4-butanediol) production exist [5, 6]. Key challenges include the limited substrate range, low maximum yields, susceptibility to contamination, fermentation instability, scale-up difficulties, and the inability to operate in a continuous mode. These challenges have prevented more rapid commercialization. Additionally, *E. coli* and yeast lack certain metabolic and cellular traits such as unique co-factors or cell structures which prevent certain reactions and pathways from being functional. Because of the challenges associated with *E. coli* and yeast, most commercial processes applied to date rely on other more robust organisms and native producers such as *Corynebacterium* for amino acid production [20], ABE (acetone-butanol-ethanol)-fermenting Clostridia [21], and propionic acid and

succinic acid producing bacteria or fungi for citric acid and itaconic acid production.

Fermentation processes with modified *E. coli* and yeast are reported to be prone to bacterial contaminations and bacteriophage infection caused by the rich substrates used in the process [22–24]. Although the use of pure sugar feedstocks and strict sterility regimes control this problem on the lab scale, continuous fermentations using modified *E. coli* and yeast on raw sugars are challenging. As a result, *E. coli* and yeast fermentations at scale are nearly always limited to batch processes and often require the addition of antibiotics. This leads to significantly increased capital and operating costs [24]. A low tolerance to acids and alcohols, and the inability to survive in low pH conditions, further limit industrial *E. coli* fermentation [25]. Production rates achieved in the lab are often challenging to reproduce at scale using industrial-grade chemicals and raw C6 sugar streams with lot-to-lot variations [24]. A considerable amount of work has been conducted to improve the substrate range and yield of *E. coli* and yeast and to allow the use of cellulosic material. To date, this work has only been moderately successful, despite the availability of advanced engineering tools and significant investment.

In general, fermentations from sugar offer low maximum theoretical yields because of the inevitable formation of significant amounts of CO_2. For example, ethanol production from sugar with *E. coli* or yeast has a maximum theoretical carbon yield of 66%, with the remaining 33% of carbon lost as greenhouse gas CO_2. There have been attempts to engineer *E. coli* and yeast strains to improve yields by preventing CO_2 production and to allow the utilization of other cheap and abundant feedstocks, including glycerol or C1 substrates such as carbon monoxide (CO), carbon dioxide (CO_2), methane (CH_4), or methanol. Although some innovative new pathways have been designed which show promise [26, 27], the lack of efficiency prevents commercialization in the foreseeable future. Besides these synthetic pathways, there has also been significant efforts to optimize glycerol utilization [28] and to engineer methane [29] and methanol [30] utilization pathways from methanotrophs and methylotrophs or the reductive acetyl-CoA pathway for CO and CO_2 utilization [31] from acetogens into *E. coli*. However, redox imbalances, lack of co-factors, difficulties expressing active protein, and the complex biochemistry of involved enzymes have only allowed low rates of carbon utilization, or prevented any utilization of carbon into the metabolism.

Therefore, there is a need to establish and advance additional, complementary model systems. These would ideally be industrially-proven organisms, such as anaerobic acetogens, with unique capabilities that cannot be achieved using the current microbial models. However, for most of these microbial strains there are at best only basic genetic tools available to enhance the native biological capability. This limitation excludes these production organisms from the enormous potential benefits made possible by advances in synthetic biology, refactoring, and high-throughput strain engineering.

4 Anaerobic Acetogens as a Microbial Chassis

Anaerobic acetogens possess unique capabilities which make them an ideal microbial chassis. In particular, their ability for autotrophic carbon assimilation via the reductive acetyl-CoA pathway, also known as the Wood–Ljungdahl (WL) pathway, makes them attractive. This pathway is argued to be the most energy-efficient process of carbon fixation as it operates in a linear fashion [32] and is speculated to be one of the first biochemical pathways on Earth [33]. Although a repertoire of acetogenic microbes (acetogens are present in at least 25 different genera) that grow chemolithoautotrophically on CO and CO_2/H_2 have been discovered, only a handful are being considered for their ability to ferment syngas and produce useful biofuels and biocommodities on an industrial scale [34–37]. Those mainly belonging to the class of Clostridia and genus *Clostridium* are particularly exploited. Clostridia are known for their enormous capacity for biotechnological applications such as fuel, chemical, and natural product synthesis. As such, they have been industrially proven over almost 100 years for production of fuels and commodity chemicals [38–40].

The exceptional substrate flexibility and metabolic diversity of Clostridia enables the production of a broad range of compounds at high yield from a variety of feedstocks [40]. Beside a range of C5 and C6 sugars, many Clostridia are able to utilize substrates such as glycerol and cellulose [41]. Acetogenic Clostridia can also utilize C1 compounds such as CO, CO_2, methanol, or formate [42]. Furthermore, acetogenic Clostridia have been shown to be able to utilize electricity as a source of energy, allowing CO_2 fixation and product synthesis [43–46], a process far more efficient than photosynthesis, with more than 85% of electrons and more than 70% of energy input recovered in produced biocommodities [44].

Acetogens currently being used for commercial syngas fermentation include *C. autoethanogenum, C. ljungdahlii*, *C. ragsdalei, C. coskatii, C. carboxidivorans, C. aceticum*, *Moorella thermoacetica* (formerly: *Clostridium thermoaceticum*), *Acetobacterium woodii*, and *Butyribacterium methylotrophicum*. Isolated from various habitats, these organisms can grow on a range of other carbon sources (Table 1). *C. aceticum*, *M. thermoacetica,* and *A. woodii* are mainly considered for acetate production, *B. methylotrophicum* and *C. carboxidivorans* for butanol production, and *C. ljungdahlii*, *C. autoethanogenum, C. coskatii*, and *C. ragsdalei* for ethanol and 2,3-butanediol production. *C. ljungdahlii, C. aceticum*, and *M. thermoacetica* can also produce other organic compounds such as 2-oxobutyrate and formate by electrosynthesis using CO_2 as the electron acceptor and electrons derived from electrodes [45].

Table 1 List of gas-fermenting acetogens currently pursued for commercial production of fuels and some of their characteristics

Microbe	Isolated from	Products	Substrates	Genome data (GenBank accession)	References
C. autoethanogenum	Rabbit feces	Acetate, ethanol, 2,3-BDO, lactate	Fructose, rhamnose, xylose, arabinose, mannose, pyruvate, sucrose, malate, glutamate, etc.	CP006763	[38, 47–49]
C. ljungdahlii	Chicken yard waste	Acetate, ethanol, 2,3-BDO, lactate	Fructose, glucose, rhamnose, xylose, arabinose, mannose, pyruvate, sucrose, malate, glutamate, etc.	CP001666	[38, 50, 51]
C. coskatii	Sediment	Ethanol, acetate	Fructose, glucose, rhamnose, xylose, arabinose, mannose, pyruvate, sucrose, malate, glutamate, etc.	NA	[52]
C. ragsdalei	Duck pond sediments	Ethanol, acetate, lactate, 2,3-BDO	Fructose, glucose, rhamnose, xylose, arabinose, mannose, pyruvate, sucrose, malate, glutamate, etc.	NA	[38, 53]
C. carboxidivorans	Agricultural settling lagoon	Ethanol, acetate, butyrate, butanol, hexanol	Fructose, glucose, rhamnose, xylose, arabinose, mannose, pyruvate, cellubiose, cellulose, malate, glutamate, etc.	CP011803	[54–56]

(continued)

Table 1 (continued)

Microbe	Isolated from	Products	Substrates	Genome data (GenBank accession)	References
C. aceticum	Soil	Acetate	Fructose, ribose, pyruvate, glutamate, fumarate, and malate	CP00698	[57–62]
M. thermoacetica	Soil, horse manure	Ethanol, acetate	Glucose, fructose, xylose, pyruvate, methanol, etc.	CP000232	[63–65]
A. woodii	Black sediment from marine estuary	Acetate	Glucose, fructose, pyruvate, methanol, lactate, 2,3-BDO	CP002987	[66, 67]
B. methylotrophicum	Sewage digester	Ethanol, acetate, butanol, butyrate, lactate	Glucose, pyruvate, methanol, etc.	NA	[68, 69]

NA not available

5 Metabolism of Acetogens

Understanding the metabolism, energy-conserving processes, and redox balance mechanisms of acetogens is crucial for their industrial exploitation for fuel and commodity chemical production. This understanding is the key to strain optimization through metabolic engineering and process optimization on the industrial scale. Acetogens are considered to thrive at the thermodynamic edge of life, and until recently it was unclear how these organisms conserve energy. Insights into energy-generating processes and redox homeostasis from industrially relevant acetogens are now becoming available.

Carbon is taken up via the reductive-acetyl-CoA or Wood–Ljungdahl (WL) pathway (Fig. 1) which is briefly discussed below. The pathway has been reviewed in detail by Wood [70], Müller [71], Ragsdale [72], Drake et al. [73], and Ragsdale and Pierce [74]. The WL pathway consists of two branches, the methyl (Eastern) and carbonyl (Western) branch (Fig 1). In the methyl branch, CO_2 is reduced by formate dehydrogenase to formate, which is then activated by condensation with tetrahydrofolate (THF) to form formyl-THF by a formate-THF synthetase. This is an energy-intense reaction and consumes one molecule of ATP. A formyl-THF cyclohydrolase then converts formyl-THF to methenyl-THF with the removal of a molecule of water. Methenyl-THF is reduced by methylenetetrahydrofolate dehydrogenase and methylene-THF reductase to methylene-THF and methyl-THF, respectively. The methyl group in the final reaction of the methyl

Acetogens

- Has a wide portfolio of natural products
- Recently catching-up in the development of genetic tools and metabolic engineering
- Industrialization of gas fermentation face scale-up and engineering challenges
- Can utilize a wide range of carbon sources starting from C1 by gas fermentation (Wood-Ljungdahl pathway)

Traditional hosts

- Needs to be genetically modified to diversify its product portfolio
- Backed by almost a century of work on genetic tools and metabolic engineering; fast growing
- Sugar fermentation, scale-up and industrialization is well-established
- Cannot utilize C1 as carbon source, depend on sugar (glycolysis)

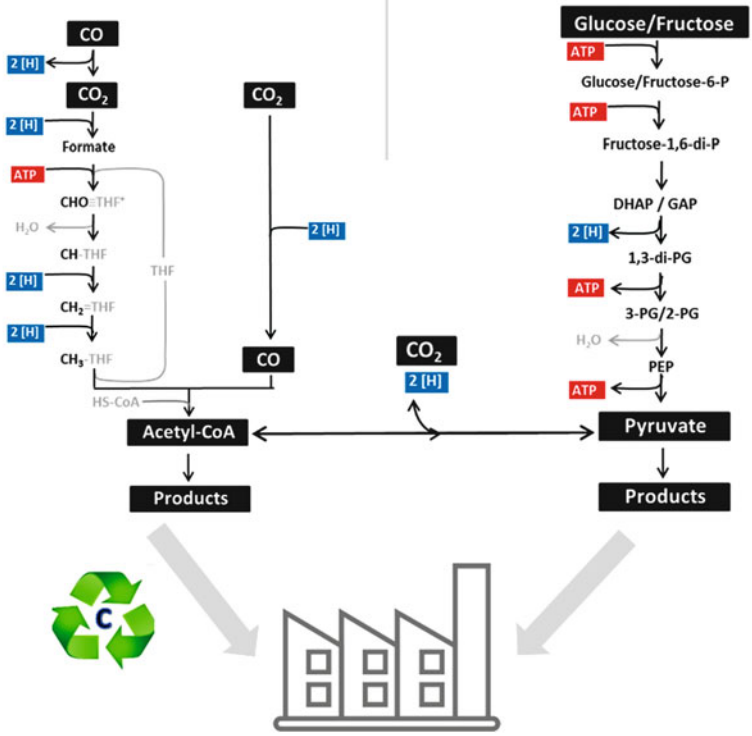


Fig. 1 Comparison between acetogens and other traditional hosts such as *E. coli* and yeast for product synthesis. Acetogens can use C1 carbon compounds as a carbon source by the Wood-Ljungdahl pathway to make a wide range of compounds that can substitute petroleum derived fuels and chemicals. Use of acetogens for such biotechnology purposes has a major benefit in recycling carbon and reducing greenhouse gas effect. Other traditional hosts mainly use sugar as carbon source by glycolysis to produce a limited number of products that can substitute petroleum derived fuels and chemicals. Industrialization of sugar fermentation is backed by years of work on genetic modification and scale-up by a wide community

branch is transferred to a corrinoid iron-sulfur-containing protein. The carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) enzyme complex catalyzes the reaction in the carbonyl branch. When grown in CO alone, CO_2 required for the methyl branch is generated by the CODH-catalyzed water-gas shift reaction. For the carbonyl branch, CO is formed from CO_2 by CODH, or CO is directly fed to the CODH/ACS complex, which then condenses with the methyl group transferred to ACS from corrinoid iron-sulfur-containing protein, forming acetyl-CoA.

By the action of phosphotransacetylase and acetate kinase, acetyl-CoA can be converted to acetate. The reaction catalyzed by acetate kinase generates a molecule of ATP via SLP. Still, there is no net energy gain by SLP via the WL pathway, as one molecule of ATP is consumed in the methyl branch. Additional energy derived from chemiosmotic mechanisms is used for energy conservation [71, 73, 75, 76].

Different energy conservation mechanisms exist across acetogenic bacteria. In *M. thermoacetica* [77] a cytochrome-mediated proton gradient is generated for energy conservation, whereas *A. woodii* relies on a membrane-integral Rnf complex (which acts as a ferredoxin:NAD oxidoreductase) to build up a sodium gradient which can be used for energy conservation [78–80]. *M. thermoacetica*, which lacks Rnf complex, may have a different membrane-bound energy-converting hydrogenase (Ech) complex. However, less energy is released by the Ech complex compared to the Rnf-catalyzed mechanism [76]. Energy conservation in *C. autoethanogenum* [81, 82] and *C. ljungdahlii* [83] is also Rnf-mediated but seems to rely on a proton-dependent chemiosmotic mechanism. Genome analysis of *C. aceticum* reveals the presence of genes encoding the Rnf complex and all the genes necessary for cytochrome synthesis [57].

In addition, several enzymes have recently been discovered in acetogens which allow coupling of exergonic reactions to the endergonic reduction of ferredoxin by flavin-based electron bifurcation [75]. The reducing equivalents gained by the reduction of ferredoxin and NAD(P) from CO or from H_2 by hydrogenases can be used to generate the chemiosmotic ion gradients required for ATP production. Acetogens can utilize a range of electron donors and acceptors because of the presence of electron carriers with a range of redox potentials, such as NADH, NADPH, ferredoxins, cytochromes, quinones, and rubredoxins [75, 76]. A recently-proposed bioenergetic classification divides acetogens into two groups based on the type of integral membrane protein complex involved in creating the electrochemical gradient: either Rnf-containing (membrane integral, multi-subunit ferredoxin–NAD+ oxidoreductase) or Ech-containing. These groups are further subdivided based on the ions involved in creating the electrochemical gradient: proton-dependent or sodium ion-dependent [76].

The WL pathway and its energetics in *C. autoethanogenum* [81, 82] and *C. ljungdahlii* [83], grown on syngas and CO_2/H_2, respectively, have recently been studied. In *C. autoethanogenum* grown on syngas, CO_2 is reversibly reduced to formate by a heptameric protein cluster consisting of a selenium-containing formate dehydrogenase in complex with an NADP and ferredoxin-specific electron bifurcating tungsten dependent [FeFe]-hydrogenase cluster, $HytABCDE_1E_2$ [82]. This is the first report of an NADP-specific electron bifurcating [FeFe]-

hydrogenase. In other acetogens, such as *A. woodii*, the same reaction is NAD specific and is composed of a different electron-bifurcating hydrogenase, HydABCD [76].

Apart from NADP and ferredoxin-dependent hydrogenase and formate dehydrogenase activities, other hydrogenase and formate dehydrogenase activities are also detected. Additional hydrogenases and formate dehydrogenases are found in the genome annotations of *C. autoethanogenum*. The methylene-THF dehydrogenase reaction of the WL pathway is NADP-dependent, whereas it is NAD specific in *A. woodii* [84]. *C. autoethanogenum* has an electron-bifurcating and ferredoxin-dependent transhydrogenase (Nfn) [81] which is absent in *A. woodii* [67]. The enzymes, acetaldehyde dehydrogenase and alcohol dehydrogenase, involved in ethanol production from acetyl-CoA, and 2,3-butanediol dehydrogenase (converts acetoin to 2,3-butanediol), involved in 2,3-BDO production, can use both NADH and NADPH as substrates, but NADPH is the preferred substrate. In addition to acetaldehyde formation by alcohol dehydrogenase from acetyl-CoA, *C. autoethanogenum* can also convert acetic acid to acetaldehyde by the activity of acetaldehyde: ferredoxin oxidoreductase (AOR). Genome analysis of *C. autoethanogenum* reveals two copies of acetaldehyde: ferredoxin oxidoreductase which are ~75% identical at protein level. *A. woodii* does not have acetaldehyde: ferredoxin oxidoreductase [81]. The reason that *C. autoethanogenum* is an ideal platform strain and can make reduced products as ethanol is based on three important distinguishing features: (1) NADPH electron-bifurcating $HytABCDE_1E_2$ hydrogenase, (2) electron-bifurcating and ferredoxin-dependent transhydrogenase Nfn and (3) acetaldehyde: ferredoxin oxidoreductase. The latter two activities are absent in *A. woodii* which only makes acetate from CO_2/H_2 and cannot grow on CO [85].

The information on energy conservation mechanisms is not only useful for further optimizing *C. autoethanogenum* and *C. ljungdahlii* for improved production of ethanol and 2,3-BDO but is also useful in the metabolic engineering of *C. autoethanogenum* and *C. ljungdahlii* for diverse fuel and bulk chemical production.

6 Genetic Manipulation of Acetogens

The ability to modify acetogens genetically is required to establish a chemical production platform via gas fermentation. Through genetic manipulation, fermentation performance can be improved, and strains can be created which would have the potential to produce non-native chemicals. However, the intrinsic difficulty in genetically manipulating these organisms has historically been a major challenge in strain engineering and optimization. Over the past two decades, research in understanding the basic physiology, cellular metabolism, genetic manipulation, and metabolic engineering of the Clostridial species has gained momentum [7, 36, 40, 86–89]. Major highlights include the genetic manipulation and pathway

engineering of *C. aceticum* for acetone production [36], *C. ljungdahlii* [90] and *C. autoethanogenum* [91] for butanol production, and *C. autoethanogenum* for isopropanol [92] and methyl ethyl ketone/2-butanol production [93].

Initially, strains have been optimized for solvent production by adapted evolution [7, 94], by chemical mutagenesis for solvent tolerance [95, 96], and by transposon-based random mutagenesis [97]. These traditional methods can be time-consuming and require a robust protocol for screening vast mutant libraries. Consequently, rational targeted metabolic engineering of strains is a preferred approach. Metabolic pathways have been optimized, and strains with desirable phenotype have been engineered using these different genetic tools. A few examples include *C. acetobutylicum* mutant strains with decoupled sporulation-solventogenesis pathways [98], aerotolerant strains to study oxidative stress response [99], strains engineered to study acid and solvent production [100–102], and strains engineered to grow on CO_2/H_2 and CO as the carbon source [103]. An example from *C. thermocellum* [104, 105] and *C. cellulolyticum* [106] includes its genetic manipulation to create strains with high ethanol yield.

C. acetobutylicum is a platform organism for the genus *Clostridium* for understanding the biology and developing basic genetic manipulation and metabolic engineering tools [107]. Transformation of Clostridia is performed either by electroporation or by conjugation by in vitro or in vivo methylation of plasmids and using *E. coli* as conjugal donor strain, respectively [108–112]. A set of replicative plasmids that work in several Clostridia are available [109, 113]. Genetic manipulation by homologous recombination at reasonable efficiency is achieved in non-acetogenic Clostridia by using one of the many methods such as (1) suicide or non-replicative plasmids [102] or replicative plasmids with segregation instability (pseudo suicide plasmids) [113] alone or in combination with helper factors such as *recU*, a Holiday junction resolvase from *Bacillus subtilis* [98, 114], (2) counter selection markers such as *codA*, a cytosine deaminase [115], and *B. subtilis mazF*, an mRNA interferase [96, 114], (3) in the absence of a suitable counter selectable marker auxotrophic mutants of *pyrE*, an orotate phosphoribosyltransferase [116], *pyrF*, an orotidine-5′-monophosphate decarboxylase [105], *upp,* uracil phosphoribosyltransferase [117], *galK*, a galactokinase [118] are used as base strains with the corresponding mutated genes as counter selectable marker, (4) by the use of I-SceI, an intron-encoded endonuclease from *Saccharomyces cerevisiae* [119], and (5) with the application of *Streptococcus pyogenes* CRISPR/cas9 for scarless genetic modifications [120, 121]. Gene disruption using group II intron directed insertional inactivation of genes has been a successful alternative to homologous recombination [106, 108, 122, 123]. Having efficient transformation and chromosomal integration strategies is a prerequisite to metabolic engineering. However, to regulate and fine-tune cellular and heterologous metabolic pathways, various other accessories such as a selection of reporter genes, a library of promoters (both constitutive and inducible promoters), ribosome binding sites, terminators, and overexpression systems are also essential. Reporter genes shown to work in Clostridia include *gusA*, a β-glucuronidase [107, 114], anaerobic fluorescent protein [120, 124], *Thermoanaerobacterium thermosulfurogenes* β-galactosidase *lacZ*

[125, 126], *Photinus pyralis* luciferase gene, *lucB* [125], oxygen-independent fluorescent reporter [96], and the most common reporter gene, *catP* for chloramphenicol acetyltransferase [127, 128]. Some promoters and terminators from *Clostridium* species have interspecies compatibility [108]. However, a library of synthetic promoters would be desirable to avoid complications of cross-regulation in using the clostridial promoters. Inducible promoter or gene expression systems applied in non-acetogenic Clostridia have only recently been developed and include those that are induced by anhydrotetracycline [129], lactose [114, 130], isopropyl β-D-1-thiogalactopyranoside (IPTG) [108], xylose [107], and arabinose [124]. Heterologous controlled expression systems, analogous to the T3/T7 system used in *E. coli*, such as *tcdR* and *botR*, sigma factors from *C. difficile* and *C. botulinum*, respectively, are successfully applied to drive the expression of genes only in the presence of their cognate transcription factor in other Clostridia [96].

The most recent progress made in extending the genetic tools developed for acetogenic Clostridia is discussed below.

A major hurdle in genetic manipulation of acetogenic syngas-fermenting Clostridia, as with other members of the genus *Clostridium*, is in transforming or introducing plasmid DNA into these organisms followed by chromosomal integration events for gene deletions and gene insertions. Of the gas-fermenting acetogenic Clostridia discussed above, a genetic system has been established only for *C. ljungdahlii*, *C. autoethanogenum*, *C. aceticum*, and *M. thermoacetica*. In particular *C. ljungdahlii* and *C. autoethanogenum* have been proved as useful chassis organisms for the production of fuels and bio-commodities using CO_2 and H_2 and/or syngas as the electron and carbon source [90, 131].

A first genetic system has been developed for *C. ljungdahlii*. Köpke et al. demonstrated the heterologous expression of butanol pathway genes on an *E. coli-Clostridium* pIMP1 shuttle plasmid in *C. ljungdahlii* [90]. The plasmid was introduced into *C. ljungdahlii* by electro-transformation. Derek Lovely's group has further optimized electro-transformation and plating protocols and obtained transformants at a much higher frequency and efficiency with *E. coli-Clostridium* shuttle plasmids having different Gram positive replicons [131]. Furthermore, they have demonstrated chromosomal modification of *C. ljungdahlii* by gene deletions via homologous recombination [131, 132]. Using suicide plasmids, they have successfully deleted *filA*, involved in flagella formation and motility, and *adhE1* and *adhE2*, bi-functional aldehyde/alcohol dehydrogenases, involved in ethanol formation. However, the frequency of double crossover recombination was only ca. 30%. The wild-type phenotype of Δ*adhE1* mutant was restored to a larger extent by complementing the mutant with a plasmid borne copy of *adhE1*. By deleting adjacent *adhE1* and *adhE2* genes simultaneously, they have shown that the genomic region spanning ~5 kb can be targeted by homologous recombination [131]. Even though this is a first big step in genetic manipulation of *C. ljungdahlii,* the drawback is that this strategy leaves the antibiotic selection marker on the chromosome. Following the same electroporation protocol and using suicide plasmid, the Rnf complex operon in *C. ljungdahlii* was disrupted [83]. However, the efficiency of electroporation was very low with suicide plasmid. Because

of the limited availability of positive selection markers, alternatives such as marker recycling or scarless gene deletion strategies to target genes at different loci on the chromosome is preferred. The *cre-lox* system has been successfully tested in *C. ljungdahlii* to remove markers from the genome, leaving behind a scar of 32 bases [132].

Genetic modification of *C. autoethanogenum* was demonstrated by introducing heterologous pathways for butanol [91] and acetone/ isopropanol [92, 133] as well as to increase ethanol tolerance [134]. For this, the native *groESL* operon, encoding heat shock proteins, was episomally overexpressed. For butanol production, biosynthetic genes thiolase A (*thlA*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*), and electron transfer protein A and B (*etfAB*) were heterologously expressed from a plasmid. Similarly, heterologous genes thiolase (*thlA*), CoA transferase (*ctfAB*), and acetoacetate decarboxylase (*adc*) were episomally expressed to obtain isopropanol producing a *C. autoethanogenum* strain. A native secondary alcohol dehydrogenase is present in the strain [135] which can be inactivated for production of acetone [136]. In all these instances, the plasmids, following in vivo methylation in *E. coli* strains harboring a *C. autoethanogenum* methyltransferase gene, were introduced into *C. autoethanogenum* by electroporation. Recently, transformation of *C. autoethanogenum* by conjugation has been achieved using *E. coli* as a plasmid donor strain [81].

Targeted gene deletion by homologous recombination has also been reported in *C. autoethanogenum* [137]. The *budA* gene coding for an acetolactate decarboxylase enzyme involved in 2,3-butanediol production was deleted by homologous recombination, but the frequency of integration was low and involves extensive screening. Gene disruption by using a mobile group II intron-based retrohoming strategy works reliably in *C. autoethanogenum* [81] but this method leaves a scar on the genome. Using group II intron, the role of genes encoding 2,3-butanediol dehydrogenase (*bdh*) and acetolactate decarboxylase (*budA*) in 2,3-butanediol pathway [137] and hydrogenases in energy conservation [81] in *C. autoethanogenum* has been investigated.

Apart from gene deletion and gene insertion tools, other important genetic tools in metabolic engineering and for constructing synthetic genetic circuits in any organism are libraries of constitutive and inducible promoters, ribosome binding sites, and terminators. There is some progress made in this direction in *C. ljungdahlii* where the lactose-inducible system is used to control the expression of *gusA*-coded β-glucuronidase reporter gene [138] and the butyrate metabolic pathway is optimized by manipulating the ribosome binding sites [132]. This is just the beginning, and these arsenals are yet to be further developed for acetogenic Clostridia.

Only with the aid of an effective genetic tool box is it possible to bring together metabolic engineering and systems biology knowledge onto a synthetic biology platform in acetogenic Clostridia. This is essential in maximizing the breadth of fuels and biocommodities that can be produced on an industrial scale.

7 Modeling of Gas-Fermenting Organisms and Fermentation Process

Genome-scale modeling promises to be a powerful tool for the systems-level characterization and metabolic manipulation of gas-fermenting acetogens. Metabolic network reconstructions describe the relationship between the metabolic reactions and genes of an organism. Genome-scale modeling uses these reconstructions to predict computationally microbial phenotypes from a specified genotype [139]. Researchers have used these genome-scale models to predict microbial metabolism for metabolic engineering, network analysis, and biological discovery applications. For example, new microbial strain designs can be tested in silico prior to creating them in the lab.

Researchers have recently published models for gas-fermenting acetogens *C. ljungdahlii, M. thermoacetica*, and *C. autoethanogenum*. As highlighted in Table 2, these models were informed by previously published models for related non-acetogenic Clostridia, including *C. thermocellum*, *C. beijerinckii*, *C. acetobutylicum*, and *C. cellulolyticum* (Fig. 2) [143–147]. Nagarajan et al. [140] published the first genome-scale model of an acetogen, *C. ljungdahlii* model iHN637. The authors used the model, alongside transcriptomic and physiological data, to characterize a nitrate reduction pathway. Furthermore, they identified the importance of flavin-based electron bifurcation in energy conservation during autotrophic

Table 2 Published acetogenic genome-scale models

Organism	Reconstruction technique	Reactions	Metabolites	Genes	References
C. ljungdahlii	Model SEED	785	698	637	[140]
	SimPheny				
	C. acetobutylicum model				
	C. thermocellum model				
	Manual curation				
M. thermoacetica	Model SEED	705	698	558	[141]
	C. ljungdahlii model				
	C. acetobutylicum model				
	C. cellulolyticum model				
	C. thermocellum model				
	Manual curation				
C. autoethanogenum	Model SEED	1,002	1,075	805	[142]
	KBase				
	C. ljungdahlii model				
	Manual curation				

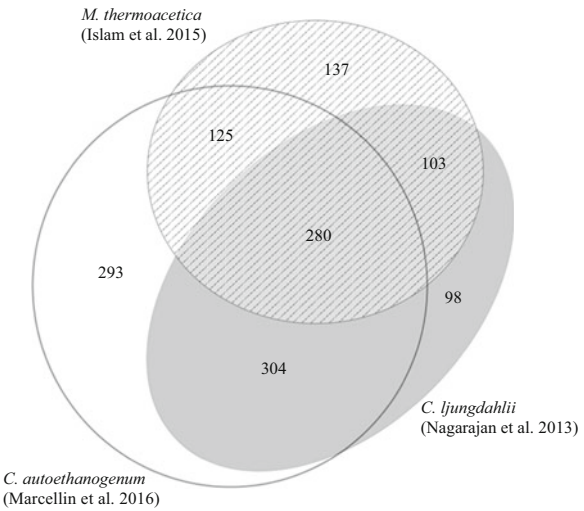


Fig. 2 Comparison of the metabolic reactions inside the three published acetogenic genome-scale models, excluding metabolite drains. *Overlapping regions* indicate shared reactions. All three models share a core set of 280 reactions. Diagram generated using eulerAPE [149]

growth. The model was validated by comparing heterotrophic growth and acetate secretion rates with experimental observations. Recently, Chen et al. [148] developed a bubble column fermentation reactor model using this *C. ljungdahlii* genome-scale model. They ran multiple instances of the genome-scale model to represent the localized behavior of cells in the bioreactor, across time. Although this approach was not validated with experimental data, spatiotemporal metabolic modeling promises to be a powerful way to link predictive metabolic models into the full context of industrial fermentation modeling.

Islam et al. [141] published iAI558, a genome-scale model of *M. thermoacetica*. The authors compared predicted growth rates with experimental data and used the model to analyze the feasibility of different energy conservation mechanisms.

Marcellin et al. [142] developed a genome-scale model of *C. autoethanogenum*. They explored growth on autotrophic and heterotrophic carbon sources and the patterns of ATP generation and redox balancing. Using the model, combined with transcriptomic and metabolomic experiments, they investigated the roles of two distinct glyceraldehyde 3-phosphate dehydrogenase genes in *C. autoethanogenum* energy metabolism.

To date, these published acetogenic genome-scale models have undergone limited validation, often using the same experimental data used to calibrate the model. Fermentation data from genetic and environmental perturbation-based studies allow researchers to validate and improve these models further.

8 Fermentation, Reactor Design, and Scale-Up

Because gas fermentation is different to traditional sugar fermentations, the technology has undergone significant development. Gas fermentation is typically operated as a fed-batch process with substrate gas continually supplied or as pure continuous chemostat process. Researchers have extensively studied parameters including reactor design, pH, temperature, and media formulation [7, 150, 151].

Gas fermentation bioreactors are designed to transfer gaseous substrates efficiently into the microbial cell. The gas-to-liquid mass transfer of substrate into the culture medium is the rate-limiting step during gas fermentation because of the low solubilities of CO and H_2 in water. Maximizing this mass transfer rate at the same time as minimizing operational cost is important; consequently, the volumetric mass transfer coefficient per unit power input ($k_L a P_g^{-1}$) is a measure of the performance of a gas fermentation bioreactor.

Continuous Stirred Tank Reactor (CSTR) is the most commonly used design for gas fermentation research. Mechanical agitation with a rotating impeller facilitates mixing and creates smaller bubbles of gaseous substrate. The decreased volume and increased overall surface area of these bubbles increases gas–liquid mass transfer rates. This design achieves high mass transfer rates. However, the power input required makes it challenging for commercial scale gas fermentation [152]. Bubble column reactors achieve efficient mass transfer by mixing the gas through gas spargers. For example, researchers achieved an ethanol concentration of 6 g/L by culturing *C. carboxidivorans* P7 in a 4-L bubble column reactor [153]. This design requires lower capital and operation costs, making it more appropriate for commercial scale reactor designs. Other proposed reactor designs include immobilized cell column reactors, where cells are affixed to insoluble materials and packed within the cell, and trickle-bed reactors, which involve trickling liquid culture through packing media that contains suspended cells. Gas substrate is delivered co-currently or counter-currently to the liquid flow. Finally, hollow fiber membrane bioreactors consist of fibers through which substrate gases are introduced. Cells are attached to the outer surface of the membrane, and the entire fiber is immersed in growth media. Munasinghe and Khanal compared the mass transfer coefficients of more than eight different reactor designs. They found that an air-lift reactor combined with a 20-μm bulb diffuser had the highest mass transfer coefficient ($k_L a$) [150]. Orgill et al. [154] compared the mass transfer coefficients of variants of trickle bed, hollow fiber membrane and stirred tank reactors and found that hollow fiber membrane reactors offered the highest volumetric mass transfer coefficients. Pressure is another parameter that has successfully been employed to improve mass transfer and production.

The cost-efficient recovery of fermentation products is an important consideration in gas fermentation. Distillation is traditionally used, although researchers have developed techniques which require lower energy input including pervaporation, adsorption, gas stripping, and liquid–liquid extraction [155].

Parameters including pH, temperature, media formulation, gas composition, and gas pressure have a significant influence on gas fermentation [156]. Acetogens have a limited pH range which supports optimum growth, and controlling this extracellular pH enables the gas fermentation product profile to be defined. Generally, a lower pH favors solventogenesis, allowing increased yields of highly reduced products such as ethanol [50, 157]. For example, Abubackar et al. [158] found that a pH of 4.75 is optimal for high ethanol yields in *C. autoethanogenum*. Temperature is important because of its effect on gas solubility and microbial activity. Most acetogens operate best at temperatures between 30 and 40 °C, although some strains such as *M. thermoacetica* are thermophiles. Finally, many media optimization studies have been carried out to identify the best formulations of vitamins, minerals, trace metal elements, and reducing agents. For example, reducing B-vitamin concentrations and eliminating yeast extract increased final ethanol yield in *C. ljungdahlii* [50] Trace metal concentrations are also needed because of their importance to many Wood–Ljungdahl pathway enzymes which require co-factors including nickel, selenium, and tungsten [72].

As a result of process optimization, high productivities of 360 g/L/day ethanol [159], 150 g/L/day acetic acid [160], and 330 g/L/day 2,3-BDO [161] have been demonstrated in CSTRs at bench scale, providing the basis for processes developed at scale.

9 Commercialization of Gas Fermentation

The majority of gas fermentation research has occurred at bench scale. However, three companies have operated demonstration plants and are seeking to commercialize gas fermentation for the production of sustainable fuels and chemicals.

The first gas fermentation patents were filed by J. L Gaddy of the University of Arkansas [162–164]. These patents and related technology using proprietary isolates of *C. ljungdahlii* were acquired in 2008 by INEOS, who created the subsidiary INEOS Bio (www.ineos.com/businesses/ineos-bio/). In 2012, they completed construction of the Indian River BioEnergy Center, a semi-commercial plant in Florida. This plant has a projected annual output of eight million gallons of ethanol, and 6 MW (gross) of power from unused syngas and recovered heat. In 2013, INEOS Bio reported that the plant was producing ethanol using gasified municipal solid waste as a feedstock (INEOS [165]). However, the plant was taken offline and has recently been upgraded to overcome difficulties associated with gas impurities such as hydrogen cyanide [166].

Coskata (http://www.coskata.com) was founded in 2006 using technology licensed from Oklahoma State University and the University of Oklahoma [167, 168]. Coskata has reported the use of acetogens *C. ragsdalei*, *C. carboxidivorans*, and a related proprietary bacterium "*C. coskatii*" [52]. Coskata operated a demonstration facility for 2 years from 2009, using syngas produced from wood biomass and municipal solid waste to make ethanol [167, 168]. However,

at the time of writing, they have not announced the implementation of a commercial project.

LanzaTech (http://www.lanzatech.com) was founded in Auckland, New Zealand in 2005 and is now based in Chicago, Illinois. In 2012, it operated a 100,000 gal/year pre-commercial plant at a Baosteel steel mill near Shanghai, China. This plant produced ethanol from steel-mill waste gases using a proprietary strain of *C. autoethanogenum* [169]. In 2013, LanzaTech operated a second 100,000 gal/year pre-commercial plant at a Shougang steel mill near Beijing, China. This facility was certified by the Roundtable on Sustainable Biomaterials (RSB), a global group which certifies biomaterials based on environmental, social, and economic principles [170]. In April 2015, China Steel Corporation approved investment for a full 50,000 MT/year of ethanol LanzaTech commercial project [171]. In July 2015, LanzaTech reported a partnership with ArcelorMittal and Primetals Technology to construct a flagship plant in Ghent, Belgium, with a total capacity 47,000 MT/year of ethanol [172]. The company has reported that it is working to diversify the product portfolio. These products include 2,3-butanediol, jet fuel, nylon and rubber precursor butadiene, and other specialty plastics [173–175].

10 Legislative Challenges and Life-Cycle Analysis

Today's biofuel legislation is largely prescriptive, often citing specific feedstock lists, qualifying the resulting fuel as a biofuel. The content of these lists were mostly written prior to the development of gas fermentation technology. As a result, legislation in some countries is either ambiguous or specifically prevents fuels made through carbon recycling technologies from being classified as a biofuel [176]. This lack of clarity makes investment in such novel technologies a problem as biofuels legislation creates the market for fuels in each jurisdiction and a secure market is needed for investor confidence. Policy makers, however, are beginning to understand the importance of creating technology and feedstock-neutral legislation. A model very similar to California's Low Carbon Fuel Standard (LCFS) or Europe's Fuel Quality Directive (FQD) focuses on the sustainability of a fuel, as measured by a reduction in greenhouse gas emissions compared to petroleum gasoline [177, 178]. This approach is more robust as it supports advanced biofuel technologies which not only give the most impactful sustainability results but also take into account the range of feedstocks or processes that can be used.

Life-cycle analysis (LCA) indicates that fuels and chemicals produced through gas fermentation are sustainable. LCA is a technique to evaluate systematically the environmental impact associated with all stages of a product's life, from creation to disposal. Key areas of analysis for fuels and chemicals include total greenhouse gas emissions and local air pollution. LCA has been carried out for gas fermentation technology to determine the sustainability of its products. For example, Ou et al. [179] carried out an LCA of the LanzaTech steel mill off-gas to ethanol

process in China. They calculated that the use of this ethanol reduces greenhouse gas emissions by approximately 50% compared with conventional petroleum gasoline.

In 2014, the UK-based environmental consultancy E4tech Ltd studied the greenhouse gas (GHG) emissions associated with the production of ethanol via the LanzaTech process in line with the European Union's Renewable Energy Directive (RED) [180]. The process considered gas from the basic oxygen furnace (BOF) of a steel mill. The study included all stages of production, i.e., the production of the feedstock gas, its fermentation, the subsequent processes leading to the final product (ethanol), and its transport to a filling station. The calculated total emissions associated with LanzaTech ethanol, following the RED requirements, represent a 76.6% saving over current (2014) EU baseline fossil fuel. This is even higher than Brazilian sugarcane ethanol which offers close to 70% savings over conventional fossil gasoline and significantly higher than savings from US corn ethanol (30%) and SE Asia cassava (25–50% depending on the energy used for production) [181, 182]. Current US mandates require >20% GHG savings from conventional fuels, >50% GHG savings from advanced biofuels, and >60% GHG savings from cellulosic biofuels, wheres EU mandates require 50% GHG savings (60% from 2018).

The study also includes a comparison of GHG emissions savings between alternative uses of BOF gas. In conclusion, ethanol production saves 30% more GHG emissions than electricity generation. Furthermore, with decreasing electrical grid carbon intensity, the production of LanzaTech ethanol becomes increasingly attractive compared to the generation of electricity from a GHG perspective.

Ethanol produced by the LanzaTech gas fermentation process at the LanzaTech Shougang Demonstration plant in China has been recognized as a sustainable bioethanol by the Roundtable on Sustainable Biomaterials (RSB) [170]. These findings confirm that gas fermentation can contribute to the displacement of fossil-based fuels and chemicals. A technology and feedstock neutral approach to legislation continues to support these technologies.

Acknowledgements We thank the following investors in LanzaTech's technology: Sir Stephen Tindall, Khosla Ventures, Qiming Venture Partners, Softbank China, the Malaysian Life Sciences Capital Fund, Mitsui, Primetals, CICC Growth Capital Fund I, L.P., and the New Zealand Superannuation Fund.

References

1. Stern N (2007) The economics of climate change: the Stern review. Cambridge University Press, New York
2. Naik SN, Goud VV, Rout PK, Dalai AK (2010) Production of first and second generation biofuels: a comprehensive review. Renew Sustain Energy Rev 14:578–597. doi:10.1016/j.rser.2009.10.003

3. Sheldon RA (2014) Green and sustainable manufacture of chemicals from biomass: state of the art. Green Chem 16:950–963. doi:10.1039/C3GC41935E
4. Balat M, Balat H (2009) Recent trends in global production and utilization of bio-ethanol fuel. Appl Energy 86:2273–2282. doi:10.1016/j.apenergy.2009.03.015
5. Yim H, Haselbeck R, Niu W et al (2011) Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. Nat Chem Biol 7:445–452. doi:10.1038/nchembio.580
6. Nakamura CE, Whited GM (2003) Metabolic engineering for the microbial production of 1,3-propanediol. Curr Opin Biotechnol 14:454–459. doi:10.1016/j.copbio.2003.08.005
7. Daniell J, Köpke M, Simpson S (2012) Commercial biomass syngas fermentation. Energies 5:5372–5417. doi:10.3390/en5125372
8. Friedlingstein P, Andrew RM, Rogelj J et al (2014) Persistent growth of CO_2 emissions and implications for reaching climate targets. Nat Geosci 7:709–715. doi:10.1038/ngeo2248
9. Kircher M (2014) The emerging bioeconomy: industrial drivers, global impact, and international strategies. Ind Biotechnol 10:11–18. doi:10.1089/ind.2014.1500
10. Zilberman D, Hochman G, Rajagopal D et al (2013) The impact of biofuels on commodity food prices: assessment of findings. Am J Agric Econ 95:275–281. doi:10.1093/ajae/aas037
11. Hussain S, Miller D (2014) The Economics of Ecosystems and Biodiversity (TEEB) for agriculture & food – concept note. http://www.teebweb.org/publication/the-economics-of-ecosystems-and-biodiversity-teeb-for-agriculture-food-concept-note/. Accessed 25 Aug 2015
12. Fargione J, Hill J, Tilman D et al (2008) Land clearing and the biofuel carbon debt. Science 319:1235–1238. doi:10.1126/science.1152747
13. 110th United States Congress (2007) Energy Independence and Security Act of 2007. US Public Law 110-140. https://www.gpo.gov/fdsys/pkg/PLAW-110publ140/pdf/PLAW-110publ140.pdf
14. Denvir B, Taylor R, Bauen A et al (2015) Novel low carbon transport fuels and the RTFO: sustainability implications. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/417650/Novel_Low_Carbon_Transport_Fuels_Scoping_paper_vFINAL5.pdf. Accessed 25 Aug 2015
15. Dürre P, Eikmanns BJ (2015) C1-carbon sources for chemical and fuel production by microbial gas fermentation. Curr Opin Biotechnol 35:63–72. doi:10.1016/j.copbio.2015.03.008
16. Kirkels AF, Verbong GPJ (2011) Biomass gasification: still promising? A 30-year global overview. Renew Sustain Energy Rev 15:471–481. doi:10.1016/j.rser.2010.09.046
17. McKendry P (2002) Energy production from biomass (part 3): gasification technologies. Bioresour Technol 83:55–63
18. Temme K, Zhao D, Voigt CA (2012) Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. Proc Natl Acad Sci 109:7085–7090. doi:10.1073/pnas.1120788109
19. Gardner TS (2013) Synthetic biology: from hype to impact. Trends Biotechnol 31:123–125. doi:10.1016/j.tibtech.2013.01.018
20. Leuchtenberger W, Huthmacher K, Drauz K (2005) Biotechnological production of amino acids and derivatives: current status and prospects. Appl Microbiol Biotechnol 69:1–8. doi:10.1007/s00253-005-0155-y
21. Köpke M, Dürre P (2010) Biochemical production of biobutanol. In: Luque R, Campelo J, Clark JH (eds) Handbook of biofuels production: processes and technologies. Woodhead Publishing, Cambridge, pp 221–257
22. Los M (2012) Minimization and prevention of phage infections in bioprocesses. Methods Mol Biol 834:305–315. doi:10.1007/978-1-61779-483-4_19
23. Skinner KA, Leathers TD (2004) Bacterial contaminants of fuel ethanol production. J Ind Microbiol Biotechnol 31:401–408. doi:10.1007/s10295-004-0159-0
24. Westfall PJ, Gardner TS (2011) Industrial fermentation of renewable diesel fuels. Curr Opin Biotechnol 22:344–350. doi:10.1016/j.copbio.2011.04.023
25. Foster JW (2004) *Escherichia coli* acid resistance: tales of an amateur acidophile. Nat Rev Microbiol 2:898–907. doi:10.1038/nrmicro1021
26. Bogorad IW, Chen C-T, Theisen MK et al (2014) Building carbon–carbon bonds using a biocatalytic methanol condensation cycle. Proc Natl Acad Sci 111:15928–15933. doi:10.1073/pnas.1413470111

27. Bogorad IW, Lin T-S, Liao JC (2013) Synthetic non-oxidative glycolysis enables complete carbon conservation. Nature 502:693–697. doi:10.1038/nature12575
28. Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R (2008) Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Appl Environ Microbiol 74:1124–1135. doi:10.1128/AEM.02192-07
29. Murrell JC, Gilbert B, McDonald IR (2000) Molecular biology and regulation of methane monooxygenase. Arch Microbiol 173:325–332
30. Müller JEN, Meyer F, Litsanov B et al (2015) Engineering *Escherichia coli* for methanol conversion. Metab Eng 28:190–201. doi:10.1016/j.ymben.2014.12.008
31. Burk M, Schilling CH, Burgard A, Trawick JD (2009) Methods and organisms for utilizing synthesis gas and other gaseous carbon sources and methanol. International Patent WO/2009/094485
32. Fast AG, Papoutsakis ET (2012) Stoichiometric and energetic analyses of non-photosynthetic CO_2-fixation pathways to support synthetic biology strategies for production of fuels and chemicals. Curr Opin Chem Eng 1–16. doi:10.1016/j.coche.2012.07.005
33. Russell MJ, Martin W (2004) The rocky roots of the acetyl-CoA pathway. Trends Biochem Sci 29:358–363. doi:10.1016/j.tibs.2004.05.007
34. Mohammadi M, Najafpour GD, Younesi H et al (2011) Bioconversion of synthesis gas to second generation biofuels: a review. Renew Sustain Energy Rev 15:4255–4273. doi:10.1016/j.rser.2011.07.124
35. Munasinghe PC, Khanal SK (2010) Biomass-derived syngas fermentation into biofuels: opportunities and challenges. Bioresour Technol 101:5013–5022. doi:10.1016/j.biortech.2009.12.098
36. Schiel-Bengelsdorf B, Dürre P (2012) Pathway engineering and synthetic biology using acetogens. FEBS Lett 586:2191–2198. doi:10.1016/j.febslet.2012.04.043
37. Tirado-Acevedo O, Chinn MS, Grunden AM (2010) Production of biofuels from synthesis gas using microbial catalysts. Adv Appl Microbiol 70:57–92. doi:10.1016/S0065-2164(10)70002-2
38. Köpke M, Mihalcea C, Liew F et al (2011) 2,3-Butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. Appl Environ Microbiol 77:5467–5475. doi:10.1128/AEM.00355-11
39. Schiel B, Dürre P (2010) *Clostridium*. In: Flickinger MC (ed) Encyclopedia of industrial biotechnology: bioprocess, bioseparation and cell technology. Wiley, Hoboken, pp 1–15
40. Tracy BP, Jones SW, Fast AG et al (2012) Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr Opin Biotechnol 23:364–381. doi:10.1016/j.copbio.2011.10.008
41. Olson DG, McBride JE, Shaw AJ, Lynd LR (2012) Recent progress in consolidated bioprocessing. Curr Opin Biotechnol 23:396–405. doi:10.1016/j.copbio.2011.11.026
42. Drake HL, Küsel K (2005) Acetogenic clostridia. In: Dürre P (ed) Handbook of clostridia. CRC, Boca Raton, pp 721–748
43. Lovley DR (2011) Powering microbes with electricity: direct electron transfer from electrodes to microbes. Environ Microbiol Rep 3:27–35. doi:10.1111/j.1758-2229.2010.00211.x
44. Lovley DR, Nevin KP (2013) Electrobiocommodities: powering microbial production of fuels and commodity chemicals from carbon dioxide with electricity. Curr Opin Biotechnol 1–6. doi:10.1016/j.copbio.2013.02.012
45. Nevin KP, Hensley SA, Franks AE et al (2011) Electrosynthesis of organic compounds from carbon dioxide is catalyzed by a diversity of acetogenic microorganisms. Appl Environ Microbiol 77:2882–2886. doi:10.1128/AEM.02642-10
46. Nevin KP, Woodard TL, Franks AE et al (2010) Microbial electrosynthesis: feeding microbes electricity to convert carbon dioxide and water to multicarbon extracellular organic compounds. MBio 1:e00103–e00110. doi:10.1128/mBio.00103-10

47. Abrini J, Naveau H, Nyns EJ (1994) *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch Microbiol 161: 345–351. doi:10.1007/BF00303591
48. Brown SD, Nagaraju S, Utturkar S et al (2014) Comparison of single-molecule sequencing and hybrid approaches for finishing the genome of *Clostridium autoethanogenum* and analysis of CRISPR systems in industrial relevant Clostridia. Biotechnol Biofuels 7:40. doi:10.1186/1754-6834-7-40
49. Utturkar SM, Klingeman DM, Bruno-Barcena JM et al (2015) Sequence data for *Clostridium autoethanogenum* using three generations of sequencing technologies. Sci Data 2:150014. doi:10.1038/sdata.2015.14
50. Phillips JR, Klasson KT, Claussen EC et al (1993) Biological production of ethanol from coal synthesis gas. Appl Biochem Biotechnol 39:559–571. doi:10.1007/BF02919018
51. Tanner RS, Miller LM, Yang D (1993) *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial rRNA homology group I. Int J Syst Bacteriol 43:232
52. Zahn JA, Saxena J (2011) Novel ethanologenic species *Clostridium coskatii*. US Patent 20110229947
53. Huhnke R, Lewis R, Tanner R (2008) Isolation and characterization of novel clostridial species. US Patent 7704723
54. Li N, Yang J, Chai C et al (2015) Complete genome sequence of *Clostridium carboxidivorans* P7(T), a syngas-fermenting bacterium capable of producing long-chain alcohols. J Biotechnol 211:44–45. doi:10.1016/j.jbiotec.2015.06.430
55. Liou JS-C, Balkwill DL, Drake GR, Tanner RS (2005) *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of the acetogen *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. nov. Int J Syst Evol Microbiol 55:2085–2091. doi:10.1099/ijs.0.63482-0
56. Phillips JR, Atiyeh HK, Tanner RS et al (2015) Butanol and hexanol production in *Clostridium carboxidivorans* syngas fermentation: medium development and culture techniques. Bioresour Technol 190:114–121. doi:10.1016/j.biortech.2015.04.043
57. Poehlein A, Bengelsdorf FR, Schiel-Bengelsdorf B et al (2015) Complete genome sequence of Rnf- and cytochrome-containing autotrophic acetogen *Clostridium aceticum* DSM 1496. Genome Announc 3:e00786–15. doi:10.1128/genomeA.00786-15
58. Sim JH, Kamaruddin AH (2008) Optimization of acetic acid production from synthesis gas by chemolithotrophic bacterium – *Clostridium aceticum* using statistical approach. Bioresour Technol 99:2724–2735. doi:10.1016/j.biortech.2007.07.004
59. Sim JH, Kamaruddin AH, Long WS (2008) Biocatalytic conversion of CO to acetic acid by *Clostridium aceticum*—medium optimization using response surface methodology (RSM). Biochem Eng J 40:337–347. doi:10.1016/j.bej.2008.01.006
60. Sim JH, Kamaruddin AH, Long WS, Najafpour G (2007) *Clostridium aceticum*—a potential organism in catalyzing carbon monoxide to acetic acid: application of response surface methodology. Enzyme Microb Technol 40:1234–1243. doi:10.1016/j.enzmictec.2006.09.017
61. Song Y, Hwang S, Cho B-K (2015) Draft genome sequence of *Clostridium aceticum* DSM 1496, a potential butanol producer through syngas fermentation. Genome Announc 3: e00258–15. doi:10.1128/genomeA.00258-15
62. Wieringa KT (1939) The formation of acetic acid from carbon dioxide and hydrogen by anaerobic spore-forming bacteria. Antonie Van Leeuwenhoek 6:251–262. doi:10.1007/BF02146190
63. Drake HL, Daniel SL (2004) Physiology of the thermophilic acetogen *Moorella thermoacetica*. Res Microbiol 155:869–883. doi:10.1016/j.resmic.2004.10.002
64. Fontaine FE, Peterson WH, Johnson MJ, George J (1942) A new type of glucose fermentation by Clostridium thermoaceticum. J Bacteriol 43:701–715
65. Pierce E, Xie G, Barabote RD et al (2008) The complete genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). Environ Microbiol 10: 2550–2573. doi:10.1111/j.1462-2920.2008.01679.x

66. Balch WE, Schoberth S, Tanner RS, Wolfe RS (1977) *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. Int J Syst Bacteriol 27: 355–361. doi:10.1099/00207713-27-4-355
67. Poehlein A, Schmidt S, Kaster A-K et al (2012) An ancient pathway combining carbon dioxide fixation with the generation and utilization of a sodium ion gradient for ATP synthesis. PLoS One 7, e33439. doi:10.1371/journal.pone.0033439
68. Worden RM, Grethlein AJ, Jain MK, Datta R (1991) Production of butanol and ethanol from synthesis gas via fermentation. Fuel 70:615–619. doi:10.1016/0016-2361(91)90175-A
69. Zeikus JG, Lynd LH, Thompson TE et al (1980) Isolation and characterization of a new, methylotrophic, acidogenic anaerobe, the Marburg strain. Curr Microbiol 3:381–386. doi:10.1007/BF02601907
70. Wood HG (1991) Life with CO or CO_2 and H_2 as a source of carbon and energy. FASEB J 5: 156–163
71. Müller V (2003) Energy conservation in acetogenic bacteria. Appl Environ Microbiol 69: 6345–6353. doi:10.1128/AEM.69.11.6345
72. Ragsdale SW (2008) Enzymology of the Wood-Ljungdahl pathway of acetogenesis. Ann N Y Acad Sci 1125:129–136. doi:10.1196/annals.1419.015
73. Drake HL, Küsel K, Matthies C et al (2006) Acetogenic prokaryotes. In: Dworkin M, Falkow S, Rosenberg E et al (eds) The prokaryotes, 3rd edn. Springer, New York, pp 354–420
74. Ragsdale SW, Pierce E (2008) Acetogenesis and the Wood-Ljungdahl pathway of CO_2 fixation. Biochim Biophys Acta 1784:1873–1898. doi:10.1016/j.bbapap.2008.08.012
75. Buckel W, Thauer RK (2013) Energy conservation via electron bifurcating ferredoxin reduction and proton/Na+ translocating ferredoxin oxidation. Biochim Biophys Acta 1827: 94–113
76. Schuchmann K, Müller V (2014) Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. Nat Rev Microbiol 12:809–821. doi:10.1038/nrmicro3365
77. Ljungdahl LG (1994) The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis. In: Acetogenesis. Springer US, Boston, pp 63–87
78. Müller V, Imkamp F, Biegel E et al (2008) Discovery of a ferredoxin:NAD+−oxidoreductase (Rnf) in *Acetobacterium woodii*: a novel potential coupling site in acetogens. Ann N Y Acad Sci 1125:137–146. doi:10.1196/annals.1419.011
79. Schmidt S, Biegel E, Müller V (2009) The ins and outs of Na(+) bioenergetics in *Acetobacterium woodii*. Biochim Biophys Acta 1787:691–696. doi:10.1016/j.bbabio.2008.12.015
80. Heise R, Müller V, Gottschalk G (1989) Sodium dependence of acetate formation by the acetogenic bacterium *Acetobacterium woodii*. J Bacteriol 171:5473–5478
81. Mock J, Zheng Y, Mueller AP et al (2015) Energy conservation associated with ethanol formation from H_2 and CO_2 in *Clostridium autoethanogenum* involving electron bifurcation. J Bacteriol. doi:10.1128/JB.00399-15
82. Wang S, Huang H, Kahnt J et al (2013) NADP-specific electron-bifurcating [FeFe]-hydrogenase in a functional complex with formate dehydrogenase in *Clostridium autoethanogenum* grown on CO. J Bacteriol 195:4373–4386. doi:10.1128/JB.00678-13
83. Tremblay P-L, Zhang T, Dar SA et al (2012) The Rnf Complex of *Clostridium ljungdahlii* is a proton-translocating ferredoxin:NAD^+ oxidoreductase essential for autotrophic growth. MBio 4:e00406–12–e00406–12. doi:10.1128/mBio.00406-12
84. Bertsch J, Öppinger C, Hess V et al (2015) Heterotrimeric NADH-oxidizing methylene-tetrahydrofolate reductase from the acetogenic bacterium *Acetobacterium woodii*. J Bacteriol 197:1681–1689. doi:10.1128/JB.00048-15
85. Bertsch J, Müller V (2015) CO metabolism in the acetogen *Acetobacterium woodii*. Appl Environ Microbiol AEM.01772–15. doi:10.1128/AEM.01772-15
86. Lee SY, Park JH, Jang SH et al (2008) Fermentative butanol production by Clostridia. Biotechnol Bioeng 101:209–228. doi:10.1002/bit.22003

87. Liew FM, Köpke M, Simpson SD (2013) Gas fermentation for commercial biofuels production. In: Fang Z (ed) Biofuel production-recent developments and prospects. InTech, Rijeka, pp 125–174
88. Lütke-Eversloh T, Bahl H (2011) Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. Curr Opin Biotechnol 22:634–647. doi:10.1016/j.copbio.2011.01.011
89. Papoutsakis ET (2008) Engineering solventogenic clostridia. Curr Opin Biotechnol 19: 420–429. doi:10.1016/j.copbio.2008.08.003
90. Köpke M, Held C, Hujer S et al (2010) *Clostridium ljungdahlii* represents a microbial production platform based on syngas. Proc Natl Acad Sci U S A 107:13087–13092. doi:10.1073/pnas.1004716107
91. Koepke M, Liew FM (2011) Recombinant microorganism and methods of production thereof. US Patent 20110256600
92. Koepke M, Simpson S, Liew FM, Chen W (2012) Fermentation process for producing isopropanol using a recombinant microorganism. US Patent 20120252083
93. Mueller A, Koepke M, Nagaraju S (2013) Recombinant microorganisms and uses therefor. US Patent 20130330809
94. Tirado-Acevedo O (2010) Production of bioethanol from synthesis gas using *Clostridium ljungdahlii*. Ph.D. thesis, North Carolina State University.
95. Hermann M, Fayolle F, Marchal R et al (1985) Isolation and characterization of butanol-resistant mutants of *Clostridium acetobutylicum*. Appl Environ Microbiol 50:1238–1243
96. Zhang Y, Grosse-Honebrink A, Minton NP (2015) A universal mariner transposon system for forward genetic studies in the genus *Clostridium*. PLoS One 10, e0122411. doi:10.1371/journal.pone.0122411
97. Blouzard J-C, Valette O, Tardif C, de Philip P (2010) Random mutagenesis of Clostridium cellulolyticum by using a Tn1545 derivative. Appl Environ Microbiol 76:4546–4549. doi:10.1128/AEM.02417-09
98. Tracy BP, Jones SW, Papoutsakis ET (2011) Inactivation of σE and σG in *Clostridium acetobutylicum* illuminates their roles in clostridial-cell-form biogenesis, granulose synthesis, solventogenesis, and spore morphogenesis. J Bacteriol 193:1414–1426. doi:10.1128/JB.01380-10
99. Hillmann F, Fischer R-J, Saint-Prix F et al (2008) PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*. Mol Microbiol 68:848–860. doi:10.1111/j.1365-2958.2008.06192.x
100. Cooksley CM, Zhang Y, Wang H et al (2012) Targeted mutagenesis of the *Clostridium acetobutylicum* acetone-butanol-ethanol fermentation pathway. Metab Eng 14:630–641. doi:10.1016/j.ymben.2012.09.001
101. Desai RP, Papoutsakis ET (1999) Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum*. Appl Environ Microbiol 65:936–945
102. Green EM, Boynton ZL, Harris LM et al (1996) Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology 142: 2079–2086. doi:10.1099/13500872-142-8-2079
103. Papoutsakis ET, Al-Hinai MA, Jones SW et al (2012) Recombinant clostridia that fix CO_2 and CO and uses thereof. US Patent 20120064587
104. Argyros DA, Tripathi SA, Barrett TF et al (2011) High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. Appl Environ Microbiol 77: 8288–8294. doi:10.1128/AEM.00646-11
105. Tripathi SA, Olson DG, Argyros DA et al (2010) Development of pyrF-based genetic system for targeted gene deletion in *Clostridium thermocellum* and creation of a pta mutant. Appl Environ Microbiol 76:6591–6599. doi:10.1128/AEM.01484-10
106. Li Y, Tschaplinski TJ, Engle NL et al (2012) Combined inactivation of the *Clostridium cellulolyticum* lactate and malate dehydrogenase genes substantially increases ethanol yield

from cellulose and switchgrass fermentations. Biotechnol Biofuels 5:2. doi:10.1186/1754-6834-5-2
107. Girbal L, Mortier-barrière I, Rouanet C et al (2003) Development of a sensitive gene expression reporter system and an inducible promoter-repressor system for *Clostridium acetobutylicum*. Appl Environ Microbiol 69:4985–4988. doi:10.1128/AEM.69.8.4985
108. Heap JT, Pennington OJ, Cartman ST et al (2007) The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods 70:452–464. doi:10.1016/j.mimet.2007.05.021
109. Jennert KC, Tardif C, Young DI, Young M (2000) Gene transfer to *Clostridium cellulolyticum* ATCC 35319. Microbiology 146(Pt 12):3071–3080
110. Mermelstein L, Welker N (1992) Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. Bio/Technology 10:190–195
111. Mermelstein LD, Papoutsakis ET (1993) In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 59:1077–1081
112. Williams DR, Young DI, Young M (1990) Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*. J Gen Microbiol 136:819–826. doi:10.1099/00221287-136-5-819
113. Heap JT, Pennington OJ, Cartman ST, Minton NP (2009) A modular system for *Clostridium* shuttle plasmids. J Microbiol Methods 78:79–85. doi:10.1016/j.mimet.2009.05.004
114. Al-Hinai MA, Fast AG, Papoutsakis ET (2012) Novel system for efficient isolation of Clostridium double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. Appl Environ Microbiol 78:8112–8121. doi:10.1128/AEM.02214-12
115. Cartman ST, Kelly ML, Heeg D et al (2012) Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the tcdC genotype and toxin production. Appl Environ Microbiol 78:4683–4690. doi:10.1128/AEM.00249-12
116. Heap JT, Ehsaan M, Cooksley CM et al (2012) Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. Nucleic Acids Res 1–10. doi:10.1093/nar/gkr1321
117. Soucaille P, Figge R, Croux C (2008) Process for chromosomal integration and DNA sequence replacement in clostridia. WO/2008/040387
118. Nariya H, Miyata S, Suzuki M et al (2011) Development and application of a method for counterselectable in-frame deletion in *Clostridium perfringens*. Appl Environ Microbiol 77:1375–1382. doi:10.1128/AEM.01572-10
119. Zhang N, Shao L, Jiang Y et al (2015) I-SceI-mediated scarless gene modification via allelic exchange in *Clostridium*. J Microbiol Methods 108:49–60. doi:10.1016/j.mimet.2014.11.004
120. Wang Y, Zhang Z-T, Seo S-O et al (2015) Markerless chromosomal gene deletion in *Clostridium beijerinckii* using CRISPR/Cas9 system. J Biotechnol 1–5. doi:10.1016/j.jbiotec.2015.02.005
121. Xu T, Li Y, Shi Z et al (2015) Efficient genome editing in *Clostridium cellulolyticum* via CRISPR-Cas9 nickase. Appl Environ Microbiol AEM.00873–15. doi:10.1128/AEM.00873-15
122. Heap JT, Kuehne SA, Ehsaan M et al (2010) The ClosTron: mutagenesis in *Clostridium* refined and streamlined. J Microbiol Methods 80:49–55. doi:10.1016/j.mimet.2009.10.018
123. Shao L, Hu S, Yang Y et al (2007) Targeted gene disruption by use of a group II intron (targetron) vector in *Clostridium acetobutylicum*. Cell Res 17:963–965. doi:10.1038/cr.2007.91
124. Zhang J, Liu Y-J, Cui G-Z, Cui Q (2015) A novel arabinose-inducible genetic operation system developed for *Clostridium cellulolyticum*. Biotechnol Biofuels 8:36. doi:10.1186/s13068-015-0214-2

125. Feustel L, Nakotte S, Durre P (2004) Characterization and development of two reporter gene systems for *Clostridium acetobutylicum*. Appl Environ Microbiol 70:798–803. doi:10.1128/AEM.70.2.798-803.2004
126. Tummala SB, Welker NE, Papoutsakis ET (1999) Development and characterization of a gene expression reporter system for *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 65:3793–3799
127. Matsushita C, Matsushita O, Koyama M, Okabe A (1994) A *Clostridium perfringens* vector for the selection of promoters. Plasmid 31:317–319
128. Steffen C, Matzura H (1989) Nucleotide sequence analysis and expression studies of a chloramphenicol-acetyltransferase-coding gene from *Clostridium perfringens*. Gene 75: 349–354
129. Dong H, Tao W, Zhang Y, Li Y (2012) Development of an anhydrotetracycline-inducible gene expression system for solvent-producing *Clostridium acetobutylicum*: a useful tool for strain engineering. Metab Eng 14:59–67. doi:10.1016/j.ymben.2011.10.004
130. Hartman AH, Liu H, Melville SB (2011) Construction and characterization of a lactose-inducible promoter system for controlled gene expression in *Clostridium perfringens*. Appl Environ Microbiol 77:471–478. doi:10.1128/AEM.01536-10
131. Leang C, Ueki T, Nevin KP, Lovley DR (2013) A genetic system for *Clostridium ljungdahlii*: a chassis for autotrophic production of biocommodities and a model homoacetogen. Appl Environ Microbiol 79:1102–1109. doi:10.1128/AEM.02891-12
132. Ueki T, Nevin KP, Woodard TL, Lovley DR (2014) Converting carbon dioxide to butyrate with an engineered strain of *Clostridium ljungdahlii*. MBio 5:e01636–14–e01636–14. doi:10.1128/mBio.01636-14
133. Simpson SD, Koepke M, Liew F, Chen WY (2013) Recombinant microorganisms and uses therefor. US Patent 20130224838
134. Simpson SD, Koepke M, Liew F (2011) Recombinant microorganisms and methods of use thereof. Recombinant microorganisms and methods of use thereof. US Patent 20110256600
135. Köpke M, Gerth ML, Maddock DJ et al (2014) Reconstruction of an acetogenic 2,3-butanediol pathway involving a novel NADPH-dependent primary-secondary alcohol dehydrogenase. Appl Environ Microbiol AEM.00301–14. doi:10.1128/AEM.00301-14
136. Koepke M, Al-Sinawi B, Jensen RO, Mueller PM, Hill RE (2014) Microorganisms and methods for the production of ketones. US Patent 20150152445
137. Koepke M, Nagaraju S, Chen W (2013) Recombinant microorganisms and methods of use thereof. US Patent 20150072395
138. Banerjee A, Leang C, Ueki T et al (2014) Lactose-inducible system for metabolic engineering of *Clostridium ljungdahlii*. Appl Environ Microbiol 80:2410–2416. doi:10.1128/AEM.03666-13
139. O'Brien EJ, Monk JM, Palsson BO (2015) Using genome-scale models to predict biological capabilities. Cell 161:971–987. doi:10.1016/j.cell.2015.05.019
140. Nagarajan H, Sahin M, Nogales J et al (2013) Characterizing acetogenic metabolism using a genome-scale metabolic reconstruction of *Clostridium ljungdahlii*. Microb Cell Fact 12:118. doi:10.1186/1475-2859-12-118
141. Islam MA, Zengler K, Edwards EA et al (2015) Investigating *Moorella thermoacetica* metabolism with a genome-scale constraint-nased metabolic model. Integr Biol. doi:10.1039/C5IB00095E
142. Marcellin E, Behrendorff JB, Nagaraju S, DeTissera S, Segovia S, Palfreyman R, Daniell J, Licona-Cassani C, Quek L, Speight R, Hodson MP, Simpson SD, Mitchell WP, Köpke M, Nielsen LK (2016) Low carbon fuels and commodity chemicals from waste gases – systematic approach to understand energy metabolism in a model acetogen. Green Chem. doi:10.1039/C5GC02708J
143. Lee J, Yun H, Feist AM et al (2008) Genome-scale reconstruction and in silico analysis of the *Clostridium acetobutylicum* ATCC 824 metabolic network. Appl Microbiol Biotechnol 80: 849–862. doi:10.1007/s00253-008-1654-4

144. Milne CB, Eddy JA, Raju R et al (2011) Metabolic network reconstruction and genome-scale model of butanol-producing strain *Clostridium beijerinckii* NCIMB 8052. BMC Syst Biol 5: 130. doi:10.1186/1752-0509-5-130
145. Roberts SB, Gowen CM, Brooks JP, Fong SS (2010) Genome-scale metabolic analysis of *Clostridium thermocellum* for bioethanol production. BMC Syst Biol 4:31. doi:10.1186/1752-0509-4-31
146. Salimi F, Zhuang K, Mahadevan R (2010) Genome-scale metabolic modeling of a clostridial co-culture for consolidated bioprocessing. Biotechnol J 5:726–738. doi:10.1002/biot.201000159
147. Senger RS, Papoutsakis ET (2008) Genome-scale model for *Clostridium acetobutylicum*: part I. Metabolic network resolution and analysis. Biotechnol Bioeng 101:1036–1052. doi:10.1002/bit.22010
148. Chen J, Gomez J, Höffner K et al (2015) Metabolic modeling of synthesis gas fermentation in bubble column reactors. Biotechnol Biofuels 8:89. doi:10.1186/s13068-015-0272-5
149. Micallef L, Rodgers P (2014) euler APE: drawing area-proportional 3-Venn diagrams using ellipses. PLoS One. doi:10.1371/journal.pone.0101717
150. Munasinghe PC, Khanal SK (2010) Syngas fermentation to biofuel: evaluation of carbon monoxide mass transfer coefficient (kLa) in different reactor configurations. Biotechnol Prog 26:1616–1621. doi:10.1002/btpr.473
151. Yasin M, Jeong Y, Park S et al (2015) Microbial synthesis gas utilization and ways to resolve kinetic and mass-transfer limitations. Bioresour Technol 177:361–374. doi:10.1016/j.biortech.2014.11.022
152. Bredwell MD, Srivastava P, Worden RM (1999) Reactor design issues for synthesis-gas fermentations. Biotechnol Prog 15:834–844. doi:10.1021/bp990108m
153. Datar RP, Shenkman RM, Cateni BG et al (2004) Fermentation of biomass-generated producer gas to ethanol. Biotechnol Bioeng 86:587–594. doi:10.1002/bit.20071
154. Orgill JJ, Atiyeh HK, Devarapalli M et al (2013) A comparison of mass transfer coefficients between trickle-bed, hollow fiber membrane and stirred tank reactors. Bioresour Technol 133:340–346
155. Vane LM (2008) Separation technologies for the recovery and dehydration of alcohols from fermentation broths. Biofuels Bioprod Biorefin 2:553–588. doi:10.1002/bbb.108
156. Abubackar HN, Veiga MC, Kennes C (2012) Biological conversion of carbon monoxide to ethanol: effect of pH, gas pressure, reducing agent and yeast extract. Bioresour Technol 114: 518–522. doi:10.1016/j.biortech.2012.03.027
157. Kundiyana DK, Wilkins MR, Maddipati P, Huhnke RL (2011) Effect of temperature, pH and buffer presence on ethanol production from synthesis gas by "*Clostridium ragsdalei*". Bioresour Technol 102:5794–5799. doi:10.1016/j.biortech.2011.02.032
158. Abubackar HN, Veiga MC, Kennes C (2015) Carbon monoxide fermentation to ethanol by *Clostridium autoethanogenum* in a bioreactor with no accumulation of acetic acid. Bioresour Technol. doi:10.1016/j.biortech.2015.02.113
159. Gaddy JL, Arora DK, Basu R et al (2012) Methods for increasing the production of ethanol from microbial fermentation. US Patent 20120122173
160. Kantzow C, Mayer A, Weuster-Botz D (2015) Continuous gas fermentation by *Acetobacterium woodii* in a submerged membrane reactor with full cell retention. J Biotechnol. doi:10.1016/j.jbiotec.2015.07.020
161. Simpson SD, Köpke M, Smart KF et al (2014) System and method for controlling metabolite production in a microbial fermentation. US Patent 20140273115
162. Gaddy JL (1997) *Clostridium* strain which produces acetic acid from waste gases. US Patent 5593886
163. Gaddy JL (1998) Biological production of acetic acid from waste gases with *Clostridium ljungdahlii*. US Patent 5807722
164. Gaddy JL, Clausen WC (1992) *Clostridium ljungdahlii*, an anaerobic ethanol and acetate producing microorganism. US Patent 5173429

165. INEOS Bio (2013) INEOS Bio produces cellulosic ethanol at commercial scale. http://www.ethanolproducer.com/articles/10096/ineos-declares-commercial-cellulosic-ethanol-online-in-florida. Accessed 25 Aug 2015
166. Florida Department of Environmental Protection (2014) Technical evaluation and preliminary determination. http://www.ascension-publishing.com/INEOS-FIX-090514.pdf. Accessed 25 Aug 2015
167. Coskata Inc. (2011) Coskata, Inc.'s semi-commercial facility demonstrates two years of successful operation. http://www.coskata.com/company/media.asp?story=8377ADFF-9DFE-4901-876B-B39FD96B213F. Accessed 24 July 2015
168. Coskata Inc. (2011) Form S-1 Coskata, Inc. http://www.sec.gov/Archives/edgar/data/1536893/000119312511343587/d267854ds1.htm. Accessed 25 Aug 2015
169. Heijstra B, Kern E, Koepke M et al (2012) Novel bacteria and methods of use thereof. WO/2012/015317
170. LanzaTech (2013a) Beijing Shougang LanzaTech New Energy Science & Technology Company Earns Roundtable on Sustainable Biomaterials (RSB) certification. http://www.lanzatech.com/beijing-shougang-lanzatech-new-energy-science-technology-company-earns-roundtable-on-sustainable-biomaterials-rsb-certification/. Accessed 26 July 2015
171. LanzaTech (2015a) China Steel Corporation approves investment in LanzaTech commercial project. http://www.lanzatech.com/china-steel-corporation-approves-investment-lanzatech-commercial-project/. Accessed 26 July 2015
172. LanzaTech (2015b) ArcelorMittal, LanzaTech and Primetals Technologies announce partnership to construct breakthrough €87 biofuel production facility. http://www.lanzatech.com/arcelormittal-lanzatech-primetals-technologies-announce-partnership-construct-break through-e87m-biofuel-production-facility/. Accessed 26 July 2015
173. LanzaTech (2013b) Evonik and LanzaTech working on bio-processed precursors for specialty plastics. http://www.lanzatech.com/evonik-and-lanzatech-working-on-bio-processed-precursors-for-specialty-plastics/. Accessed 26 July 2015
174. LanzaTech (2013c) LanzaTech Partners with Korea's SK Innovation on development of process technology for Green Chemicals. LanzaTech Partners with Korea's SK Innovation on development of process technology for Green Chemicals. Accessed 26 July 2015
175. LanzaTech (2014) Virgin Atlantic announces HSBC to join unique partnership in development of low carbon fuel. http://www.lanzatech.com/virgin-atlantic-announces-hsbc-join-unique-partnership-development-low-carbon-fuel/. Accessed 26 July 2015
176. Kircher M (2015) Sustainability of biofuels and renewable chemicals production from biomass. Curr Opin Chem Biol 29:26–31. doi:10.1016/j.cbpa.2015.07.010
177. California Energy Commission (2015) Low carbon fuel standard. http://www.energy.ca.gov/low_carbon_fuel_standard/. Accessed 13 Aug 2015
178. The European Parliament and the Council of the European Union (2009) Directive 2009/30/EC of the European Parliament and of the Council of 23 April 2009 amending Directive 98/70/EC as regards the specification of petrol, diesel and gas-oil and introducing a mechanism to monitor and reduce greenhouse gas emissions and amend. Off J Eur Union L 140: 88–113
179. Ou X, Zhang X, Zhang Q, Zhang X (2013) Life-cycle analysis of energy use and greenhouse gas emissions of gas-to-liquid fuel pathway from steel mill off-gas in China by the LanzaTech process. Front Energy 7:263–270. doi:10.1007/s11708-013-0263-9
180. Fleischanderl A, Plattner T, Puschitz P et al (2015) The circular economy: carbon recycling and the steel industry. http://www.metec-estad2015.com/papers2015final/P292.pdf. Accessed 25 Aug 2015
181. GREET (2013) The greenhouse gases, regulated emissions, and energy use in transportation model. https://greet.es.anl.gov/. Accessed 25 Aug 2015
182. Liu B, Wang F, Zhang B, Bi J (2013) Energy balance and GHG emissions of cassava-based fuel ethanol using different planting modes in China. Energy Policy 56:210–220. doi:10.1016/j.enpol.2012.12.052

Adv Biochem Eng Biotechnol (2016) 156: 323–362
DOI: 10.1007/10_2015_5009

Published online: 24 February 2016

Anaerobic Fermentation for Production of Carboxylic Acids as Bulk Chemicals from Renewable Biomass

Jufang Wang, Meng Lin, Mengmeng Xu, and Shang-Tian Yang

Abstract Biomass represents an abundant carbon-neutral renewable resource which can be converted to bulk chemicals to replace petrochemicals. Carboxylic acids have wide applications in the chemical, food, and pharmaceutical industries. This chapter provides an overview of recent advances and challenges in the industrial production of various types of carboxylic acids, including short-chain fatty acids (acetic, propionic, butyric), hydroxy acids (lactic, 3-hydroxypropionic), dicarboxylic acids (succinic, malic, fumaric, itaconic, adipic, muconic, glucaric), and others (acrylic, citric, gluconic, pyruvic) by anaerobic fermentation. For economic production of these carboxylic acids as bulk chemicals, the fermentation process must have a sufficiently high product titer, productivity and yield, and low impurity acid byproducts to compete with their petrochemical counterparts. System metabolic engineering offers the tools needed to develop novel strains that can meet these process requirements for converting biomass feedstock to the desirable product.

Keywords Anaerobic fermentation · Biomass · Bulk chemical · Carboxylic acid · Metabolic engineering

J. Wang
School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, P.R. China

William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH 43210, USA

M. Lin
Bioprocessing Innovative Company, 4734 Bridle Path Ct., Dublin, OH 43017, USA

M. Xu and S.-T. Yang (✉)
William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH 43210, USA
e-mail: yang.15@osu.edu

Contents

Abbreviations

1,3-PDO	1,3-Propanediol
3-HP	3-Hydroxypropionic acid
ACK	Acetate kinase
AroY	Protocatechuic acid decarboxylase
AroZ	3-Dehydroshikimic acid dehydratase
B/A	Butyrate to acetate ratio
BUK	Butyrate kinase
CAD	*cis*-Aconitic acid decarboxylase
CatA	Catechol 1,2-dioxygenase
DO	Dissolved oxygen
EDI	Electrodeionization
EMP	Embden–Meyerhof–Parnas
FOC	Formate transporter
GDH	Glycerol dehydrogenase
GDR	Glycerol dehydratase reactivase
GHG	Greenhouse gas
GlpF	Glycerol facilitator
GlpK	Glycerol kinase
HMP	Hexose monophosphate
KGSADH	Ketoglutaric semialdehyde dehydrogenase
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
MGS	Methylglyoxal synthase
MMC	Methylmalonyl-CoA carboxyltransferase
MMD	Methylmalonyl-CoA decarboxylase

MV	Methyl viologen
ORP	Oxidoreduction potential
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin oxidoreductase
PPC	Phosphoenolpyruvate carboxylase
PTA	Phosphotransacetylase
PTB	Phosphotransbutyrylase
PuuC	NAD^+-dependent γ-glutamyl-γ-aminobutyraldehyde dehydrogenase
PYC	Pyruvate carboxylase
rTCA	Reductive tricarboxylic acid
TCA	Tricarboxylic acid
YqhD	NADPH-dependent aldehyde reductase/alcohol dehydrogenase

1 Introduction

More than 80 million tons of industrial chemicals valued at over $2 trillion are manufactured annually from petroleum-based feedstock, which, however, is not sustainable because of the depletion of oil and serious environmental pollution, especially greenhouse gas (GHG) emissions, caused by the current petrochemical industry. In recent years we have seen increasing interests and commercial activities in producing bulk chemicals from renewable biomass by microbial fermentation. Table 1 lists some bio-based bulk chemicals that are already in or should soon be in commercial production. These bio-based chemicals are more environmentally friendly and sustainable in their manufacturing and can replace those manufactured from traditional petroleum-based feedstocks (e.g., ethylene, propylene, and butadiene).

In this chapter we provide an overview on bio-based carboxylic acids that have wide applications in chemical, food, and pharmaceutical industries, focusing on recent advances and challenges in their industrial production by anaerobic fermentation. Presently, some carboxylic acids, such as citric acid and itaconic acid, are exclusively produced from sugar by fermentation, and some (e.g., acetic and propionic acids) are produced mainly chemically but also by fermentation for food applications. Historically, some carboxylic acids (e.g., fumaric acid) were produced by fermentation but their industrial manufacturing was phased out because of the rise of the petrochemical industry. In fact, many carboxylic acids are or can be produced in large quantities by naturally occurring or genetically engineered microorganisms in anaerobic fermentation. In recent years, metabolic engineering and synthetic biology have been applied to developing novel microbial strains which can produce these chemicals economically from renewable resources, including agricultural commodities and residues, industrial wastes, and plant biomass, for commercial applications. Figure 1 shows anaerobic metabolic pathways

Table 1 Bio-based bulk chemicals already in or soon to be in commercial production

Chemical	Status/applications	Company
1,3-Propanediol	In commercial production	DuPont/Tate & Lyle
1,4-Butanediol	For butadiene and other chemicals	Genomatica
Iso-butanol	In commercial production	Gevo
Succinic acid	Production plant in start-up testing	Myriant, DSM, BASF
Lactic acid	For polylactic acid production	Cargill, NatureWorks
3-Hydroxypropionic acid	For acrylic acid production; still in development stage	Cargill/Novozymes; OPX/Dow Chemical
Isoprene	For natural rubber production	Genencor/Goodyear
Polyhydroxybutyrate	For biodegradable plastics	Metabolix and ADM

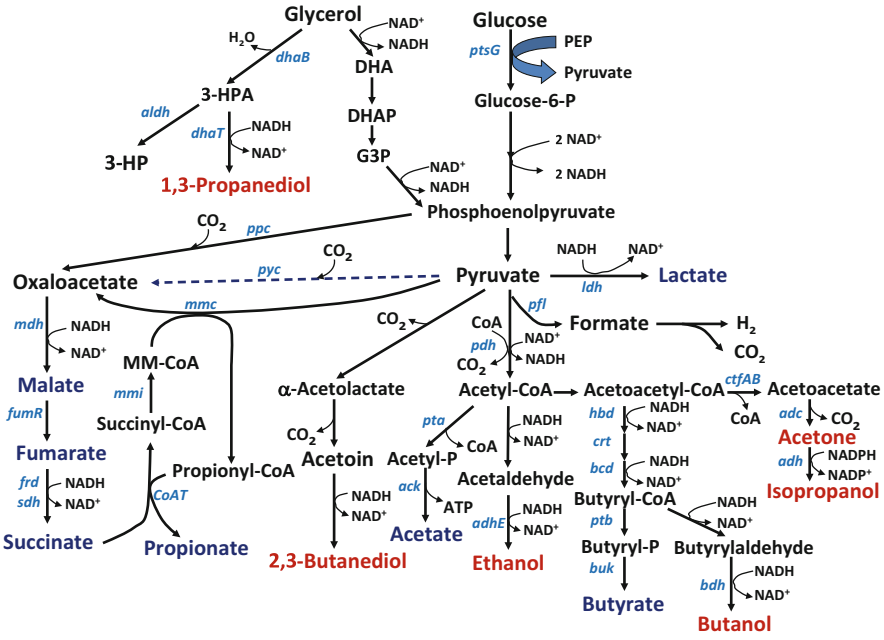


Fig. 1 Anaerobic metabolic pathways for carboxylic acid and alcohol biosynthesis in microorganisms. *DHA* dihydroxyacetone, *DHAP* dihydroxyacetone-P, *G3P* glyceraldehyde-3-P, *3-HP* 3-hydroxypropionate, *3-HPA* 3-hydroxypropionaldehyde. Key enzymes and genes in the pathway: *adc* alcohol decarboxylase, *adh* alcohol dehydrogenase, *adhE* aldehyde/alcohol dehydrogenase, *aldh* aldehyde dehydrogenase, *bcd* butyryl-CoA dehydrogenase, *bdh* butanol dehydrogenase, *buk* butyrate kinase, *crt* crotonase, *ctfAB* CoA transferase, *dhaB* glycerol dehydratase, *dhaT* 1,3-PDO oxidoreductase, *frd* fumarate reductase, *fumR* fumarase, *hbd* β-hydroxybutyryl-CoA dehydrogenase, *ldh* lactate dehydrogenase, *mdh* malate dehydrogenase, *mmc* methylmalonyl-CoA carboxyltransferase, *mmi* methylmalonyl isomerase, *pdh* pyruvate dehydrogenase, *pfl* pyruvate formate lyase, *ppc* PEP carboxylase, *pta* phosphotransacetylase, *ptb* phosphotransbutyrylase, *ptsG* glucose phosphotransferase, *pyc* pyruvate carboxylase, *sdh* succinate dehydrogenase

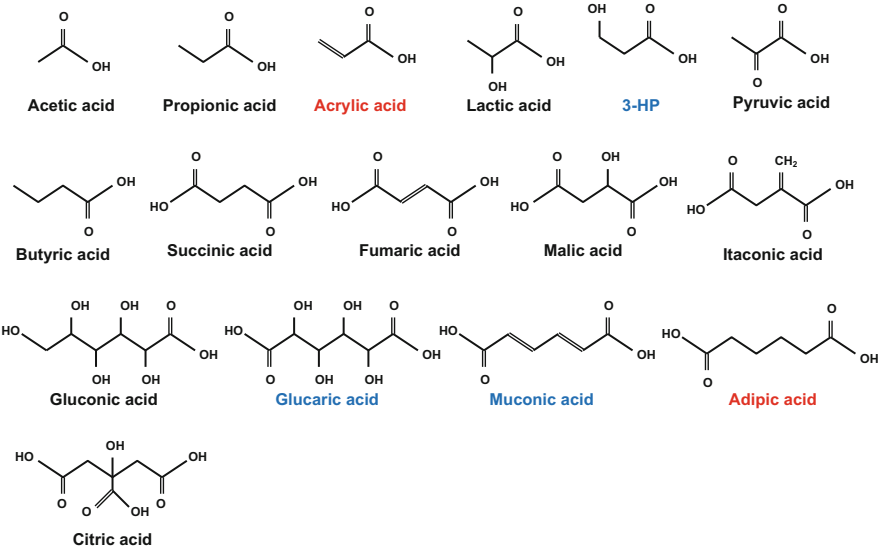


Fig. 2 Chemical structures of some carboxylic acids. Most of them can be produced by natural (*black*) or engineered microorganisms (*blue*); some cannot be produced directly by any microorganism in a significant amount (*red*)

leading to the biosynthesis of various carboxylic acids and alcohols. Compared to aerobic fermentation, higher product yields can usually be obtained in anaerobic fermentation because less carbon substrate would be used for cell growth and energy generation. Certain anaerobes can produce lactic or acetic acid with nearly 100% of substrate-to-product conversion yield without emitting any CO_2. Some species can fix CO_2, which can not only increase product yield from sugar but also reduce GHG emissions.

Figure 2 shows chemical structures of various carboxylic acids in the following groups: short-chain fatty acids (acetic, propionic, and butyric), hydroxy acids (lactic acid and 3-hydroxypropionic), dicarboxylic acids (succinic, malic, fumaric, adipic, muconic, and glucaric), and others (acrylic, pyruvic, citric, and gluconic). We briefly discuss their applications and bio-production, highlighting recent advances in strain engineering and fermentation process development and performance. Challenges and future prospects for industrial production of these carboxylic acids as bulk chemicals are also presented in this chapter.

2 Short-Chain Fatty Acids

2.1 Acetic Acid

Acetic acid, the smallest short-chain volatile fatty acid, is an important bulk chemical with an annual global demand of ~10 million tons. Acetic acid used in the chemical industry is mainly produced by the carbonylation of methanol with carbon monoxide. Currently, only about 10% of acetic acid on the market is produced by *Acetobacter* in aerobic vinegar fermentation, with ~60 wt% yield from sugar. However, many homoacetogenic anaerobes, including *Clostridium formicoaceticum*, *Moorella thermoacetica* (*C. thermoaceticum*), *Clostridium aceticum*, *Acetobacterium woodii*, and *Acetogenium kivui* can produce acetic acid as the sole fermentation product at a theoretical yield of 3 mol acetate per mol glucose – see (1) – or ~100 wt% from a variety of hexoses, pentoses, and lactic acid [1]. In addition, most of the homoacetogens can also use CO and CO_2/H_2, as carbon and energy sources for their growth – see (2, 3) [2].

$$C_6H_{12}O_6 \rightarrow 3\,CH_3COOH, \tag{1}$$

$$2\,CO_2 + 4\,H_2 \rightarrow CH_3COOH + 2\,H_2O, \tag{2}$$

$$4\,CO + 2\,H_2O \rightarrow CH_3COOH + 2\,CO_2. \tag{3}$$

As shown in Fig. 3, via the Embden–Meyerhof–Parnas (EMP) pathway, 1 mol glucose is converted into 2 mol pyruvate, which is decarboxylated into acetyl-CoA

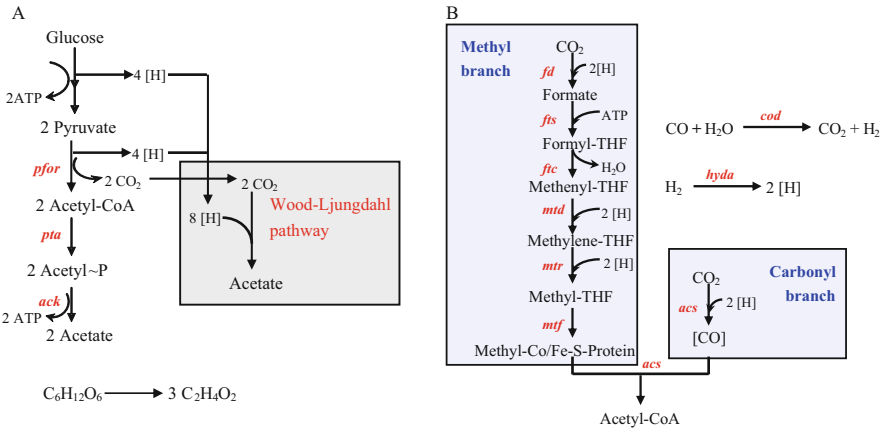


Fig. 3 Homoacetogenic conversion of glucose to acetate (**a**) with Wood-Ljungdahl pathway for CO_2 fixation (**b**). Key enzymes and genes in the pathway: *ack* acetate kinase, *acs* acetyl-CoA synthase, *cod* CO dehydrogenase, *fd* formate dehydrogenase, *ftc* formyl-THF cyclohydrolase, *fts* formyl-THF synthase, *hyda* hydrogenase, *mtd* methylene-THF dehydrogenase, *mtf* methyltransferase, *mtr* methylene-THF reductase, *pfor* pyruvate: ferredoxin oxidoreductase, *pta* phosphotransacetylase

by pyruvate ferredoxin oxidoreductase (PFOR) and then converted to acetate by phosphotransacetylase (PTA) and acetate kinase (ACK). One additional acetate is also formed by reducing 2 mol CO_2 arising from pyruvate decarboxylation with eight hydrogens via the Wood–Ljungdahl pathway (Fig. 3b), which uses CO_2 as the terminal electron acceptor.

Homoacetogenic fermentation is of high interest because nearly all the substrate carbon can be recovered in the product acetate without releasing CO_2 and any byproduct, which can greatly reduce GHG emissions and ease the downstream processing for product recovery and purification. A high final titer of 10% (w/v) acetic acid was produced from glucose by *M. thermoacetica* in fed-batch fermentation with cell recycle, which gave a reactor productivity of ~0.8 g/L h and acetate yield of ~0.8 g/g glucose consumed [3]. With *C. formicoaceticum*, acetic acid was produced from fructose at a final titer of ~78 g/L, yield of ~1.0 g/g, and reactor productivity of ~0.95 g/L h in fed-batch fermentation with cells immobilized in a fibrous bed bioreactor (FBB) [4]. *C. formicoaceticum* co-immobilized with *Lactococcus lactis* in a FBB produced 75 g/L of acetic acid from whey lactose with an overall yield of 0.9 g/g lactose and productivity of 1.23 g/L h [5]. More recently, *A. woodii* was genetically engineered to increase its ability to grow auxotrophically on CO_2 and H_2, producing 50 g/L acetate in 4 days in a pH-controlled stirred-tank reactor [6]. In addition, *Escherichia coli* was also engineered to produce acetate as the main product from glucose by inactivating oxidative phosphorylation, disrupting the tricarboxylic acid cycle, and removing native fermentation pathways, producing 51.8 g/L acetate with a yield of ~0.5 g/g in fed-batch fermentation with micro-aeration (5% dissolved oxygen) [7]. However, these homoacetogenic fermentations require a pH of >5.0 and the acetate produced in the salt form is difficult to separate from the fermentation broth, hindering its commercial application.

2.2 Propionic Acid

Propionic acid, a three-carbon volatile fatty acid, is an important chemical with a global market of ~377,000 tons in 2006 and a stable annual growth rate of ~2.3% [8]. Its industrial applications include uses in food and feed preservatives, pharmaceuticals, cellulose acetate-propionate plastics, perfumes, alkyl propionate esters, artificial flavorings, and herbicides. Current industrial production of propionic acid is mainly through petrochemical routes: carbonylation of ethylene, oxidation of propanal, and direct oxidation of hydrocarbons (mainly naphtha) [8]. Some propionic acid used in the food industry is produced by fermentation with propionibacteria, which are widely used in the dairy industry for Swiss cheese and vitamin B_{12} production [9]. Although the petrochemical routes are generally more economical for propionic acid production, bio-based propionic acid has attracted wide attention in recent years because of environmental concerns of petro-chemical

processes, unstable supplies and increased prices of crude oils, and consumer demands for natural products.

Propionibacterium acidipropionici, *Propionibacterium freudenreichii*, and *Propionibacterium shermanii* are the most commonly used bacteria in propionic acid fermentation. They are Gram-positive, nonspore-forming, and anaerobic or facultative anaerobic. They can grow on lactic acid, glycerol, and various mono- and di-saccharides, producing propionic acid as the main product with acetic acid, succinic acid, and CO_2 as byproducts via the dicarboxylic acid pathway [9]. In the fermentation, the carbon source such as glucose is first converted to pyruvate through either the EMP or hexose monophosphate (HMP) pathway – see (4, 5). Pyruvate is then oxidized to acetic acid with ATP generation or enters the Wood–Werkman cycle, in which a carboxyl group is transferred from methylmalonyl-CoA to pyruvate, leading to the formation of oxaloacetate and propionyl-CoA. The latter reacts with succinate, producing propionate and succinyl-CoA, which is isomerized to methylmalonyl-CoA, thus completing the cycle (see Fig. 1). Theoretically, each mol of pyruvate generated from glucose can be converted to 2/3 mol propionic acid and 1/3 mol acetic acid – see (6).

$$\text{EMP}: \ C_6H_{12}O_6 \rightarrow 2\,CH_3COCOOH + 2\,NADH_2 + 2\,ATP, \tag{4}$$

$$\text{HMP}: \ C_6H_{12}O_6 \rightarrow 5/3\,CH_3COCOOH + CO_2 + 11/3\,NADH_2 + 5/3\,ATP, \tag{5}$$

$$3\,CH_3COCOOH + 3\,NADH_2 \rightarrow 2\,CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O + ATP. \tag{6}$$

In propionic acid fermentation, acetate is co-produced with propionate for balancing the redox ($NADH_2/NAD^+$). The theoretical maximum propionic acid yield from glucose is 0.548 g/g if EMP or 0.68 g/g if HMP pathway is used in glycolysis. The degree of involvement of each pathway varies greatly with the substrate and fermentation conditions. When a more reduced substrate is used as carbon source, more propionate and less acetate are produced. For example, when glycerol was used as the substrate, propionate yield can be as high as 0.8 g/g with little acetate produced [10]. Depending on the growth conditions, a certain amount of succinate is also produced as a byproduct.

Propionibacteria have complex nutritional requirements, and usually grow rather slowly because of the inhibition by propionic acid. Various carbon sources, including low-cost biomass feedstocks such as corn meal [11], corn mash [12], corncob molasses [13], cane molasses [14], wheat flour [15], Jerusalem artichoke [16], sugarcane bagasse [17], whey [18, 19], and crude glycerol [10, 20, 21], have been evaluated for propionic acid production. In general, good productivity (0.22–2.1 g/L h) and yield (~0.5 g/g substrate) with a final propionic acid concentration of ~50 g/L can be obtained at the optimal pH of ~6.5. For economic production, extensive research efforts have focused on strategies to enhance product yield, productivity, and final product titer and purity, which greatly affect downstream recovery and purification costs. In general, increasing cell density in the fermentor also increases reactor productivity. Cell recycle, retention, and immobilization have

thus been widely used to achieve high cell density and reactor productivity in fermentation [11, 16, 19, 22–25]. The highest volumetric productivity of 14.3 g/L h ever reported for propionic acid fermentation was achieved when the cell density was maintained at 100 g/L in a continuous stirred-tank reactor with cell recycle by ultrafiltration, but the substrate conversion and final product titer were low [19]. In situ propionic acid separation with solvent extraction [26] or ion exchanger adsorption [27] can also greatly increase reactor productivity by alleviating propionic acid inhibition. Compared to conventional propionic acid fermentation, extractive fermentation also greatly increased product titer, yield, and purity [26].

For commodity and specialty chemicals such as propionic acid, the feedstock cost may account for ~50% of the product cost. Therefore, it is important to have a high product yield close to the theoretical yield. In propionic acid fermentation, a significant amount of the substrate (usually glucose) is consumed for cell biomass and acetate production, which is inevitable for redox balance and ATP generation to support cell growth and maintenance. In general, a higher propionic acid yield could be obtained when cell growth was restricted or reduced by nutrients limitation or a low pH [28], which, however, would compromise productivity and final product titer because of the stronger inhibition caused by the undissociated propionic acid [26]. Because acetate production (from glucose) is mainly for NADH or redox balance, acetate biosynthesis can be reduced, thereby increasing propionic acid yield, by manipulating the redox balance through oxidoreduction potential (ORP) shift [29] or the use of an artificial electron donor [30] or a more reduced substrate, such as glycerol [21] in the fermentation. Although glycerol could give a high yield for propionic acid production (up to 0.8 g/g), cell growth on glycerol is usually very poor because of redox imbalance, and the fermentation would suffer from low productivity [10]. This problem can be solved by co-fermenting glycerol with glucose, resulting in both a high propionic acid yield of ~0.6 g/g and productivity [31, 32]. CO_2 supplementation was also found to be beneficial to cell growth on glycerol and propionic acid production [33].

It is important to have a high final product titer before downstream processing for economical recovery of propionic acid from the fermentation broth. The final product titer in the fermentation is limited by the acid tolerance of the cells. In-process adaptation of cells by exposing them to gradually increased propionate concentrations such as in recycle-batch and fed-batch fermentations increased the final propionic acid concentration to ~70 g/L [13, 18]. A high final titer of >100 g/L was obtained in fed-batch fermentation with cells immobilized and adapted in a fibrous bed bioreactor (FBB) [10]. Cells adapted in the FBB had higher tolerance to propionic acid, which could be attributed to increased activities of H^+-ATPase and key enzymes in the Wood–Werkman cycle, increased growth rate and survival with decreased membrane fluidity, and increased cellular surface area for better mass transfer [34].

Commercially, only a small amount of propionate is produced by fermentation and used, in a mixture with the co-produced acetic acid, for food application. For use as a precursor or intermediate chemical, propionic acid in the fermentation

broth must be separated and purified. However, it is difficult to separate propionic acid from acetic and succinic acids by conventional methods such as precipitation and distillation [35]. Recent efforts have thus focused on solvent extraction [26], adsorption with ion exchange resins [27], and electrodialysis with composite membranes [36] which, nonetheless, are expensive to use on an industrial scale. It is thus desirable to reduce or eliminate acetate (and succinate) production in propionic acid fermentation. Attempts to knock out the acetate biosynthesis pathway in propionibacteria were not successful [37]. However, when glycerol was used to produce propionic acid, either as a co-substrate with glucose or as the sole carbon source, the propionate to acetate (P/A) ratio was greater than 10–20 (w/w), compared to only ~4 (w/w) with glucose as the substrate [32, 38].

Metabolic engineering can be used to shift the carbon flux distribution between the two branch pathways at the pyruvate node, leading to either acetate or propionate biosynthesis, and thus offers a useful tool to increase the P/A ratio, propionic acid yield, and productivity. Several propionibacteria's genomes have been fully sequenced [39–41], which facilitated metabolic engineering studies of propionibacteria. Overexpressing propionyl-CoA:succinate CoA transferase in *P. freudenreichii* subsp. *shermanii* decreased acetate and succinate production and the mutant produced more propionate with up to 10% increase in yield and 46% increase in productivity [42]. Overexpressing methylmalonyl-CoA carboxyltransferase (MMC) or methylmalonyl-CoA decarboxylase (MMD) also resulted in a significant increase in the metabolic flux toward propionic acid biosynthesis [43]. Overexpressing phosphoenolpyruvate (PEP) carboxylase (PPC) from *E. coli* in *P. freudenreichii* resulted in faster cell growth and higher propionate titer and productivity, but had negligible effect on propionate and acetate yields [41], and pyruvate carboxylase (PYC) overexpression led to slowed cell growth, reduced propionic acid production, and increased succinic acid accumulation [43]. Overexpressing glycerol dehydrogenase (GDH) and malate dehydrogenase (MDH) in *Propionibacterium jensenii* increased propionate production from glycerol to 39.43 g/L, a 46% increase compared to the wild type [44, 45]. Interestingly, overexpressing *E. coli* aldehyde/alcohol dehydrogenase (*adhE*) in *P. freudenreichii* not only produced ~1 g/L *n*-propanol (from propionyl-CoA) but also significantly increased propionic acid productivity [46].

Most propionibacteria, including *P. freudenreichii* and *P. shermanii*, produced acetic acid from acetyl-CoA through acetyl phosphate via the reactions catalyzed by phosphotransacetylase (PTA) and acetate kinase (ACK). However, neither *pta* nor *ack* gene was found in the annotated genome of *P. acidipropionici* [39]. Instead, it contains acetate-CoA ligase or acetyl-CoA synthetase, which catalyzes the reversible reaction between acetate and acetyl-CoA and is probably responsible for the acetic acid biosynthesis in the absence of *ack* or *pta*.

Some obligate anaerobes such as *Clostridium propionicum* use acrylic acid pathway to produce propionic acid from pyruvate, which is reduced to D-lactate and then to propionate via D-lactyl-CoA, acrylyl-CoA, and propionyl-CoA, involving three enzymes: propionate-CoA transferase, lactyl-CoA dehydratase, and acrylyl-CoA reductase [9]. Seven genes encoding these three enzymes were

expressed in *E. coli*, and the mutant produced 0.27 g/L propionic acid from glucose, demonstrating a novel way to produce propionic acid by using a non-native organism through synthetic biology [47].

2.3 Butyric Acid

Butyric acid, a four-carbon volatile fatty acid commonly found in rumen and anaerobic digestion, is commercially produced mainly by the oxidation of butyraldehyde obtained from oxosynthesis of propylene [48]. It has wide applications in the food, chemical, and pharmaceutical industries [49, 50], with a global market of >80,000 tons per year. Because of its many health benefits, including antineoplastic effects on the large intestine and colon [51, 52], butyrate and its derivatives have rapidly growing, emerging applications as prebiotic supplements to animal feeds and as drugs for treating hemoglobinopathies, colon cancer, and gastrointestinal diseases [1]. The production of bio-based butyric acid by fermentation has become an attractive alternative to the current petrochemical synthesis, especially for applications in the food and pharmaceutical industries [1].

Many anaerobic bacteria in the genera *Clostridium*, *Butyribacterium*, *Butyrivibrio*, *Sarcina*, *Eubacterium*, *Fusobacterium*, *Megasphera*, *Roseburia*, and *Coprococcus* can produce butyric acid along with other acids and some also with ethanol [1]. *Clostridium tyrobutyricum* and *Clostridium butyricum* are the two most studied species with the former possessing the highest commercial potential for butyric acid production from glucose and other carbon sources. They are Gram-positive, chemo-organotrophic, spore forming, strict anaerobes. They can ferment various hexoses and pentoses, and form acetic acid, CO_2, and H_2 in addition to butyric acid as major fermentation products – see (7). Lactate is also produced by *Clostridium thermobutyricum* [53]. *C. tyrobutyricum* can also convert acetate and lactate to butyrate. *Clostridium cellulovorans* can use cellulose and xylan for butyrate synthesis [54], and thus has the potential for use in consolidated bioprocessing (CBP). *Clostridium carboxidivorans* can autotrophically grow on CO and CO_2 with H_2 as the energy source via the Wood–Ljungdahl pathway [55, 56]. *Butyribacterium methylotrophicum* is also of interest because it can ferment methanol in addition to hexose, lactic acid, and H_2/CO_2. In batch culture of *B. methylotrophicum*, butyric acid was the only product from methanol, whereas acetate was the major product on H_2/CO_2 [57]. Butyric acid also can be produced as a major fermentation product from ethanol and acetate by *Clostridium kluyveri* – see (8), which produced caproate, instead of butyrate when ethanol was present in excess of acetate – see (9) [2].

$$C_6H_{12}O_6 \rightarrow 0.8\,CH_3CH_2CH_2COOH + 0.4\,CH_3COOH + 2\,CO_2 + 2.4\,H_2, \quad (7)$$

$$C_2H_5OH + CH_3COOH \rightarrow CH_3CH_2CH_2COOH + H_2O, \quad (8)$$

$$2C_2H_5OH + CH_3COOH \rightarrow CH_3(CH_2)_4COOH + 2H_2O. \quad (9)$$

In butyric acid fermentation, the substrate such as glucose is first catabolized to pyruvate, usually via EMP pathway. Pyruvate is then decarboxylated to acetyl-CoA and CO_2 by pyruvate:ferredoxin oxidoreductase (PFOR), generating the reduced ferredoxin, which is re-oxidized by hydrogenase with electrons passing to hydrogen ions to form H_2. Some pyruvate may also be converted to lactate by lactate dehydrogenase (LDH) under certain conditions [58]. Acetyl-CoA is converted either to acetate by PTA and ACK, or to butyryl-CoA, which is further converted to butyrate by phosphotransbutyrylase (PTB) and butyrate kinase (BUK) in most butyrate-producing clostridia including *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and *C. butyricum*. However, *C. kluyveri* and many colonic bacteria utilize butyryl-CoA:acetate CoA transferase to produce butyrate by transferring the CoA moiety from butyryl-CoA to acetate [59, 60]. Only a few microorganisms have genes or enzymes for both butyrate biosynthesis pathways.

Theoretically, 0.8 mol butyrate and 0.4 mol acetate are produced from 1 mol glucose – see (7). To increase butyrate and reduce acetate production, *ack* and *pta* genes in the acetate biosynthesis pathway were knocked down in *C. tyrobutyricum*, and the mutants produced ~30% more butyrate (~42 g/L vs ~30 g/L) with a higher yield of 0.42 g/g (vs 0.33 g/g) and butyrate to acetate ratio (B/A) of 5.4–6.6 g/g (vs ~4.0 g/g) compared to the wild type strain [61, 62]. The solventogenic *C. acetobutylicum* was also engineered to produce butyrate as the main product by knocking down *pta*, *ctfB*, and *adhE1*, and the mutant produced 30.8 g/L butyrate with a high B/A of 6.6 g/g [63]. Further knocking down *buk* increased butyrate production to 32.5 g/L with a high B/A of 31.3 g/g [64]. Synthetic biology has also been applied to construct butyric acid biosynthesis pathways in *E. coli*, producing 4.3–10 g/L butyric acid from glucose with a yield of ~0.4 g/g and high B/A of up to 143 [65–67].

Butyric acid production from various biomass feedstocks, including wheat flour, cane molasses, corn meal, corn fiber, sugarcane bagasse, and Jerusalem artichoke, by *C. tyrobutyricum* has been studied in batch fermentation [11, 68–72]. Up to 62.8 g/L of butyric acid and productivity of 6.78 g/L h, and yield of 0.47 g/g were obtained [1]. Adaptive evolution of cells immobilized in a fibrous bed bioreactor (FBB) increased glucose consumption rate and butyric acid tolerance and production [73, 74]. A high butyric acid titer of 86.9 g/L was obtained in fed-batch fermentation after adaptation in an FBB [75]. Acid tolerance of *C. tyrobutyricum* was also enhanced to improve butyric acid production from 22.7 to 33.4 g/L after heavy ion irradiation [76]. Continuous fermentation with cell recycling or immobilization improved the reactor productivity to as high as 9.3 g/L h [77]. However, a continuous process usually gave a lower product concentration and incomplete substrate conversion. Extractive fermentation with solvent to remove butyric acid selectively from the fermentation broth could alleviate product inhibition and significantly improve butyric acid productivity to 7.37 g/L h and yield to 0.45 g/g

[78]. The process also gave a highly concentrated sodium butyrate product (>300 g/L) with a high purity (91% butyrate, 9% acetate). Continuous butyric acid production from glucose by *C. tyrobutyricum* in fermentation with electrodeionization (EDI) for butyric acid separation to control the butyrate level at 2.5 g/L also achieved a butyric acid productivity of 2.15 g/L h, yield of 0.45 g/g, and butyric acid titer of ~200 g/L and purity of ~92% in the recovered concentrate stream [79].

Similar to propionic acid fermentation, acetic acid as a byproduct makes it difficult to produce chemical-grade butyric acid from fermentation. So far, metabolic engineering to knock out acetate biosynthesis has not been successful. Acetate was co-produced with butyrate mainly for redox balance. Recently, it was shown that using an electron mediator or artificial electron carrier such as methyl viologen (MV) could shift the metabolic flux toward butyrate biosynthesis and significantly reduce or even eliminate acetate production, resulting in a highly pure butyrate production in the fermentation [80]. Thus, by combining metabolic and process engineering approaches, it is possible to produce butyric acid economically from glucose and low-cost biomass feedstocks for chemical use.

3 Hydroxy Acids

3.1 Lactic Acid

Lactic acid, a three-carbon hydroxy acid, occurs widely in nature. It is produced from pyruvate by the lactate dehydrogenase (LDH) in cells. Commercial production of lactic acid is either by chemical synthesis via hydrolysis of lactonitrile or by fermentation of sugars with lactic acid bacteria. The biological production route has the advantage of producing optically pure L- or D-lactic acid, whereas chemical methods produce racemic mixtures of DL-lactic acid that is difficult to use in the manufacturing of poly-lactic acid (PLA), a biodegradable polymer (plastic) with wide applications in the packaging and textile industries [81]. The worldwide market for lactic acid is about 450,000 tons and is rapidly growing because of its increased uses in processed foods and PLA manufacturing.

Lactic acid bacteria (LAB) are facultative anaerobic or microaerophilic, Gram-positive, and can be classified as homofermentative (produce lactic acid only) and heterofermentative (produce lactic acid and other metabolic products such as acetic acid, ethanol, and CO_2) [82]. Homolactic acid bacteria, such as *Lactobacillus* and *Lactococci*, utilize the EMP pathway for glycolysis and produce lactic acid as the sole end product without any gas production. Theoretically, 1 mol glucose can yield 2 mol lactic acid – see (10).

$$C_6H_{12}O_6 \rightarrow 2\,CH_3CHOHCOOH. \tag{10}$$

Homolactic acid fermentation can generally give high final product titer (>100 g/L), yield (>90 wt%), and productivity (1 g/L h) [83], and has been widely used in

commercial lactic acid production from sugar. However, most of the lactic acid bacteria require complex nutrients, such as yeast extract and skimmed milk powder, and a relatively high pH of >5.5 for growth, which cause difficulties in product recovery and purification and are the main drawbacks of industrial lactic acid fermentation processes. Also, only a few lactic acid bacteria, usually after metabolic engineering, can produce optically pure lactic acid [84]. The complex medium components such as amino acids and optical isomers co-produced in the fermentation are difficult to separate to produce pure or polymer-grade lactic acid. Optically pure L(+)- or D(−)-lactic acid may be biologically produced by utilizing LDH with desirable stereospecificity [85]. Yeasts, which naturally produce ethanol, have been metabolically engineered to produce L-lactic acid (99.9% optical purity) from pyruvate by overexpressing a heterologous *ldh* gene and knocking out pyruvate decarboxylase (PDC) to eliminate ethanol production [86] and pyruvate dehydrogenase (PDH) to reduce channeling pyruvate into the tricarboxylic acid (TCA) cycle, resulting in a high yield of up to 0.85 g/g in fed-batch fermentation [87]. The engineered yeast is better than LAB for lactic acid production because of its higher acid tolerance and simpler medium in fermentation, and the process has been commercialized by A.E. Staley (Decatur, IL; now part of Tate & Lyle).

E. coli has also been engineered to produce optically pure lactic acid from glucose and xylose [88]. A strain with knockout mutations in four genes (*pflB*, *ackA*, *adhE*, and *frdBC*) produced high-yield D-lactic acid from sugars in a mineral salt medium [89]. Another metabolically engineered *E. coli* strain produced 138 g/L D-lactic acid from glucose with a yield of 0.86 g/g and productivity of 3.5 g/L h in fed-batch fermentation [90]. More recently, an engineered *Sporolactobacillus* produced 207 g/L D-lactate from glucose with an optical purity of 99.3%, a yield of 0.93 g/g, and productivity of 3.8 g/L h in fed-batch fermentation supplemented with peanut meal as the nitrogen source [91]. Other bacteria, such as *Corynebacterium glutamicum*, have also been engineered to produce optically pure lactic acid under anaerobic conditions [92]. In addition, the filamentous fungus *Rhizopus oryzae* can also produce optically pure L-lactic acid from glucose and starch with a high yield of >0.9 g/g in a simple mineral medium under aerobic conditions [93].

3.2 3-Hydroxypropionic Acid

3-Hydroxypropionic acid (3-HP) is a promising bio-based platform chemical which can be used as a monomer to make biodegradable polymer similar to PLA. It can also be converted to bulk chemicals including acrylic acid, 1,3-propanediol (1,3-PDO), propiolactone, and malonic acid [94]. The primary commercial interest in bio-based 3-HP is to dehydrate it to acrylic acid, which has a worldwide market of 4.5 million tons annually. Current 3-HP production relies mainly on chemical synthesis routes. Although no naturally occurring microorganism can produce 3-HP as a significant metabolite, *E. coli* and *Klebsiella pneumonia* have been metabolically engineered to produce 3-HP from glycerol [95–99]. Theoretically, 1 mol

glycerol can generate 1 mol 3-HP – see (11), or a yield of 0.97 g/g could be obtained in the bioconversion, which makes bio-based 3-HP economically attractive as a precursor for acrylic acid production because the dehydration yield is 0.8 g/g. Based on a crude glycerol cost of $0.35/kg, the cost for bio-acrylic acid can be as low as $0.98/kg, compared to the price of $2.0/kg for petroleum-based acrylic acid [96, 100].

$$C_3H_8O_3 \rightarrow CH_2OHCH_2COOH. \quad (11)$$

An engineered *E. coli* strain overexpressing vitamin B_{12} dependent glycerol dehydratase (DhaB), aldehyde dehydrogenase (AldH), and glycerol dehydratase reactivase (GDR), which stabilized DhaB activity, converted glycerol to 3-HP, along with 1,3-PDO and acetate [95]. Replacing AldH with α-ketoglutaric semialdehyde dehydrogenase (KGSADH), the recombinant *E. coli* produced 38.7 g/L 3-HP from glycerol with a yield of 0.35 g/g and productivity of 0.54 g/L h in aerobic fed-batch fermentation at pH 7 [95]. The strain with inactivated Pta-AckA (acetate biosynthesis genes) and YqhD (NADPH-dependent aldehyde reductase/alcohol dehydrogenase) produced 3-HP as the only main product from glycerol [96]. Further engineering of the glycerol metabolic pathway, including overexpressing glycerol kinase (GlpK) and glycerol facilitator (GlpF) and knocking out glycerol pathway repressor GlpR, in *E. coli* improved 3-HP production to 42.1 g/L with a productivity of 1.2 g/L h, but the yield was only 0.268 g/g [96]. However, the *E. coli* process is aerobic and needs the addition of expensive coenzyme B_{12} required for DhaB activity. In contrast, *K. pneumonia* natively synthesizes vitamin B_{12} and is thus a more suitable host to produce 3-HP [97]. *K. pneumonia* overexpressing an *E. coli* AldH produced 24.4 g/L 3-HP and 49.3 g/L 1,3-PDO from glycerol in 24 h in an anaerobic fed-batch bioreactor with a yield of 0.176 g/g for 3-HP and 0.355 g/g for 1,3-PDO [98]. By deleting the two 1,3-propanediol oxidoreductases (DhaT and YqhD) and overexpressing DhaB and the NAD^+-dependent γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (PuuC), 28 g/L 3-HP was produced from glycerol with a yield of 0.4 g/g and productivity of 0.58 g/L h in a fed-batch bioreactor with dissolved oxygen (DO) controlled at 5% [99]. A higher or lower DO greatly reduced 3-HP production.

4 Dicarboxylic Acids

Dicarboxylic acids are organic acids with two carboxyl groups. Many of them have wide applications as starting materials for products in the chemical, food, agricultural, and pharmaceutical industries. Some dicarboxylic acids, including fumaric acid, malic acid, succinic acid, and itaconic acid, are naturally produced by microorganisms. They can be produced from abundant renewable biomass and used as building-block chemicals. However, currently only itaconic acid is predominantly produced by filamentous fungi in large-scale industrial fermentation,

although fumaric acid was also once produced in industrial fermentation. Recent advancements in metabolic engineering of *E. coli* and other microorganisms have made it possible to produce succinic acid, malic acid, fumaric acid, muconic acid, and glucaric acid in anaerobic fermentation.

4.1 Succinic Acid

Succinic acid, a four-carbon dicarboxylic acid, is a common intermediate in the metabolic pathway of some anaerobic and facultative microorganisms. It is a potential chemical building block for the synthesis of various important chemicals, including 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, and 1,4-diaminobutane [101]. Currently, it is produced mainly from maleic anhydride. Commercially, maleic anhydride derived by vapor-phase oxidation of *n*-butane is the precursor for the production of fumaric (*trans*-butenedioic acid), malic (hydroxysuccinic acid), and succinic acids. Annual US production of maleic anhydride is ~260,000 tons, not including maleic anhydride produced as an intermediate in the synthesis of 1,4-butanediol.

Naturally, succinate is produced along with other acids (i.e., acetic, formic, and propionic) by various anaerobic bacteria, including *Actinobacillus succinogenes* [102], *Anaerobiospirillum succiniproducens* [103], and *Mannheimia succiniciproducens* [104], and a few anaerobic fungi, *Neocallimastix*, and yeasts. Succinic acid bacteria can metabolize simple sugars, including glucose, fructose, sucrose, lactose, and maltose, and glycerol. Only a few succinic acid bacteria, such as *Fibrobacter succinogenes* (previously named *Bacteroides succinogenes*) and *Ruminococcus flavefaciens*, can use cellulose to produce high yields of acids (i.e., succinic, acetic, and formic). The biochemical pathways for succinate production by rumen bacteria are quite similar. In general, equal molar quantities of succinate, acetate, and formate are formed per mol glucose and CO_2 fermented – see (12).

$$C_6H_{12}O_6 + CO_2 \rightarrow HOOC{-}CH_2CH_2COOH + CH_3COOH + HCOOH. \quad (12)$$

In batch fermentation at the optimum pH of ~6.0 and with gassing of 1% CO_2, *A. succiniciproducens* produced 50.3 g/L succinic acid from glucose and corn steep liquor as the nitrogen source in 24 h, with a yield of 0.9 g/g glucose and productivity of 2.09 g/L h [105]. About 13.6 g/L acetate was also produced. At pH higher than 6.4, lactate became the major acid product with greatly reduced production of both succinate and acetate. Increasing the CO_2 partial pressure in the bioreactor also increased succinate yield, probably because CO_2 fixation was enhanced at increased CO_2 concentration. Continuous fermentation in an integrated membrane-bioreactor-electrodialysis process produced 83 g/L succinic acid with a productivity of 10.4 g/L h and yield of 0.88 g/g [106]. Glycerol as a more reduced carbon source, compared to glucose, usually resulted in a higher succinic acid yield (1.6 g/g) and reduced production of byproducts in fermentation [103].

A. succinogenes isolated by directed evolution showed higher succinic acid tolerance and production compared to *A. succiniciproducens*. One strain was able to produce more than 60 g/L succinic acid from glucose in a complex medium in less than 36 h, and continued incubation reached 79 g/L in serum vial [107]. This bacterium is a facultative anaerobe, so not as sensitive to oxygen as the strict anaerobic *A. succiniciproducens*. It also had a wider pH range for succinate production, but had a slightly lower succinate yield of 0.83 g/g. Another strain of this species produced 103.4 g/L succinic acid in ~52 h with a high productivity of ~2 g/L h [108]. Cell growth and succinic acid production were enhanced by ~20% and acetic acid production was reduced by ~50% when the reduced neutral red was used as the sole electron donor [109]. *M. succiniciproducens* isolated from cow's rumen could grow in a simple, chemically defined medium which increased succinic acid production by 17%, productivity by 36%, and yield by 15%, with 30% reduction in acetic acid production, as compared with fermentation in a complex medium [110]. The complete genome sequences of *A. succinogenes* and *M. succiniciproducens* are available, enabling metabolic engineering to overexpress critical enzymes in succinic acid biosynthesis and to eliminate competing byproduct formation pathways [104, 111, 112].

A metabolically engineered *Corynebacterium glutamicum* strain with disrupted *ldhA* encoding L-lactate dehydrogenase and *pyc* (pyruvate carboxylase) overexpression produced 146 g/L succinic acid and 16 g/L acetic acid in 46 h under oxygen deprivation with intermittent addition of glucose and sodium bicarbonate [113]. The yields of succinic acid and acetic acid from glucose were 0.92 and 0.10 g/g, respectively. Bicarbonate was used to supply CO_2 for succinic acid synthesis, and its concentration affected both succinic acid production rate and yield.

E. coli has also been metabolically engineered to produce succinic acid from glucose under anaerobic and aerobic conditions [114, 115]. Naturally, *E. coli* produced a mixture of organic acids, including succinic acid, under anaerobic conditions. Strategies for enhancing succinic acid production included overexpressing PEP carboxylase (*ppc*), pyruvate carboxylase (*pyc*), fumarate reductase (*frd*), and malic enzyme, inactivating pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldhA*) to shut down competing pathways, and inactivating the *ptsG* gene and the glucose phosphotransferase system (PTSG) to increase the availability of phosphoenolpyruvate [115]. A high titer of 87 g/L succinic acid was achieved by removing pathways for by-product synthesis [116]. An *E. coli* strain with mutations in *pfl*, *ldhA*, *pyc*, and *ptsG* produced 99.2 g/L succinic acid from glucose with a productivity of 1.3 g/L h and yield of 1.1 g/g in a dual-phase (aerobic for cell growth followed by anaerobic for production) fed-batch fermentation using a complex medium containing yeast extract and tryptone [117, 118]. The higher than 1.0 g/g succinate yield from glucose was attributed to CO_2 fixation and possibly the additional carbon sources present in the complex medium. Fermentative production of succinic acid by *E. coli* could be limited by the available NADH and poor cell growth and slow metabolism under anaerobic conditions [119, 120].

E. coli and the afore-mentioned bacteria require a fermentation pH close to neutral which can cause difficulties in succinic acid recovery and purification [121]. Metabolic engineering of *Saccharomyces cerevisiae*, which can grow at a low pH of ~4.0, has thus also been studied for succinic acid production [122, 123]. However, the highest succinic acid titer so far was only ~13 g/L with a low yield of less than 0.14 g/g glucose [123].

4.2 Malic Acid

Malic acid, a food additive and an intermediate in the synthesis of fine chemicals, is presently manufactured as a racemic mixture of D-(−) and L-(+) isomers through the hydration of maleic or fumaric acid [124]. On the other hand, optically pure L-(+)-malic acid, which can be produced from glucose by microbial fermentation, is desirable for applications in foods and pharmaceuticals. Malic acid is an intermediate in the TCA cycle in aerobic metabolism and is found naturally in fruits, such as apples. However, the over-production of malic acid is only found in some fungal cells under aerobic conditions. Its overproduction by these microorganisms is mainly from oxaloacetate produced from the carboxylation of pyruvate in the cytoplasm, known as the reductive tricarboxylic acid (rTCA) pathway, and does not involve the TCA cycle. The maximum theoretical yield is 2 mol/mol glucose or 1.49 g/g – see (13). The actual yield in fermentation is much lower because of the formation of cell biomass and co-production of acetate and succinate [125–129].

$$C_6H_{12}O_6 + 2\,CO_2 \rightarrow 2\,HOOC-CH_2CHOHCOOH. \tag{13}$$

The fermentative production of malic acid has been demonstrated with *Aspergillus flavus*, attaining a high production of 113 g/L from glucose with a productivity of 0.59 g/L h and yield of 1.26 mol/mol or 0.94 g/g [125]. However, commercial application of *A. flavus* is limited by its ability to produce toxic aflatoxin, a safety concern in the food industry. A natural yeast isolate *Zygosaccharomyces rouxii* produced up to 74.9 g/L malic acid with a yield of 0.40 g/g [126]. Other fungi, including *Aspergillus niger*, *Monascus araneosus*, and *Schizophyllum commune*, have also been reported to produce malic acid, but at lower levels (18–28 g/L) [127–129]. Interestingly, the yeast *Aureobasidium pullulans* was able to produce a large amount of malic acid in the polymeric form, poly-malic acid (PMA), which upon hydrolysis gave malic acid [130, 131]. As high as 123.7 g/L PMA or 142.2 g/L malic acid can be produced from glucose at a productivity of 0.74 g/L h and yield of 0.55 g/g in a fed-batch bioreactor with aeration [131].

Metabolic engineering has been used for enhancing malic acid biosynthesis. Overexpression of native C4-dicarboxylate transporter and cytosolic alleles of pyruvate carboxylase (PYC) and malate dehydrogenase (MDH) in *Aspergillus oryzae* increased its malate production from glucose to 154 g/L with a productivity of 0.94 g/L h and yield of 1.03 g/g [132]. Similarly, a malate biosynthesis pathway

was constructed in *S. cerevisiae* by overexpressing the native PYC2 gene (encoded for PYC), an allele of the MDH3 gene retargeted to the cytosol with the deletion of the C-terminal peroxisomal targeting sequence, and the malate transporter gene SpMAE1 from *Schizosaccharomyces pombe*, and the engineered yeast produced up to 59 g/L malic acid from glucose with a productivity of 0.19 g/L h and yield of 0.31 g/g [133]. Several recombinant *E. coli* strains have also been constructed for malic acid production [134–136]. *E. coli* C [116] was engineered by deleting three genes (*ldhA*, *adhE*, *ackA*) in the central anaerobic fermentation pathway to produce succinate or malate as the primary metabolite in mineral salts media in an anaerobic stirred bioreactor with pH control. Evolution and selection of strains with improved growth resulted in increased production of these dicarboxylic acids. Additional deletions of three genes encoding FOC (*focA*), PFL (*pflB*), and MGS (*mgsA*), respectively, resulted in the best malate-producing strain, which produced 69 g/L malate with a productivity of 0.69 g/L h and yield of 1.04 g/g glucose metabolized [135]. *E. coli* strains previously developed for succinate production were also modified for malate production. It was found that a mutation in fumarate reductase (*frd*) alone redirected carbon flow into malate even in the presence of fumarase, whereas deleting fumarase isoenzymes did not affect succinate production. A strain produced 34 g/L malate from glucose with a high yield of 1.06 g/L and productivity of 0.47 g/L h in a two-stage process with aerobic cell growth and anaerobic malate production [136].

4.3 Fumaric Acid

Fumaric acid, a four-carbon dicarboxylic acid with a carbon–carbon double bond, is a starting material for the synthesis of polymers and resins. It is currently produced by petrochemical synthesis through catalytic isomerization of maleic acid [137]. However, commercial production of fumaric acid by fermentation was once practiced using a strain of *Rhizopus arrhizus*. Naturally, *Rhizopus* species (*arrhizus*, *oryzae*, *formosa*, *nigricans*) are the best microorganisms for fumaric acid production [138], although other fungi, including *Penicillium griseofulvum*, *Aspergillus glaucus*, and *Caldariomyces fumago*, can also produce fumaric acid. Similar to malic acid, fumaric acid biosynthesis in *R. oryzae* is mainly by the reductive TCA pathway (rTCA), including pyruvate carboxylation with CO_2 fixation, oxaloacetate hydrogenation, and malate dehydration, with a high theoretical yield of 2 mol/mol or 1.29 g/g glucose – see (14).

$$C_6H_{12}O_6 + 2\,CO_2 \rightarrow 2\,HOOC{-}CH{=}CHCOOH + 2\,H_2O. \qquad (14)$$

However, the energy and reducing power are not balanced in the rTCA pathway, and the oxidative TCA cycle, which limits the theoretical yield to 1 mol/mol glucose, is also used for fumaric acid biosynthesis [139]. With optimized DO and $CaCO_3$ concentrations, up to 130 g/L fumaric acid was produced from glucose at a

productivity of 0.92 g/L h and yield of 1.0 g/g by *A. arrhizus* [140]. With simultaneous production and recovery of fumaric acid, a rotary biofilm contactor with immobilized *R. oryzae* gave a high fumarate productivity of 4.25 g/L h and yield of 0.85 g/g [141]. Good fumarate production from cheap feedstocks such as brewery wastewater [142] and crude glycerol from biodiesel wastes [143] has also been reported with these *Rhizopus* species.

The effects of overexpressing fumarase (*fumR*), pyruvate carboxylase (*pyc*), and PEP carboxylase (*ppc*) in *R. oryzae* have been studied; and the results showed that overexpressing *ppc* increased fumarate production by ~26%, whereas *fumR* overexpression increased malate, instead of fumarate, production, and *pyc* overexpression caused poor cell growth and fumarate production [144, 145]. Metabolic engineering has also been used to create fumarate-producing *E. coli*, *S. cerevisiae*, and *Torulopsis glabrata*; however, fumaric acid production by these mutants was relatively poor with a low product titer (5.6–28.2 g/L), yield (0.13–0.38 g/g), and productivity (0.059–0.448 g/L h) [139, 146, 147].

4.4 Other Dicarboxylic Acids

Itaconic acid is a five-carbon dicarboxylic acid used as a precursor to several widely used polymers, with a worldwide market of over 80,000 tons [148]. The current commercial production of itaconic acid is by fermentation with *Aspergillus terreus*, which is the most efficient producer of itaconic acid from glucose with a final titer as high as 82.3 g/L [149]. However, productivity and yield are generally lower compared to four-carbon dicarboxylic acids discussed before (succinic, malic, fumaric). Some yeasts such as *Candida* and *Rhodotorula* strains obtained by mutagenesis were also capable of producing itaconic acid [150]. The key enzyme in the itaconic acid biosynthesis pathway is *cis*-aconitic acid decarboxylase (CAD). Attempts to engineer *S. cerevisiae* for itaconic acid production using sequential, in silico computational genome-scanning to identify beneficial genetic perturbations improved itaconic acid production titer, but the best strain only produced 168 mg/L itaconic acid in high-cell density fermentation [151]. A better host, *Yarrowia lipolytica*, which can produce a large amount of citric acid, has also been metabolically engineered for itaconic acid production by overexpressing a heterologous CAD, and the mutant produced 4.6 g/L itaconic acid from glucose with a yield of 0.058 g/g and maximum productivity of 0.045 g/L h in bioreactor fermentation [152].

Adipic acid is a six-carbon dicarboxylic acid primarily used for the production of nylon-6,6 polyamide, polyurethanes, and polyester polyols in the chemical industry, with a worldwide market of 2.6 million tons per year and an annual growth rate of 3.0–3.5% [153]. It is also used in the food industry for acidulation. Currently, adipic acid is produced from benzene or butadiene via chemical synthesis, but there is also great interest in producing bio-based adipic acid. Although adipic acid is found in some tissues as a result of the degradation of longer aliphatic dicarboxylic

acids, *n*-alkylcyclohexanes, and adiponitrile, no known microorganism or bioreaction can produce adipic acid directly from glucose. Recently, a synthetic pathway for adipic acid biosynthesis was constructed in *E. coli*, but the mutant produced only <1 mg/L adipic acid from glucose in fermentation [154]. Alternatively, biobased adipic acid can be produced from *Muconic acid* or *Glucaric acid* derived from glucose in fermentation using metabolically engineered *E. coli*. A recombinant *E. coli* expressing a heterologous pathway composed of 3-dehydroshikimic acid dehydratase (*aroZ*) and protocatechuic acid decarboxylase (*aroY*) from *K. pneumoniae* and catechol 1,2-dioxygenase (*catA*) from *Acinetobacter calcoaceticus* produced 36.8 g/L muconic acid from glucose with a productivity of 0.77 g/L h and yield of 0.18 g/g [155]. Upon hydrogenation with H_2 under pressure, the muconic acid in the fermentation broth was converted to adipic acid with 97 mol% yield. Synthetic biology was also used to construct glucaric acid biosynthesis pathway in *E. coli*, which produced 1.13–2.37 g/L glucaric acid [156, 157].

5 Other Carboxylic Acids and Bulk Chemicals

Acrylic acid (2-propenoic acid) is a commodity chemical widely used in polymeric flocculants, dispersants, coatings, paints, adhesives, and binders, with an annual worldwide market of 4.5 million tons. Current commercial production of acrylic acid is mainly by partial oxidation of propene and by a newer two-step process via acrolein [158]. The bioproduction of acrylic acid from glucose is possible, because acrylyl-CoA is an intermediate in the 3-HP cycle [159] and in the acrylic acid pathway found in *C. propionicum* [9], but would be very difficult to achieve because of its high toxicity to cells [158]. Alternatively, bio-based acrylic acid can be produced from the dehydration of 3-HP [158] produced from glycerol in *E. coli* [96] or *K. pneumonia* [99] discussed earlier.

Pyruvate, a central intermediate in the carbon and energy metabolism in almost all organisms, is used mainly in food, cosmetics, agrochemical, and pharmaceutical industries [160]. Commercial production of pyruvate is by chemical synthesis or fermentation using a multi-vitamin auxotrophic yeast, *Torulopsis glabrata*, which produced 60.4 g/L pyruvate from glucose at a productivity of 1.28 g/L h and yield of 0.68 g/g [161]. *C. glutamicum*, *E. coli*, and *S. cerevisiae* have also been engineered to produce pyruvate at titers of 44.5 g/L [162], 89 g/L [163], and 135 g/L [164], respectively.

Citric acid, a tricarboxylic acid formed in the TCA cycle, is extensively used in foods, pharmaceuticals, detergents, and cosmetics, with a worldwide market of more than 1.2 million tons per year [165]. Current commercial production of citric acid is mainly from molasses or sucrose by aerobic fermentation with *Aspergillus niger* [166]. Some yeasts such as *Candida oleophila* [167] and *Y. lipolytica* [168, 169] can also produce large amounts of citric acid, with isocitrate as a byproduct, which can be reduced or eliminated by deleting ATP-citrate lyase and

expressing isocitrate lyase [170]. In general, a high citric acid titer of 140 g/L, productivity of >1.0 g/L h, and yield up to 0.9 g/g can be obtained in fermentation under aerobic conditions. There is no known anaerobic pathway that can produce citric acid from sugar. *Gluconic acid*, a strong chelating agent widely used in foods and various industrial products, is currently produced by oxidizing the aldehyde group of glucose with either bacteria or filamentous fungi in fermentation with aeration, with a high titer of 140–260 g/L, productivity of ~10 g/L h, and yield of >0.9 g/g [171, 172].

Other carboxylic acids such as α-ketoglutaric acid [173] and many other bulk chemicals, including various alcohols (ethanol, propanol, butanol, etc.), diols (1,3-PDO, 1,4-butanediol, 2,3-butanediol), diamines (putrescine, cadaverine), and hydrocarbons (isoprene, styrene), can also be produced by fermentation with natural or engineered microorganisms (see review articles [174–177]).

6 Challenges and Future Prospects

Successful commercialization of bio-based chemicals depends on the production cost or process economics. Figure 4 shows a general bioprocess involving feedstock that may require pretreatments and hydrolysis with enzymes, fermentation with selected microorganisms for converting the substrate to the product, and separation to get the final purified product. Industrial production of the carboxylic acids discussed in this chapter thus requires low-cost feedstock, robust producing strains, and high process performance with respect to the product titer, yield, productivity, and purity. For economical production of biobased carboxylic acids and bulk chemicals in general, the fermentation process must have a final product titer of >50–100 g/L, productivity of >1–2 g/L h, yield of >0.5 g/g, and minimal or no impurity byproducts [35]. As can be seen in Table 2, not all carboxylic acids can be produced in fermentation at a sufficiently high titer, productivity, or yield for commercial application, largely because of the toxicity of the chemical to cells [179]. Non-native producers with synthetic biosynthesis pathways created through metabolic engineering usually suffer from low product tolerance as compared to native producers, such as in the cases of propionic acid, butyric acid, and fumaric acid production using recombinant *E. coli* [47]. Low product tolerance results in poor cell growth or activity and low product titer and reactor productivity. Strain engineering through mutagenesis, adaptive evolutionary engineering, and metabolic engineering has been applied successfully to increase cell tolerance to toxic metabolite [74, 179–181], which can partially solve the low titer and productivity issues, and may be used in future strain development. Reactor productivity can also be greatly enhanced by increasing (viable) cell density through cell immobilization [11, 70] or recycling [21, 22] and by in situ removal of toxic metabolite to alleviate product inhibition [35, 78, 182]. Figure 5 illustrates two widely studied high-cell-density fermentation processes which can greatly increase reactor productivity and final product titer and yield. One is free-cell fermentation with cell recycling via

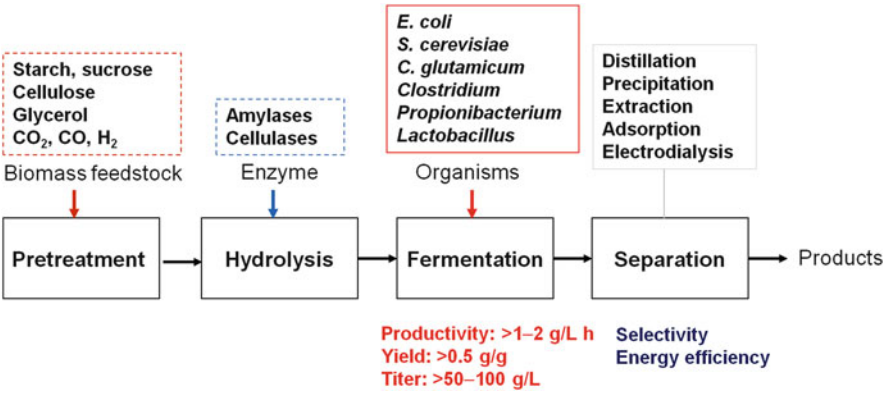


Fig. 4 Bioprocess for production of bulk chemicals involving different types of biomass feedstocks, enzymes, microorganisms, and separation technologies

filtration or centrifugation to separate cells from the effluent, and the other is immobilized-cell fermentation with cells retained in the bioreactor via adsorption on and entrapment in a solid support matrix. As discussed earlier, cell immobilization in an FBB not only increased cell density but also allowed cells to adapt and evolve to become more tolerant and productive for the inhibitory metabolite they produced [17, 22, 75]. However, FBB and other similar immobilized cell bioreactors have not yet been scaled up for industrial production of bulk chemicals.

Fermentation processes for lower-value bulk chemicals, such as acetic, butyric, propionic, and malic acids, cannot compete very well with petrochemical processes in the chemical market because fermentation usually also produces a significant amount of other byproducts which are difficult and costly to separate. Metabolic engineering has been used to knock out pathways leading to undesirable metabolites, which, however, often have to be produced by cells for redox balance and ATP generation, and their knockouts could result in poor cell growth and metabolic activities [62, 115]. Metabolic process engineering, which manipulates metabolic flux distribution through controlling the fermentation conditions such as pH, and substrate and other medium components, can also be used to increase product yield and purity [183]. For example, a higher product yield and purity can be obtained when glycerol, instead of glucose, is used as the carbon source for propionic acid production [21, 32]. Adding an artificial electron carrier in the medium also shifted the metabolic flux in *C. tyrobutyricum* from acetate to butyrate biosynthesis, resulting in a higher-purity butyric acid product in the fermentation broth [80].

Nevertheless, for bio-based carboxylic acids to be competitive in the chemical market, substantial improvements in separation technologies are needed. Separation costs could account for more than 50% of the final product cost [182]. Carboxylic acids are non-volatile or have very low vapor pressures compared to alcohols and hydrocarbons, and thus cannot be separated economically by conventional distillation. Current separation methods for recovering and purifying carboxylic acids, such as lactic acid and citric acid, from fermentation broth include

Table 2 Microbial production of some carboxylic acids and their market size

Chemical	Market (tons)	Microorganism/substrate[a] or process	Titer (g/L)	Productivity (g/L h)	Yield (g/g)	Reference
Acetic acid	10,000,000	*C. thermoaceticum*	100	0.8	0.8	[3]
		C. formicoaceticum/fructose	78	0.95	0.95	[4]
		A. woodii/CO_2, H_2	50	0.52	NA	[6]
		E. coli	51.8	0.95	0.5	[7]
Propionic acid	~450,000	*P. acidipropionici*/and glycerol	68.9	1.55	0.48	[16]
			97	0.05	0.54	[10]
		E. coli	0.27	NA	NA	[47]
Butyric acid	80,000	*C. tyrobutyricum*	86.9	1.10	0.46	[75]
		E. coli/+ acetate	10	0.21	0.36	[65]
Lactic acid	450,000	*L. delbrueckii*	135	3.4	0.9	[83]
		E. coli	138	3.54	0.99	[90]
		Sporolactobacillus sp.	207	3.8	0.93	[91]
3-HP	Precursor for acrylic acid	*K. pneumonia*/glycerol	28	0.58	0.4	[99]
		E. coli/glycerol	38.7	0.54	0.35	[95]
			42.1	1.2	0.268	[96]
Succinic acid	30,000	*A. succiniciproducens*/+ CO_2	83	10.4	0.88	[106]
		A. succinogenes/+ CO_2	103.4	2.0	0.83	[108]
		C. glutamicum	146	3.2	0.9	[113]
		E. coli	99.2	1.31	1.10	[117]
Malic acid	200,000	*A. flavus*	113	0.59	0.94	[125]
		A. oryzae	154	0.94	1.03	[132]
		Z. rouxii	75	0.52	0.40	[126]
		A. pullulans	142.2[b]	0.74	0.55	[131]
		S. cerevisiae	59	0.19	0.31	[133]
		E. coli	69	0.69	1.04	[135]

Fumaric acid	90,000	*R. arrhizus*	130	0.92	1.0	[140]
		R. oryzae	92	4.25	0.85	[141]
		S. cerevisiae	5.64	0.059	0.13	[139]
		E. coli	28.2	0.448	0.38	[146]
Itaconic acid	80,000	*Aspergillus terreus*	82.3	0.57	0.54	[149]
Adipic acid	2,600,000	Hydrogenation of muconic acid	–	–	–	[153]
		Dehydration of glucaric acid				
Muconic acid	Precursor for adipic acid	*E. coli*	36.8	0.77	0.18	[155]
Glucaric acid	42,000	*E. coli*	1.1–2.4	0.016–0.049	0.15	[156, 157]
Acrylic acid	4,500,000	Dehydration of lactic acid or 3-HP	–	–	–	[158]
Pyruvic acid	>1,000	*Torulopsis glabrata*	60.4	1.28	0.68	[161]
		C. glutamicum	44.5	0.49	0.72	[162]
		E. coli	88.9	2.08	0.67	[163]
		S. cerevisiae	135	1.35	0.54	[164]
Citric acid	1,200,000	*A. niger*	140	0.5–0.8	0.7–0.9	[166, 178]
		Candida oleophila	166.5	1.28	0.50	[167]
		Yarrowia lipolytica/Glycerol	139	1.16	0.69	[168, 169]
Gluconic acid	50,000	*A. niger*	140–230	3–8	0.9	[171]
		A. pullulans	208–260	10–19.3	0.93	[172]

[a]Glucose unless otherwise noted
[b]Malic acid obtained after acid hydrolysis of PMA
NA not available

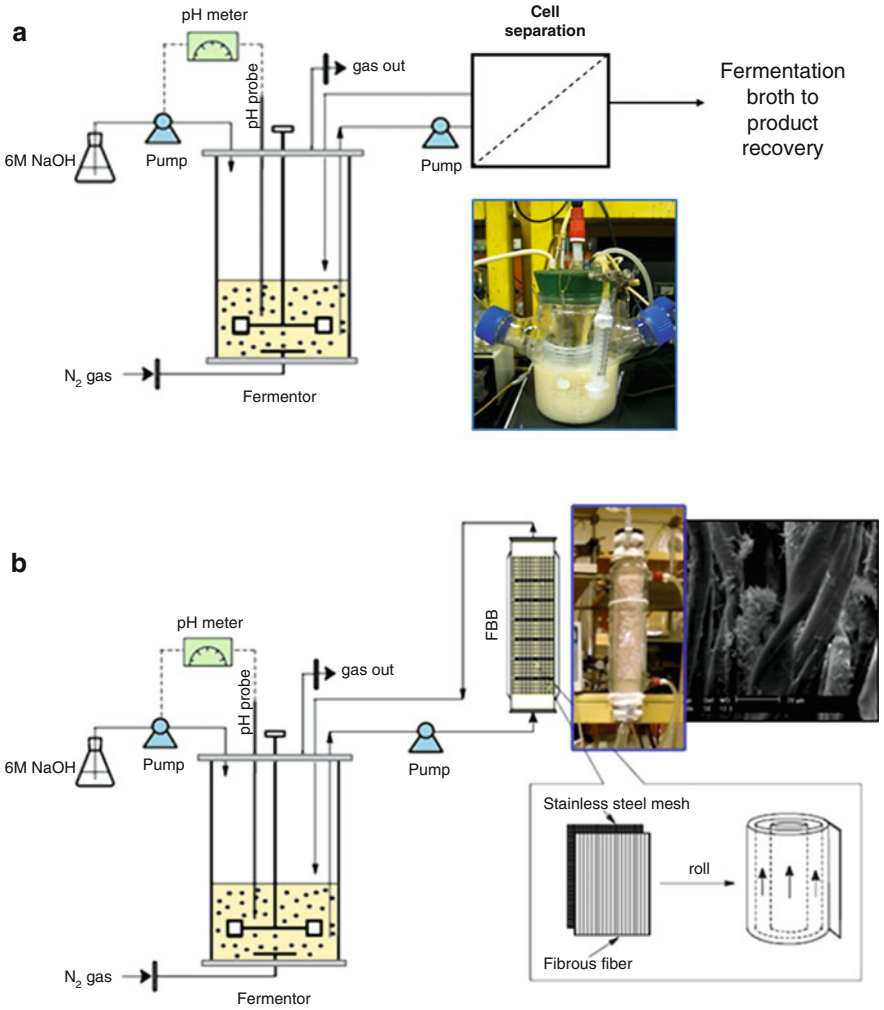


Fig. 5 Fermentation processes with cell recycle via cell separation by filtration or centrifugation (**a**) and immobilization via adsorption and entrapment in a porous support matrix (**b**) for organic acid production. Photos show high density of cells in bioreactor either as free cells (**a**) or immobilized cells (**b**). The construction of the fibrous bed bioreactor (FBB) with free flow channels avoiding bed clogging is also illustrated

precipitation and solvent extraction. Adsorption with ion-exchange resins and electrodialysis with bipolar membranes have also been developed for separating some carboxylic acids, although they are not yet widely used in industry. Table 3 compares the pros and cons of these separation methods. The choice of the separation method would depend on the type of carboxylic acid and its concentration and purity in the fermentation broth. More development work would be needed

Table 3 Separation methods for recovering carboxylic acids from fermentation broth

Method	Operating principle	Advantages	Disadvantages
Precipitation	Based on low solubility of the calcium salts of carboxylic acids	Low capital costs, performed with simple equipment, high yield	Produce solid waste $CaSO_4$; require the addition of H_2SO_4 to release carboxylic acid
Ion exchange adsorption	Adsorption of undissociated organic acids to ionic exchange resins followed with desorption	High selectivity, high yield for charged molecules; can be used for in situ recovery	Resins are expensive and regeneration of the resin requires additional chemicals and/or energy
Solvent extraction	Distribution between organic and aqueous phases (two immiscible phases) based on different solubilities of the carboxylic acid	Widely used in industry for recovery of lactic acid and citric acid; easy to operate and scale up	Toxicity of solvent to cells; extractant requires regeneration
Electrodialysis	Use electric current to move negatively charged carboxylate ions through an anion-exchange membrane towards the anode in the electrodialyzer	Carboxylate is concentrated in aqueous solution, does not require acid addition to adjust the solution pH	Low product purity and needs further purification; high energy input; membrane fouling; difficult to scale up

Yang and Lu [182]

to improve their selectivity and lower operating costs for industrial production of carboxylic acids.

Although glucose (from starch) or sucrose (from sugarcane and molasses) is usually the substrate used in fermentation, non-food material such as lignocellulosic biomass is the desirable feedstock for biobased chemicals in biorefinery because of its low cost, large-scale availability, environmentally benign production, and near-zero GHG emission when used for biofuels and chemicals production [184, 185]. However, using lignocellulosic biomass as substrate in fermentation presents additional technological challenges and economic hurdles to overcome, although progress has been made on ethanol production from cellulosic biomass such as corn stover and switch grass. Glycerol is another alternative feedstock for biobased chemicals production [186]. It was estimated that about 4 billion gallons or 38.85 billion lb of crude glycerol would be produced in 2016 as a byproduct in the biodiesel industry [187]. Crude glycerol could therefore be available at a much lower cost compared to sugar, and, meanwhile, could provide some unique advantages such as higher product yields for more reduced products in a heterofermentative pathway, although cell growth on glycerol could be compromised [32]. Recently, there has also been increased interest in using synthesis and process waste gases containing CO, CO_2, and H_2 for biofuels and chemicals production [188, 189]. However, poor mass transfer properties of the gaseous substrates (mainly CO and H_2), slow cell growth, and low productivity and yield are common

problems in industrial syngas fermentation [190]. Because CO_2 is a by-product in many fermentation processes, it is desirable to utilize CO_2 produced in fermentation for biofuels and chemicals production using carboxydotrophic bacteria, such as homoacetogens, which use the Wood–Ljungdahl pathway to fix CO_2 to produce acetate. The acetogenic clostridia can also be metabolically engineered to produce higher-value bulk chemicals such as *n*-butanol, which can not only improve the economics of bio-based chemicals but also further reduce GHG emission [191].

Although advances in process engineering and cell engineering have significantly improved the bioconversion efficiency and reduced the product costs, most of the current petroleum-based chemicals still cannot be economically produced from biomass or via fermentation. Metabolic pathway engineering, synthetic biology, and systems biology offer powerful tools for developing novel strains for the production of bulk chemicals [192–195]. In silico genome-scale modeling and omic analysis of genes, enzymes and metabolites in the metabolic pathways can provide the information needed for systems metabolic engineering. Through the design of responsive, selective, and controllable metabolic systems, metabolic flux and gene regulation can be precisely predicted and controlled in the future [196]. To date, *E. coli* and *S. cerevisiae* have been the most engineered cell factories for chemicals and fuels production [197, 198]. Other robust organisms such as *C. glutamicum* [199] and *Clostridium* [200] have also been successfully engineered to produce carboxylic acids with high titers and yields.

However, many native carboxylic acid-producing microorganisms, including *Propionibacterium* and *Clostridium*, are difficult to engineer genetically because of the lack or limitation of cloning tools, which must be developed first to facilitate the metabolic engineering of less studied microorganisms [9, 201]. Furthermore, a hybrid biological/chemical process may be more efficient in producing some biobased bulk chemicals, such as in the cases of adipic acid and acrylic acid discussed earlier [153, 158]. It should be mentioned that carboxylic acids produced in anaerobic fermentation can be converted to alcohols, either biologically or chemically. For example, the butyric acid-producing *C. tyrobutyricum* was engineered to produce *n*-butanol as the main product by overexpressing an aldehyde/alcohol dehydrogenase (*adhE2*) [202]. Similarly, fermentation-produced propionic acid and acetic acid can be converted to *n*-propanol and ethanol, respectively. These alcohols can be catalytically dehydrated to the corresponding alkenes, which are major feedstock chemicals in current petroleum refineries [185]. In Brazil, "green" polyethylene and ethylene are produced from bioethanol obtained from sugarcane, a move from petroleum-based feedstock toward bio-based feedstock for sustainability and carbon credit in the traditional petrochemical industry.

Acknowledgements This work was supported in part by the National Science Foundation STTR program (IIP-1026648), Advanced Research Projects Agency–Energy (DE-AR0000095), the Department of Energy, EERE Bioenergy Technologies Incubator program (DE-EE0007005), and the National Science Foundation of China (21276093).

References

1. Yang ST, Yu M, Chang WL, Tang IC (2013) Anaerobic fermentations for the production of acetic and butyric acids. In: Yang ST, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. Wiley, Hoboken, pp 351–373
2. Ljungdahl LG, Hugenholtz J, Wiegel J (1989) Acetogenic and acid-producing Clostridia. In: Minton NP, Clarke DJ (eds) Clostridia. Plenum, New York, pp 145–180
3. Parekh SR, Cheryan M (1994) Continuous production of acetate by *Clostridium thermoaceticum* in a cell-recycle membrane bioreactor. Enzyme Microb Technol 16:104–109
4. Huang YL, Mann K, Novak JM, Yang ST (1998) Acetic acid production from fructose by *Clostridium formicoaceticum* in a fibrous-bed bioreactor. Biotechnol Prog 14:800–806
5. Huang Y, Yang ST (1998) Acetate production from whey lactose using co-immobilized cells of homolactic and homoacetic bacteria in a fibrous-bed bioreactor. Biotechnol Bioeng 60:498–507
6. Strauba M, Demlerb M, Weuster-Botzb D, Dürre P (2014) Selective enhancement of autotrophic acetate production with genetically modified *Acetobacterium woodii*. J Biotechnol 178:67–72
7. Causey TB, Zhou S, Shanmugam KT, Ingram LO (2003) Engineering the metabolism of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products: homoacetate production. Proc Natl Acad Sci U S A 100:825–832
8. Samel U-R, Kohler W, Gamer AO, Keuser U, Yang ST, Jin Y, Lin M, Wang Z (2014) Propionic acid and derivatives. In: ULLMANN'S encyclopedia of industrial chemistry. Wiley, Weinheim. doi:10.1002/14356007.a22_223.pub2
9. Wang Z, Sun J, Zhang A, Yang ST (2013) Propionic acid fermentation. In: Yang ST, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. Wiley, Hoboken, pp 331–349
10. Zhang A, Yang ST (2009) Engineering *Propionibacterium acidipropionici* for enhanced propionic acid tolerance and fermentation. Biotechnol Bioeng 45:79–386
11. Huang YL, Wu Z, Zhang L, Cheung CM, Yang ST (2002) Production of carboxylic acids from hydrolyzed corn meal by immobilized cell fermentation in a fibrous-bed bioreactor. Bioresour Technol 82:51–59
12. Stowers CC, Cox BM, Rodriguez BA (2014) Development of an industrializable fermentation process for propionic acid production. J Ind Microbiol Biotechnol 41:837–852
13. Liu Z, Ma C, Gao C, Xu P (2012) Efficient utilization of hemicellulose hydrolysate for propionic acid production using *Propionibacterium acidipropionici*. Bioresour Technol 114:711–714
14. Feng X, Chen F, Xu H, Wu B, Li H, Li S, Ouyang P (2011) Green and economical production of propionic acid by *Propionibacterium freudenreichii* CCTCC M207015 in plant fibrous-bed bioreactor. Bioresour Technol 102:6141–6146
15. Kagliwal LD, Survase SA, Singhal RS, Granström T (2013) Wheat flour based propionic acid fermentation: an economic approach. Bioresour Technol 129:694–699
16. Liang Z, Li L, Li S, Cai Y, Yang ST, Wang J (2012) Enhanced propionic acid production from Jerusalem artichoke hydrolysate by immobilized *Propionibacterium acidipropionici* in a fibrous-bed bioreactor. Bioprocess Biosyst Eng 35:915–921
17. Zhu L, Wei P, Cai J, Zhu X, Wang Z, Huang L, Xu Z (2012) Improving the productivity of propionic acid with FBB-immobilized cells of an adapted acid-tolerant *Propionibacterium acidipropionici*. Bioresour Technol 112:248–253
18. Yang ST, Huang Y, Hong G (1995) A novel recycle batch immobilized cell bioreactor for propionate production from whey lactose. Biotechnol Bioeng 45:379–386
19. Boyaval P, Corre C (1987) Continuous fermentation of sweet whey permeate for propionic acid production in a CSTR with UF recycle. Biotechnol Lett 9:801–806

20. Chen F, Feng XH, Liang JF, Xu H, Ouyang PK (2013) An oxidoreduction potential shift control strategy for high purity propionic acid production by *Propionibacterium freudenreichii* CCTCC M207015 with glycerol as sole carbon source. Bioprocess Biosyst Eng 36:1165–1176
21. Dishisha T, Alvarez MT, Hatti-Kaul R (2012) Batch and continuous propionic acid production from glycerol using free and immobilized cells of *Propionibacterium acidipropionici*. Bioresour Technol 118:553–562
22. Wang Z, Jin Y, Yang ST (2015) High cell density propionic acid fermentation with an acid tolerant strain of *Propionibacterium acidipropionici*. Biotechnol Bioeng 112:502–511
23. Dishisha T, Ståhl A, Lundmark S, Hatti-Kaul R (2013) An economical biorefinery process for propionic acid production from glycerol and potato juice using high cell density fermentation. Bioresour Technol 135:504–512
24. Dishisha T, Ibrahim MH, Cavero VH, Alvarez MT, Hatti-Kaul R (2015) Improved propionic acid production from glycerol: combining cyclic batch and sequential batch fermentations with optimal nutrient composition. Bioresour Technol 176:80–87
25. Rickert DA, Glatz CE, Glatz BA (1998) Improved organic acid production by calcium alginate-immobilized propionibacteria. Enzyme Microb Technol 22:409–414
26. Jin Z, Yang ST (1998) Extractive fermentation for enhanced propionic acid production from lactose by *Propionibacterium acidipropionici*. Biotechnol Prog 14:457–465
27. Wang P, Wang Y, Liu Y, Shi H, Su Z (2012) Novel *in situ* product removal technique for simultaneous production of propionic acid and vitamin B_{12} by expanded bed adsorption bioreactor. Bioresour Technol 104:652–659
28. Hsu ST, Yang ST (1991) Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: effects of pH. Biotechnol Bioeng 38:571–578
29. Zhuge X, Li J, Shin H, Liu L, Du G, Chen J (2015) Improved propionic acid production with metabolically engineered *Propionibacterium jensenii* by an oxidoreduction potential-shift control strategy. Bioresour Technol 175:606–612
30. Emde R, Schink B (1990) Enhanced propionate formation by *P. freudenreichii* subsp. *freudenreichii* in a three-electrode amperometric culture system. Appl Environ Microbiol 56:2771–2776
31. Wang P, Jiao Y, Liu S (2014) Novel fermentation process strengthening strategy for production of propionic acid and vitamin B12 by *Propionibacterium freudenreichii*. J Ind Microbiol Biotechnol 41:1811–1815
32. Wang Z, Yang ST (2013) Propionic acid production in glycerol/glucose co-fermentation by *Propionibacterium freudenreichii* subsp. *shermanii*. Bioresour Technol 137:116–123
33. Zhang A, Sun J, Wang Z, Yang ST, Zhou H (2015) Effects of carbon dioxide on cell growth and propionic acid production from glycerol and glucose by *Propionibacterium acidipropionici*. Bioresour Technol 175:374–381
34. Suwannakham S, Yang ST (2005) Enhanced propionic acid fermentation by *Propionibacterium acidipropionici* mutant obtained by adaptation in a fibrous-bed bioreactor. Biotechnol Bioeng 91:325–337
35. Yang ST, Huang H, Tay A, Qin W, De Guzman L, San Nicolas EC (2006) Extractive fermentation for the production of carboxylic acids. In: Yang ST (ed) Bioprocessing for value-added products from renewable resources. Elsevier, Amsterdam, pp 421–446
36. Wódzki R, Nowaczyk J, Kujawski M (2000) Separation of propionic and acetic acid by pertraction in a multimembrane hybrid system. Sep Purif Technol 21:39–54
37. Suwannakham S, Huang Y, Yang ST (2006) Construction and characterization of *ack* knockout mutants of *Propionibacterium acidipropionici* for enhanced propionic acid fermentation. Biotechnol Bioeng 94:383–395
38. Ammar EM, Jin Y, Wang Z, Yang ST (2014) Metabolic engineering of *Propionibacterium freudenreichii*: effect of expressing phosphoenolpyruvate carboxylase on propionic acid production. Appl Microbiol Biotechnol 98:7761–7772

39. Parizzi LP, Grassi MC, Llerena LA, Carazzolle MF, Queiroz VL, Lunardi I, Zeidler AF, Teixeira PJPL, Mieczkowski P, Rincones J, Pereira GAG (2012) The genome sequence of *Propionibacterium acidipropionici* provides insights into its biotechnological and industrial potential. BMC Genomics 13:562
40. Falentin H, Deutsch SM, Jan G, Loux V, Thierry A, Parayre S, Maillard MB, Dherbécourt J, Cousin FJ, Jardin J, Siguier P, Couloux A, Barbe V, Vacherie B, Wincker P, Gibrat JF, Gaillardin C, Lortal S (2010) The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1, a hardy *actinobacterium* with food and probiotic applications. PLoS One 5(7): e11748
41. Horváth B, Hunyadkürti J, Vörös A, Fekete C, Urbán E, Kemény L, Nagy I (2012) Genome sequence of *Propionibacterium acnes* type II strain ATCC 11828. J Bacteriol 194:202–203
42. Wang Z, Ammar EM, Zhang A, Wang L, Lin M, Yang ST (2015) Engineering *Propionibacterium freudenreichii* subsp. *shermanii* for enhanced propionic acid fermentation: effects of overexpressing propionyl-CoA:Succinate CoA transferase. Metab Eng 27:46–56
43. Wang Z, Lin M, Wang L, Ammar EM, Yang ST (2015) Metabolic engineering of *Propionibacterium freudenreichii subsp. shermanii* for enhanced propionic acid fermentation: effects of overexpressing three biotin-dependent carboxylases. Process Biochem 50:194–204
44. Zhuge X, Liu L, Shin HD, Chen RR, Li J, Du G, Chen J (2013) Development of a *Propionibacterium-Escherichia coli* shuttle vector as a useful tool for metabolic engineering of *Propionibacterium jensenii*, an efficient producer of propionic acid. Appl Environ Microbiol 79:4595–4602
45. Liu L, Zhuge X, Shin H, Chen RR, Li J, Du G, Chen J (2015) Improved production of propionic acid in *Propionibacterium jensenii* via combinational overexpression of glycerol dehydrogenase and malate dehydrogenase from *Klebsiella pneumoniae*. Appl Environ Microbiol 81:2256–2264
46. Ammar EM, Wang Z, Yang ST (2013) Metabolic engineering of *Propionibacterium freudenreichii* for n-propanol production. Appl Microbiol Biotechnol 97:4677–4690
47. Kandasamy V, Vaidyanathan H, Djurdjevic I, Jayamani E, Ramachandran KB, Buckel W, Jayaraman G, Ramalingam S (2013) Engineering *Escherichia coli* with acrylate pathway genes for propionic acid synthesis and its impact on mixed-acid fermentation. Appl Microbiol Biotechnol 97:1191–1200
48. Kroschwitz JI (1997) Kirk-Othmer encyclopedia of chemical technology V23: sugar to thin films, 4th edn. Wiley, New York, p 1118
49. Zhang C, Yang H, Yang F, Ma Y (1989) Current progress on butyric acid production by fermentation. Curr Microbiol 59:656–663
50. Dwidar M, Park JY, Mitchell RJ, Sang BI (2012) The future of butyric acid in industry. Sci World J 2012:471417
51. Zuo L, Lu M, Zhou Q, Wei W, Wang Y (2013) Butyrate suppresses proliferation and migration of RKO colon cancer cells though regulating endocan expression by MAPK signaling pathway. Food Chem Toxicol 62:892–900
52. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ (2008) The role of butyrate on colonic function. Aliment Pharmacol Ther 27:104–119
53. Canganella F, Kuk SU, Morgan H, Wiegel J (2002) *Clostridium thermobutyricum*: growth studies and stimulation of butyrate formation by acetate supplementation. Microbiol Res 157:149–156
54. Tamaru Y, Miyake H, Kuroda K, Nakanishi A, Kawade Y, Yamamoto K, Uemura M, Fujita Y, Doi RH, Ueda M (2010) Genome sequence of the cellulosome-producing mesophilic organism *Clostridium cellulovorans* 743B. J Bacteriol 192:901–902
55. Paul D, Austin FW, Arick T, Bridges SM, Burgess SC, Dandass YS, Lawrence ML (2010) Genome sequence of the solvent-producing bacterium *Clostridium carboxidivorans* strain P7. J Bacteriol 192:5554–5555

56. Ukpong MN, Atiyeh HK, De Lorme MJ, Liu K, Zhu X, Tanner RS, Wilkins MR, Stevenson BS (2012) Physiological response of *Clostridium carboxidivorans* during conversion of synthesis gas to solvents in a gas-fed bioreactor. Biotechnol Bioeng 109:2720–2728
57. Zeikus JG (1980) Chemical and fuel production by anaerobic-bacteria. Annu Rev Microbiol 34:423–464
58. Zhu Y, Yang ST (2004) Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. J Biotechnol 110:143–157
59. Charrier C, Duncan GJ, Reid MD, Rucklidge GJ, Henderson D, Young P, Russell VJ, Aminov RI, Flint HJ, Louis P (2006) A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria. Microbiology 152:179–185
60. Duncan SH, Barcenilla A, Stewart CS, Pryde SE, Flint HJ (2002) Acetate utilization and butyryl coenzyme A (CoA): acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. Appl Environ Microbiol 68:5186–5190
61. Liu X, Zhu Y, Yang ST (2006) Construction and characterization of *ack* deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid and hydrogen production. Biotechnol Prog 22:1265–1275
62. Zhu Y, Liu XG, Yang ST (2005) Construction and characterization of *pta* gene-deleted mutant of *Clostridium tyrobutyricum* for enhanced butyric acid fermentation. Biotechnol Bioeng 90:154–166
63. Jang YS, Woo HM, Im JA, Kim IH, Lee SY (2013) Metabolic engineering of *Clostridium acetobutylicum* for enhanced production of butyric acid. Appl Microbiol Biotechnol 97:9355–9363
64. Jang YS, Im JA, Choi SY, Lee JI, Lee SY (2014) Metabolic engineering of *Clostridium acetobutylicum* for butyric acid production with high butyric acid selectivity. Metab Eng 23:165–174
65. Saini M, Wang ZW, Chiang CJ, Chao YP (2014) Metabolic engineering of *Escherichia coli* for production of butyric acid. J Agric Food Chem 62:4342–4348
66. Lim JH, Seo SW, Kim SY, Jung GY (2013) Refactoring redox cofactor regeneration for high-yield biocatalysis of glucose to butyric acid in *Escherichia coli*. Bioresour Technol 135:568–573
67. Baek JM, Mazumdar S, Lee SW, Jung MY, Lim JH, Seo SW, Jung GY, Oh MK (2013) Butyrate production in engineered *Escherichia coli* with synthetic scaffolds. Biotechnol Bioeng 110:2790–2794
68. Fayolle F, Marchal R, Ballerini D (1990) Effect of controlled substrate feeding on butyric acid production by *Clostridium tyrobutyricum*. J Ind Microbiol 6:179–183
69. Huang J, Cai J, Wang J, Zhu X, Huang L, Yang S-T, Xu Z (2011) Efficient production of butyric acid from Jerusalem artichoke by immobilized *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. Bioresour Technol 102:3923–3926
70. Jiang L, Wang J, Liang S, Wang X, Cen P, Xu Z (2009) Butyric acid fermentation in a fibrous bed bioreactor with immobilized *Clostridium tyrobutyricum* from cane molasses. Bioresour Technol 100:3403–3409
71. Zhu Y, Wu ZT, Yang ST (2002) Butyric acid production from acid hydrolysate of corn fibre by *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. Process Biochem 38:657–666
72. Wei D, Liu X, Yang ST (2013) Butyric acid production from sugarcane bagasse hydrolysate by *Clostridium tyrobutyricum* immobilized in a fibrous-bed bioreactor. Bioresour Technol 129:553–560
73. Zhu Y, Yang ST (2003) Adaptation of *Clostridium tyrobutyricum* for enhanced tolerance to butyric acid in a fibrous-bed bioreactor. Biotechnol Prog 19:365–372
74. Jiang L, Li S, Hu Y, Xu Q, Huang H (2012) Adaptive evolution for fast growth on glucose and the effects on the regulation of glucose transport system in *Clostridium tyrobutyricum*. Biotechnol Bioeng 109:708–718

75. Jiang L, Wang J, Liang S, Cai J, Xu Z, Cen P, Yang S, Li S (2011) Enhanced butyric acid tolerance and bioproduction by *Clostridium tyrobutyricum* immobilized in a fibrous bed bioreactor. Biotechnol Bioeng 108:31–40
76. Zhou X, Lu XH, Li XH, Xin ZJ, Xie JR, Zhao MR, Wang L, Du WY, Liang JP (2014) Radiation induces acid tolerance of *Clostridium tyrobutyricum* and enhances bioproduction of butyric acid through a metabolic switch. Biotechnol Biofuels 7:22
77. Michel-Savin D, Marchal R, Vandecasteele JP (1990) Butyric fermentation: metabolic behavior and production performance of *Clostridium tyrobutyricum* in a continuous culture with cell recycle. Appl Microbiol Biotechnol 34:172–177
78. Wu ZT, Yang ST (2003) Extractive fermentation for butyric acid production from glucose by *Clostridium tyrobutyricum*. Biotechnol Bioeng 82:93–102
79. Du J, Lorenz N, Beitle RR, Hestekin JA (2011) Application of wafer-enhanced electrodeionization in a continuous fermentation process to produce butyric acid with *Clostridium tyrobutyricum*. Sep Sci Technol 47:43–51
80. Choi O, Um Y, Sang BI (2012) Butyrate production enhancement by *Clostridium tyrobutyricum* using electron mediators and a cathodic electron donor. Biotechnol Bioeng 109:2494–2502
81. Thongchul N (2013) Production of lactic acid and polylactic acid for industrial applications. In: Yang ST, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. Wiley, Hoboken, pp 293–316
82. Kascak JS, Kominek J, Roehr M (1996) Lactic acid. In: Rehm H-J, Reed G, Puhler A, Stadler P (eds) Biotechnology, 2nd edn. VCH Verlagsgesellschaft mbH, Weinheim, pp 293–306
83. Kadam SR, Patil SS, Bastawde KB, Khire JA, Gokhale DV (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. Process Biochem 41:120–126
84. Benthin S, Villadsen J (1995) Production of optically pure D-lactate by *Lactobacillus bulgaricus* and purification by crystallization and liquid/liquid extraction. Appl Microbiol Biotechnol 42:426–429
85. Singh SK, Ahmed SU, Pandey A (2006) Metabolic engineering approaches for lactic acid production. Process Biochem 41:991–1000
86. Saitoh S, Ishida N, Onishi T, Tokuhiro K, Nagamori E, Kitamoto K, Takahashi H (2005) Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. Appl Environ Microbiol 71:2789–2792
87. Bianchi MM, Brambilla L, Protani F, Liu CL, Lievense J, Porro D (2001) Efficient homolactic fermentation by *Kluyveromyces lactis* strains defective in pyruvate utilization and transformed with the heterologous LDH gene. Appl Environ Microbiol 67:5621–5625
88. Zhou S, Shanmugam KT, Ingram LO (2003) Functional replacement of the *Escherichia coli* D-(−)-lactate dehydrogenase gene (*ldhA*) with the L-(+)-lactate dehydrogenase gene (*ldhL*) from *Pediococcus acidilactici*. Appl Environ Microbiol 69:2237–2244
89. Zhou S, Causey TB, Hasona A, Shanmugam KT, Ingram LO (2003) Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *Escherichia coli* W3110. Appl Environ Microbiol 69:399–407
90. Zhu Y, Eiteman MA, DeWitt K, Altman E (2007) Homolactate fermentation by metabolically engineered *Escherichia coli* strains. Appl Environ Microbiol 73:456–464
91. Wang L, Zhao B, Li F, Xu K, Ma C, Tao F, Li Q, Xu P (2011) Highly efficient production of D-lactate by *Sporolactobacillus* sp. CASD with simultaneous enzymatic hydrolysis of peanut meal. Appl Microbiol Biotechnol 89:1009–1017
92. Okino S, Suda M, Fujikura K, Inui M, Yukawa H (2008) Production of D-lactic acid by *Corynebacterium glutamicum* under oxygen deprivation. Appl Microbiol Biotechnol 78:449–454
93. Tay A, Yang ST (2002) Production of L(+)-lactic acid from glucose and starch by immobilized cells of *Rhizopus oryzae* in a rotating fibrous bed bioreactor. Biotechnol Bioeng 80:1–12

94. Kumar V, Ashok S, Park S (2013) Recent advances in biological production of 3-hydroxypropionic acid. Biotechnol Adv 31:945–961
95. Rathnasingh C, Raj SM, Jo JE, Park S (2009) Development and evaluation of efficient recombinant *Escherichia coli* strains for the production of 3-hydroxypropionic acid from glycerol. Biotechnol Bioeng 104:729–739
96. Jung WS, Kang JH, Chu HS et al (2014) Elevated production of 3-hydroxypropionic acid by metabolic engineering of the glycerol metabolism in *Escherichia coli*. Metab Eng 23:116–122
97. Ashok S, Raj SM, Rathnasingh C, Park S (2011) Development of recombinant *Klebsiella pneumoniae* dhaT strain for the co-production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol. Appl Microbiol Biotechnol 90:1253–1265
98. Huang Y, Li Z, Shimizu K, Ye Q (2012) Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol by a recombinant strain of *Klebsiella pneumoniae*. Bioresour Technol 103:351–359
99. Ashok S, Sankaranarayanan M, Ko Y, Jae KE, Ainala SK, Kumar V, Park S (2013) Production of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumoniae* Δ*dhaT*Δ*yqhD* which can produce vitamin B_{12} naturally. Biotechnol Bioeng 110:511–524
100. Quispe CAG, Coronado CJR, Carvalho JA Jr (2013) Glycerol: production, consumption, prices, characterization and new trends in combustion. Renew Sustain Energy Rev 27:475–493
101. Yi J, Choi S, Han M-S, Lee JW, Lee SY (2013) Production of succinic acid from renewable resources. In: Yang ST, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. Wiley, Hoboken, pp 317–330
102. Zeikus JG, Jain MK, Elankovan P (1999) Biotechnology of succinic acid production and markets for derived industrial products. Appl Microbiol Biotechnol 51:545–552
103. Lee PC, Lee WG, Lee SY, Chang HN (2001) Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. Biotechnol Bioeng 72:41–48
104. Lee SJ, Song H, Lee SY (2006) Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. Appl Environ Microbiol 72:1939–1948
105. Glassner DA, Datta R (1992) Process for the production and purification of succinic acid. US Patent 5,143,834
106. Meynial-Salles I, Dorotyn S, Soucaille P (2008) A new process for the continuous production of succinic acid from glucose at high yield, titer, and productivity. Biotechnol Bioeng 99:129–135
107. Guettler MV, Jain MK, Soni BK (1998) Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US Patent 5,723,322
108. Guettler MV, Jain MK, Rumler D (1996) Method for making succinic acid, bacterial variants for use in process, and methods for obtaining variants. US Patent 5,573,931
109. Park DH, Laivenieks M, Guettler MV, Jain MK, Zeikus JG (1999) Microbial utilization of electrically reduced neutral red as the sole electron donor for growth and metabolite production. Appl Environ Microbiol 65:2912–2917
110. Song H, Kim TY, Choi BK, Choi SJ, Nielsen LK, Chang HN, Lee SY (2008) Development of chemically defined medium for *Mannheimia succiniciproducens* based on its genome sequence. Appl Microbiol Biotechnol 79:263–272
111. Kim P, Laivenieks M, Vieille C, Zeikus JG (2004) Effect of overexpression of *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase on succinate production in *Escherichia coli*. Appl Environ Microbiol 70:1238–1241
112. Kim TY, Kim HU, Park JM, Song H, Kim JS, Lee SY (2007) Genome-scale analysis of *Mannheimia succiniciproducens* metabolism. Biotechnol Bioeng 97:657–671

113. Okino S, Noburyu R, Suda M, Jojima T, Inui M, Yukawa H (2008) An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl Microbiol Biotechnol 81:459–464
114. Hong SH (2007) Systems approaches to succinic acid-producing microorganisms. Biotechnol Bioprocess Eng 12:73–79
115. YangST LX, Zhang Y (2007) Metabolic engineering – applications, methods, and challenges. In: Yang ST (ed) Bioprocessing for value-added products from renewable resources: new technologies and applications. Elsevier, Amsterdam, pp 73–118
116. Jantama K, Zhang X, Moore JC, Shanmugam KT, Svoronos SA, Ingram LO (2008) Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. Biotechnol Bioeng 101:881–893
117. Vemuri GN, Eiteman MA, Altman E (2002) Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J Ind Microbiol Biotechnol 28:325–332
118. Vemuri GN, Eiteman MA, Altman E (2002) Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol 68:1715–1727
119. Lin H, Bennett GN, San K-Y (2005) Effect of carbon sources differing in oxidation state and transport route on succinate production in metabolically engineered *Escherichia coli*. J Ind Microbiol Biotechnol 32:87–93
120. San K-Y, Bennett GN, Berríos-Rivera SJ, Vadali RV, Yang Y-T, Horton E, Rudolph FB, Sariyar B, Blackwood K (2002) Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli*. Metab Eng 4:182–192
121. Thakker C, Martínez I, San K-Y, Bennett GN (2012) Succinate production in *Escherichia coli*. Biotechnol J 7(2):213–224
122. Otero JM, Cimini D, Patil KR, Poulsen SG, Olsson L, Nielsen J (2013) Industrial systems biology of *Saccharomyces cerevisiae* enables novel succinic acid cell factory. PLoS One 8 (1):e54144
123. Yan D, Wang C, Zhou J, Liu Y, Yang M, Xing J (2014) Construction of reductive pathway in *Saccharomyces cerevisiae* for effective succinic acid fermentation at low pH value. Bioresour Technol 156:232–239
124. Zhang K, Zhang B, Yang ST (2013) Production of citric, itaconic, fumaric and malic acids in filamentous fungal fermentations. In: Yang ST, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. Wiley, Hoboken, pp 375–397
125. Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I (1991) Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. Biotechnol Bioeng 37:1108–1116
126. Taing O, Taing K (2007) Production of malic and succinic acids by sugar-tolerant yeast *Zygosaccharomyces rouxii*. Eur Food ResTechnol 224:343–347
127. West TP (2011) Malic acid production from thin stillage by *Aspergillus species*. Biotechnol Lett 33:2463–2467
128. Lumyong S, Tomita F (1993) L-malic acid production by an albino strain *Monascus araneosus*. World J Microbiol Biotechnol 9:383–384
129. Kawagoe M, Hyakumura K, Suye SI, Miki K, Naoe K (1997) Application of bubble column fermenters to submerged culture of *Schizophyllum commune* for production of L-malic acid. J Ferment Bioeng 84:333–336
130. Liu SJ, Steinbuchel A (1997) Production of poly(malic acid) from different carbon sources and its regulation in *Aureobasidium pullulans*. Biotechnol Lett 19:11–14
131. Zou X, Zhou YP, Yang ST (2013) Production of polymalic acid and malic acid by *Aureobasidium pullulans* fermentation and acid hydrolysis. Biotechnol Bioeng 110:2105–2113

132. Brown SH, Bashkirova L, Berka R, Chandler T, Doty T, McCall K et al (2013) Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of L-malic acid. Appl Microbiol Biotechnol 97:8903–8912
133. Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, Winkler AA et al (2008) Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. Appl Environ Microbiol 74:2766–2777
134. Moon SY, Hong SH, Kim TY, Lee SY (2008) Metabolic engineering of *Escherichia coli* for the production of malic acid. Biochem Eng J 40:312–320
135. Jantama K, Haupt MJ, Svoronos SA, Zhang XL, Moore JC, Shanmugam KT, Ingram LO (2008) Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. Biotechnol Bioeng 99:1140–1153
136. Zhang X, Wang X, Shanmugam KT, Ingram LO (2011) L-Malate production by metabolically engineered *Escherichia coli*. Appl Environ Microbiol 77:427–434
137. Yang ST, Zhang K, Zhang B, Huang H (2011) Bio-based chemicals - fumaric acid. In: Moo-Young M (ed) Comprehensive biotechnology, vol 3, 2nd edn. Elsevier, Burlington, pp 163–177
138. Xu Q, Li S, Huang H, Wen J (2012) Key technologies for the industrial production of fumaric acid by fermentation. Biotechnol Adv 30:1685–1696
139. Xu G, Chen X, Liu L, Jiang L (2013) Fumaric acid production in *Saccharomyces cerevisiae* by simultaneous use of oxidative and reductive routes. Bioresour Technol 148:91–96
140. Ling LB, Ng TK (1989) Fermentation process for carboxylic acids. US 4,877,731
141. Cao NJ, Du JX, Gong CS, Tsao GT (1996) Simultaneous production and recovery of fumaric acid from immobilized *Rhizopus oryzae* with a rotary biofilm contactor and an adsorption column. Appl Environ Microbiol 62:2926–2931
142. Das RK, Brar SK (2014) Enhanced fumaric acid production from brewery wastewater and insight into the morphology of *Rhizopus oryzae* 1526. Appl Biochem Biotechnol 172:2974–2988
143. Zhou Y, Nie K, Zhang X, Liu S, Wang M, Deng L et al (2014) Production of fumaric acid from biodiesel-derived crude glycerol by *Rhizopus arrhizus*. Bioresour Technol 163:48–53
144. Zhang BH, Yang ST (2012) Metabolic engineering of *Rhizopus oryzae*: effects of overexpressing *fumR* gene on cell growth and fumaric acid biosynthesis from glucose. Process Biochem 47:2159–2165
145. Zhang BH, Skory CD, Yang ST (2012) Metabolic engineering of *Rhizopus oryzae*: effects of overexpressing *pyc* and *pepc* genes on fumaric acid biosynthesis from glucose. Metab Eng 14:512–520
146. Song CW, Kim DI, Choi S, Jang JW, Lee SY (2013) Metabolic engineering of *Escherichia coli* for the production of fumaric acid. Biotechnol Bioeng 110:2025–2034
147. Chen X, Wu J, Song W, Zhang L, Wang H, Liu L (2015) Fumaric acid production by *Torulopsis glabrata*: engineering the urea cycle and the purine nucleotide cycle. Biotechnol Bioeng 112:156–167
148. Okabe M, Lies D, Kanamasa S, Park EY (2009) Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus*. Appl Microbiol Biotechnol 84:597–606
149. Yahiro K, Takahama T, Park YS, Okabe M (1995) Breeding of *Aspergillus terreus* mutant Tn-484 for itaconic acid production with high-yield. J Ferment Bioeng 79:506–508
150. Kawamura D, Furuhashi M, Saito O, Matsui H (1981) Production of itaconic acid by fermentation. Japan Patent 56,137,893
151. Blazeck J, Miller J, Pan A, Gengler J, Holden C, Jamoussi M, Alper HS (2014) Metabolic engineering of *Saccharomyces cerevisiae* for itaconic acid production. Appl Microbiol Biotechnol 98:8155–8164
152. Blazeck J, Hill A, Jamoussi M, Pan A, Miller J, Alper HS (2015) Metabolic engineering of *Yarrowia lipolytica* for itaconic acid production. Metab Eng 32:66–73

153. Polen T, Spelberg M, Bott M (2012) Toward biotechnological production of adipic acid and precursors from biorenewables. J Biotechnol 167:75–84
154. Yu J-L, Xia X-X, Zhong J-J, Qian Z-G (2014) Direct biosynthesis of adipic acid from a synthetic pathway in recombinant *Escherichia coli*. Biotechnol Bioeng 111:2580–2586
155. Niu W, Draths KM, Frost JW (2002) Benzene-free synthesis of adipic acid. Biotechnol Prog 18:201–211
156. Moon TS, Yoon SH, Lanza AM, Roy-Mayhew JD, Prather KLJ (2009) Production of glucaric acid from a synthetic pathway in recombinant *Escherichia coli*. Appl Environ Microbiol 75:589–595
157. Moon TS, Dueber JE, Shiue E, Prather KLJ (2010) Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*. Metab Eng 12:298–305
158. Straathof AJJ, Sie S, Franco TT, van der Wielen LAM (2005) Feasibility of acrylic acid production by fermentation. Appl Microbiol Biotechnol 67:727–734
159. Ishii M, Chuakrut S, Arai H, Igarashi Y (2004) Occurrence, biochemistry and possible biotechnological application of the 3-hydroxypropionate cycle. Appl Microbiol Biotechnol 64:605–610
160. Li Y, Chen J, Lun S-Y (2001) Biotechnological production of pyruvic acid. Appl Microbiol Biotechnol 57:451–459
161. Miyata R, Yonehara T (1999) Breeding of high-pyruvate-producing *Torulopsis glabrata* with acquired reduced pyruvate decarboxylase. J Biosci Bioeng 88:173–178
162. Wieschalka S, Blombach B, Eikmanns BJ (2012) Engineering *Corynebacterium glutamicum* for the production of pyruvate. Appl Microbiol Biotechnol 94:449–459
163. Zhu Y, Eiteman MA, Altman R, Altman E (2008) High glycolytic flux improves pyruvate production by a metabolically engineered *Escherichia coli* strain. Appl Environ Microbiol 74:6649–6655
164. Maris AJ, Geertman JM, Vermeulen A, Groothuizen MK, Winkler AA, Piper MDW, Van Dijken JP, Pronk JT (2004) Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl Environ Microbiol 70:159–166
165. Roehr M, Kubicek CP, Kominek J (1996) Citric acid. In: Roehr M (ed) Biotechnology, vol 6, 2nd edn, Products of primary metabolism. Verlag Chemie, Weinheim, pp 308–345
166. Max B, Salgado JM, Rodriguez N, Cortes S, Converti A, Dominguez JM (2010) Biotechnological production of citric acid. Braz J Microbiol 41:862–875
167. Anastassiadis S, Rehm HJ (2006) Citric acid production from glucose by yeast *Candida oleophila* ATCC 20177 under batch, continuous and repeated batch cultivation. Electron J Biotechnol 9:26–39
168. Rywinska A, Rymowicz W, Larowska B, Wojtatowicz M (2009) Biosynthesis of citric acid from glycerol by acetate mutants of *Yarrowia lipolytica* in fed-batch fermentation. Food Technol Biotechnol 47:1–6
169. Forster A, Aurich A, Mauersberger S, Barth G (2007) Citric acid production from sucrose using a recombinant strain of the yeast *Yarrowia lipolytica*. Appl Microbiol Biotechnol 75:1409–1417
170. Liu XY, Chi Z, Liu GL, Madzak C, Chi ZM (2013) Both decrease in ACL1 gene expression and increase in ICL1 gene expression in marine-derived yeast *Yarrowia lipolytica* expressing INU1 gene enhance citric acid production from inulin. Mar Biotechnol 15:26–36
171. Roehr M, Kubicek CP, Kominek J (1996) Gluconic acid. In: Roehr M (ed) Biotechnology, vol 6, 2nd edn, Products of Primary Metabolism. Verlag Chemie, Weinheim, pp 347–362
172. Anastassiadis S, Aivasidis A, Wandrey C (2003) Continuous gluconic acid production by isolated yeast-like mould strains of *Aureobasidium pullulans*. Appl Microbiol Biotechnol 61:110–117
173. Yin X, Li J, Shin H-D, Du G, Liu L, Chen J (2015) Metabolic engineering in the biotechnological production of organic acids in the tricarboxylic acid cycle of microorganisms: advances and prospects. Biotechnol Adv 33:830–841

174. Sun J, Alper HS (2015) Metabolic engineering of strains: from industrial-scale to lab-scale chemical production. J Ind Microbiol Biotechnol 42:423–436
175. Jang Y-S, Kim B, Shin JH, Choi YJ, Choi S, Song CW, Lee J, Park HG, Lee SY (2012) Bio-based production of C2–C6 platform chemicals. Biotechnol Bioeng 109:2437–2459
176. Lee JW, Kim HU, Choi S, Yi J, Lee SY (2011) Microbial production of building block chemicals and polymers. Curr Opin Biotechnol 22:758–767
177. Shin JH, Kim HU, Kim DI, Lee SY (2013) Production of bulk chemicals via novel metabolic pathways in microorganisms. Biotechnol Adv 31:925–935
178. Karaffa L, Kubicek CP (2003) *Aspergillus niger* citric acid accumulation: do we understand this well working black box? Appl Microbiol Biotechnol 61:189–196
179. Nicolaou SA, Gaida SM, Papoutsakis ET (2010) A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng 12:307–331
180. Xu M, Zhao J, Yu L, Tang I-C, Xue C, Yang ST (2015) Engineering *Clostridium acetobutylicum* with a histidine kinase knockout for enhanced n-butanol tolerance and production. Appl Microbiol Biotechnol 99:1011–1022
181. Royce LA, Yoon JM, Chen Y, Rickenbach E, Shanks JV, Jarboe LR (2015) Evolution for exogenous octanoic acid tolerance improves carboxylic acid production and membrane integrity. Metab Eng 29:180–188
182. Yang ST, Lu C (2013) Extraction-fermentation hybrid (extractive fermentation). In: Ramaswamy S, Ramarao BV, Huang H (eds) Separation and purification technologies in biorefineries. Wiley, Chichester, pp 409–437
183. Du Y, Jiang W, Yu M, Tang I-C, Yang S-T (2015) Metabolic process engineering of *Clostridium tyrobutyricum* Δ*ack-adhE2* for enhanced n-butanol production from glucose: effects of methyl viologen on NADH availability, flux distribution and fermentation kinetics. Biotechnol Bioeng 112:705–715
184. Parisutham V, Kim TH, Lee SK (2014) Feasibilities of consolidated bioprocessing microbes: from pretreatment to biofuel production. Bioresour Technol 161:431–440
185. Yang ST, Yu M (2013) Integrated biorefinery for sustainable production of fuels, chemicals and polymers. In: Yang ST, El-Enshasy HA, Thongchul N (eds) Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. Wiley, Hoboken, pp 1–26
186. Almeida JRM, Fávaro LCL, Quirino BF (2012) Biodiesel biorefinery: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. Biotechnol Biofuels 5:48
187. Anand P, Saxena RK (2012) A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from *Citrobacter freundi*. N Biotechnol 29:199–205
188. Schiel-Bengelsdorf B, Durre P (2012) Pathway engineering and synthetic biology using acetogens. FEBS Lett 586:2191–2198
189. Fast AG, Papoutsakis ET (2012) Stoichiometric and energetic analyses of non-photosynthetic CO_2-fixation pathways to support synthetic biology strategies for production of fuels and chemicals. Curr Opin Chem Eng 1:380–395
190. Munasinghe PC, Khanal SK (2010) Biomass-derived syngas fermentation into biofuels: opportunities and challenges. Bioresour Technol 101:5013–5022
191. Wang J, Yang X, Chen C-C, Yang ST (2014) Engineering clostridia for butanol production from biorenewable resources: from cells to process integration. Curr Opin Chem Eng 6:43–54
192. Jang Y-S, Park JM, Choi S, Choi YJ, Seung DY, Cho JH, Lee SY (2012) Engineering of microorganisms for the production of biofuels and perspectives based on systems metabolic engineering approaches. Biotechnol Adv 30:989–1000
193. Lee JW, Na D, Park JM, Lee J, Choi S, Lee SY (2012) Systems metabolic engineering of microorganisms for natural and non-natural chemicals. Nat Chem Biol 8:536–546

194. Seo SW, Yang J, Min BE, Jang S, Lim JH, Lim HG, Kim SC, Kim SY, Jeong JH, Jung GY (2013) Synthetic biology: tools to design microbes for the production of chemicals and fuels. Biotechnol Adv 31:811–817
195. Jullesson D, David F, Pfleger B, Nielsen J (2015) Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. Biotechnol Adv. doi:10.1016/j.biotechadv.2015.02.011 (in press)
196. McNerney MP, Watstein DM, Styczynski MP (2015) Precision metabolic engineering: the design of responsive, selective, and controllable metabolic systems. Metab Eng 31:123–131
197. Yu C, Cao Y, Zou H, Xian M (2011) Metabolic engineering of *Escherichia coli* for biotechnological production of high-value organic acids and alcohols. Appl Microbiol Biotechnol 89:573–583
198. Borodina I, Nielsen J (2014) Advances in metabolic engineering of yeast *Saccharomyces cerevisiae* for production of chemicals. Biotechnol J 9:609–620
199. Wieschalka S, Blombach B, Bott M, Eikmanns BJ (2013) Bio-based production of organic acids with *Corynebacterium glutamicum*. Microb Biotechnol 6:87–102
200. Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET (2012) Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr Opin Biotechnol 23:364–381
201. Yu M, Du Y, Jiang W, Chang W-L, Yang ST, Tang I-C (2012) Effects of different replicons in conjugative plasmids on transformation efficiency, plasmid stability, gene expression and n-butanol biosynthesis in *Clostridium tyrobutyricum*. Appl Microbiol Biotechnol 93:881–889
202. Yu M, Zhang Y, Tang IC, Yang ST (2011) Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production. Metab Eng 13:373–382

Adv Biochem Eng Biotechnol (2016) 156: 363–396
DOI: 10.1007/10_2015_5012

Published online: 9 March 2016

Development of Anaerobic High-Rate Reactors, Focusing on Sludge Bed Technology

J.B. van Lier, F.P. van der Zee, C.T.M.J. Frijters, and M.E. Ersahin

Abstract In the last 40 years, anaerobic sludge bed reactor technology has evolved from localized laboratory-scale trials to worldwide successful implementations in a variety of industries. High-rate sludge bed reactors are characterized by a very small footprint and high applicable volumetric loading rates. Best performances are obtained when the sludge bed consists of highly active and well settleable granular sludge. Sludge granulation provides a rich microbial diversity, high biomass concentration, high solids retention time, good settling characteristics, reduction in both operation costs and reactor volume, and high tolerance to inhibitors and temperature changes. However, sludge granulation cannot be guaranteed on every

This chapter is based on: van Lier JB, van der Zee FP, Frijters CTMJ, Ersahin ME. Celebrating 40 years anaerobic sludge bed reactors for industrial wastewater treatment, Rev. Env. Sci and Bio/techn., 14(4), 681–702.

J.B. van Lier (✉)
Delft University of Technology, Faculty of Civil Engineering and Geosciences, Department of Water Management, Sanitary Engineering Section, Stevinweg 1, 2628 CN Delft, The Netherlands

Unesco – IHE, 3015, 2601 DA Delft, The Netherlands
e-mail: j.b.vanlier@tudelft.nl

F.P. van der Zee
Biothane Systems International, Tanthofdreef 21, 2600 GB Delft, The Netherlands

C.T.M.J. Frijters
Paques BV, T. de Boerstraat 24, 8561 EL Balk, The Netherlands

M.E. Ersahin
Delft University of Technology, Faculty of Civil Engineering and Geosciences, Department of Water Management, Sanitary Engineering Section, Stevinweg 1, 2628 CN Delft, The Netherlands

Istanbul Technical University, Civil Engineering Faculty, Environmental Engineering Department, Maslak, 34469 Istanbul, Turkey

type of industrial wastewater. Especially in the last two decades, various types of high-rate anaerobic reactor configurations have been developed that are less dependent on the presence of granular sludge, and many of them are currently successfully used for the treatment of various kinds of industrial wastewaters worldwide. This study discusses the evolution of anaerobic sludge bed technology for the treatment of industrial wastewaters in the last four decades, focusing on granular sludge bed systems.

Keywords Anaerobic biotechnology, Flocculent sludge, Granulation, High-rate reactor technology, Industrial wastewater treatment, Sludge bed reactors

Contents

Abbreviations

ABR	Anaerobic baffled reactor
ACP	Anaerobic contact process
AF	Anaerobic filter
AnMBR	Anaerobic membrane bioreactor
CSTR	Completely stirred tank reactor
EGSB	Expanded granular sludge bed
EPS	Exopolymeric substances
FB	Fluidized bed
FOG	Fats, oil, and grease
GLSS	Gas–liquid–solids separation
HRT	Hydraulic retention time
IC	Internal circulation
NSSC	Neutral sulfite semichemical
SRT	Solids/sludge retention time
SS	Suspended solids
TA	Terephthalic acid

UASB Upflow anaerobic sludge blanket
VLR Volumetric loading rate
VSS Volatile suspended solids

1 Introduction

As an energy generating process, anaerobic treatment technology has been receiving growing interest since its first application; primarily because of the simplicity of the technology, low space requirement, low excess sludge production, and the positive energy balance in comparison to the conventional aerobic treatment technologies [1]. Notably, by using anaerobic treatment instead of activated sludge, about 1 kWh (fossil energy) kg^{-1} COD removed is saved, depending on the system, which is used for aeration of activated sludge. Moreover, under anaerobic conditions, the organic matter is converted to the gaseous energy carrier CH_4, producing about 13.5 MJ CH_4 energy kg^{-1} COD removed, giving 1.5 kWh electricity (assuming 40% electric conversion efficiency). In countries such as the Netherlands, the over 90% reduction in sludge production significantly contributed to the economics of the plant, whereas the high loading capacities of anaerobic high-rate reactors allowed for 90% reduction in space requirement, both compared to conventional activated sludge systems. These striking advantages led to the rapid development of anaerobic high-rate technology for industrial wastewater treatment. In this development, the group of Prof. Gatze Lettinga at Wageningen University, in close cooperation with the contractors Paques BV and Biothane Systems International, played a crucial role as recently outlined by Lettinga [2]. Anaerobic high-rate technology has improved significantly in the last few decades with the applications of differently configured high-rate reactors, especially for the treatment of industrial wastewaters.

The relatively rapid implementation of high-rate anaerobic treatment coincided with the implementation of the new environmental laws in Western Europe and the co-occurrence of very high energy prices in the 1970s. High amounts of highly concentrated wastewaters from the food processing and beverages industries, distilleries, pharmaceutical industries, and pulp and paper mills suddenly required treatment. The first anaerobic full-scale installations confirmed that, when treating the effluents, considerable amounts of useful energy in the form of biogas could be obtained for possible use in the production process [1, 3]. As mentioned, the extremely low excess sludge production was another very important asset of high-rate anaerobic treatment systems. Interestingly, the production of granular sludge even gave a market value to excess sludge, because granular sludge is nowadays sold on the market for re-inoculating or starting up new reactor systems. From the 1970s onwards, high-rate anaerobic treatment is particularly applied to organically polluted industrial wastewaters coming from the agro-food sector and the beverage industries (Table 1). Currently, in more than 90% of these

Table 1 Worldwide application of anaerobic technology for industrial wastewater treatment. Total number of registered installed reactors = 2,266, census January 2007 (adapted from [1])

Industrial sector	Type of wastewater	Installed reactors[a] (% of total)
Agro-food industry	Sugar, potato, starch, yeast, pectin, citric acid, cannery, confectionery, fruit, vegetables, dairy, bakery	36
Beverage	Beer, malting, soft drinks, wine, fruit juices, coffee	29
Alcohol distillery	Cane juice, cane molasses, beet molasses, grape wine, grain, fruit	10
Pulp and paper industry	Recycle paper, mechanical pulp, NSSC process, sulphite pulp, straw, bagasse	11
Miscellaneous	Chemical, pharmaceutical, sludge liquor, landfill leachate, acid mine water, municipal sewage	14

[a]Various types of high-rate anaerobic reactor systems

applications, anaerobic sludge bed technology is applied, for which the presence of granular sludge is of eminent importance. Interestingly, both the number of anaerobic reactors installed and the application potential of anaerobic wastewater treatment are expanding rapidly. Authors estimate that the current number of installed anaerobic high-rate reactors exceeds 4,000, whereas nowadays wastewaters are treated that were previously not considered for anaerobic treatment, such as chemical wastewaters containing toxic compounds or wastewaters with a complex composition. For the more extreme types of wastewaters, novel high-rate reactor systems have been developed as discussed below.

This chapter presents a comprehensive evaluation of anaerobic sludge bed technology for the treatment of industrial wastewaters with a focus on different types of high-rate reactors developed in the last 40 years.

2 Development of High-Rate Anaerobic Reactor Technology

Many different reactor configurations have been used and are used for the anaerobic treatment of wastewaters, as reviewed by McCarty [4]. One of the first continuous flow anaerobic reactors was designed in 1905 by Karl Imhoff, who developed a single flow-through tank for enhanced settling and concomitant digestion of settled solids. The innovative Imhoff tank was particularly applied for municipal wastewaters and is still in use, particularly in warm climate regions [5]. Anaerobic treatment of industrial wastewaters was for the first time seriously investigated by Arthur M. Buswell and co-workers starting in the 1920s [6, 7]. In fact, Buswell unraveled the biochemical oxidation–reduction reactions occurring during anaerobic digestion [8], thus advancing the basic process understanding enormously. By using Buswell's formula one can easily calculate the expected methane generation from known biochemical compounds. In their reactor studies, they made use of

completely mixed systems in which the hydraulic retention time (HRT) was similar to the solids retention time (SRT). In such systems, the anaerobic conversion capacity is fully linked to the growth rate of bacteria. Because these growth rates are very low, reactor systems are very large. Completely stirred tank reactor (CSTR) designs were the predominant systems used for anaerobic treatment until the 1960s. The most striking disadvantage of these low-rate anaerobic reactors is the requirement of large reactor volumes to provide enough biomass concentration in the reactor [9, 10]. By then it was clearly understood that any increase in treatment capacity can only be achieved by increasing the concentration of biocatalysts, i.e., the methanogenic sludge, in the anaerobic reactor. Therefore, the terminology 'high-rate' reactor generally refers to systems in which the SRT is uncoupled from the HRT. With the introduction of high-rate reactors, the required reactor volumes and concomitant capital costs were distinctly reduced, making anaerobic treatment of practical interest for cost-effective industrial wastewater treatment.

Anaerobic high-rate reactors can be classified by the way SRT is uncoupled from HRT. Immobilization of anaerobic sludge via granule and/or biofilm formation represents the traditional way to achieve the necessary biomass retention, enabling bioreactor operation at high biomass concentrations, and therefore at high volumetric loading rates (VLRs) [9, 11]. Besides, physical retention can also be used to achieve the essential sludge retention in situations where biofilm and granule formation does not proceed well. The latter is frequently the case when treating wastewaters with large amounts of suspended solids or when wastewaters are characterized by high salinity and/or high temperature. Physical retention can be achieved using a secondary clarifier with sludge return, similar to the activated sludge process, or by using a physical filtration barrier or a membrane.

Depending on the applied sludge retention mechanism, various high-rate anaerobic treatment configurations have been developed in the past four decades, such as the anaerobic contact process (ACP), anaerobic filter (AF), upflow anaerobic sludge blanket (UASB) reactor, fluidized bed (FB) reactor, expanded granular sludge bed (EGSB) reactor, internal circulation (IC) reactor, anaerobic baffled reactor (ABR), membrane coupled high-rate (UASB/EGSB/FB) reactors, and membrane coupled CSTR systems. The latter are better known as anaerobic membrane bioreactors (AnMBR). In addition, a number of variations in the basic designs have been proposed in the literature of which some made it to full-scale application. Figure 1 shows various examples of high-rate anaerobic reactor configurations. At present, the high-rate sludge bed reactors, i.e., UASB and EGSB reactors and their derivatives, are most widely implemented for the anaerobic treatment of industrial wastewater, having about 90% of the market share of all installed systems [1]. Their popularity for treating industrial wastewaters can be attributed to their compactness and ease of operation when applying high VLRs at low HRTs [1, 12]. More recently, membrane-coupled high-rate anaerobic reactor configurations are increasingly being researched because of the large amount of comparable knowledge from aerobic MBR operations and the application niche which clearly exists for these systems [13]. Membrane-assisted sludge retention ensures the

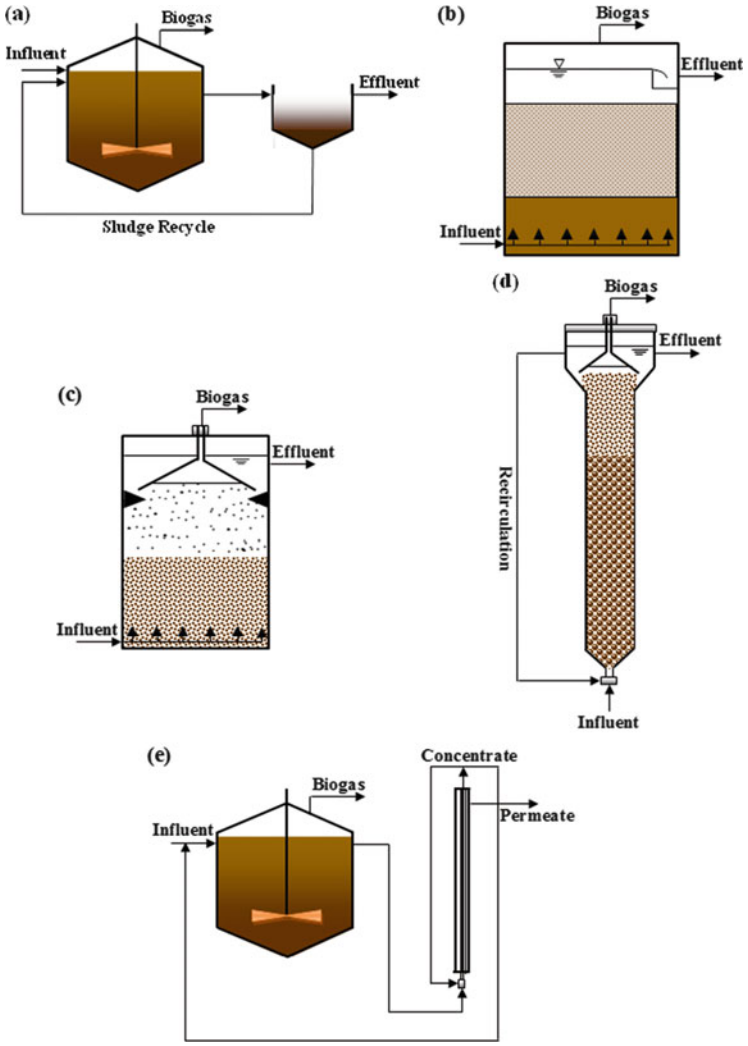


Fig. 1 Examples of high-rate anaerobic reactors. (**a**) Anaerobic contact process. (**b**) Anaerobic filter. (**c**) Upflow anaerobic sludge blanket reactor. (**d**) Expanded granular sludge bed reactor. (**e**) Membrane coupled CSTR reactor (AnMBR)

accumulation of very slowly growing microorganisms with inferior adherence properties which are frequently needed for the anaerobic treatment of toxic and recalcitrant wastewaters. In this way, the aggregation property of the biomass loses its importance for substrate degradation capacity, and cell washout risk is non-existent. Membrane coupled systems also offer a cost-effective alternative to produce nutrient-rich and solids-free effluents. Although not yet applied in practice,

these effluents would be suitable for agricultural and landscaping irrigation [14, 15].

3 First Anaerobic High-Rate Reactors: ACP and AF

Following the historic development of high-rate reactors, the ACP process is the first configuration in which the SRT was uncoupled from the HRT. The reactor biomass concentration was increased by employing a secondary clarifier with return flow, similar to its aerobic homologue. The first ACP process was reported for the treatment of dilute packing house waste that has a COD of about 1,300 mg L^{-1} [16]. The various versions of the first generation of these high-rate ACP systems for medium strength wastewaters were not very successful. In practice, the main difficulty was a poor separation of the active anaerobic sludge from the treated water in the secondary clarifier. Biogas formation and attachment in the settling tank were the other major problems [9]. The poor sludge separation was attributed to the applied very intensive agitation in the bioreactor, creating very small sludge particles with poor settleability. In addition, supersaturation of solubilized gases resulted in buoyant upward forces in the clarifier. The idea of the very intensified mixing was to ensure optimized contact between the sludge and the wastewater. Modern ACP systems apply much milder mixing conditions, whereas degasifying units are often used prior to secondary clarification. In fact, modern ACP systems are very effective for concentrated wastewaters with relatively high concentrations of suspended solids. As such, ACP has a consolidated market share within the full-scale applied anaerobic high-rate systems [1]. Nonetheless, ACP effluents require a subsequent treatment step in order to comply with effluent restrictions.

An alternative way of sludge retention was found by applying inert support material into the bioreactor on which the anaerobic organisms can adhere. Whereas the earliest anaerobic filters were already applied in the nineteenth century [4], the application for industrial wastewater treatment started in the 1960s in the US [17, 18]. The AF, also called packed bed process, has been developed as a biofilm system in which biomass is retained based on (1) the attachment of a biofilm to the solid (stationary) carrier material, (2) entrapment of sludge particles between the interstices of the packing material, and (3) the sedimentation and formation of very well settling sludge aggregates. AF technology can be applied in upflow and downflow reactors [19]. Various types of synthetic packing materials, as well as natural packing materials, such as gravel, coke, and bamboo segments, have been investigated to be used in AFs. Research results indicated that the shape, size, weight, specific surface area, and porosity of the packing material are important aspects. The surface adherence properties with regard to bacterial attachment are also important. Applying proper support material, AF systems can be rapidly started, because of the efficient adherence of anaerobic organisms to the inert carrier. The ease of starting up the AFs was the main reason for its popularity in the 1980s and 1990s. Problems with AF systems generally occur during long-term

operation. The major disadvantage of the AF concept is the difficulty in maintaining the required contact between sludge and wastewater, because clogging of the "bed" easily occurs. This is particularly the case for partly soluble wastewaters. These clogging problems can be overcome – at least partly – by applying a primary settler and/or a pre-acidification step [20]. However, this would require the construction and operation of additional units. Moreover, apart from the higher costs, it would not completely eliminate the problem of short-circuiting (clogging of the bed), leading to disappointing treatment efficiencies.

AF technology has been widely used for treatment of wastewaters from the beverage, food-processing, pharmaceutical, and chemical industries because of its high capability for biosolids retention [3]. Since 1981, about 130–140 full-scale upflow AF installations have been put in operation for the treatment of various types of wastewater, which is about 6% of the total amount of installed high-rate reactors. The experiences with the system certainly are rather satisfactory, with modest to relatively high loading rates of up to 10 kg COD m^{-3} day^{-1}. The AF system remains attractive for treatment of mainly non-particulate wastewaters, particularly when the sludge granulation process cannot occur satisfactorily. On the other hand, long-term problems related to system clogging and the stability of filter material caused a decline in the number of installed full-scale AF systems.

4 Sludge Granulation

The key for modern high-rate biotechnology, whatever system is considered, is immobilization of proper bacteria and archaea. In fact, the required high sludge retention in anaerobic sludge bed systems is based on immobilization, which generally leads to the formation of well-balanced microbial consortia. The presence of these consortia is considered a prerequisite for proper anaerobic process operation, particularly considering the occurrence of various syntrophic conversion reactions in the anaerobic degradation of most organic compounds, the detrimental effect of higher concentrations of specific intermediates, and the strong effect of environmental factors such as pH and redox potential. Significant progress in the knowledge of the fundamentals of the immobilization process has been made since the development and successful implementation of high-rate anaerobic treatment systems in the 1970s [21]. In the absence of fixed or free floating inert support material, a so-called "auto-immobilization" occurs, which is understood to be the immobilization of bacteria on themselves or on very fine inert or organic particles present in the wastewater, forming dense bacterial conglomerates. The bacterial conglomerates mature in due course and form round shaped granular sludge.

The phenomenon of sludge granulation has puzzled many researchers from very different disciplines. Granulation, in fact, is a completely natural process and proceeds in all systems where the basic conditions for its occurrence are met, i.e., on mainly soluble substrates applying HRTs lower than the bacterial doubling times. Because of the very low growth rate of the crucial acetoclastic methanogenic

bacteria, particularly under sub-optimal conditions, the latter conditions are easily met. Anaerobic granule formation is mostly observed in anaerobic bioreactors which are operated in upflow mode [21]. However, successful granulation was also observed in anaerobic sequencing batch reactors [22, 23]. Maybe for the first time, sludge granulation was found to occur in the up-flow Dorr Oliver Clarigesters have used in South Africa since the 1950s. However, this only became apparent by observation of sludge samples taken from such a digester in 1979 [2]. Surprisingly enough, no attention was given to the characteristics of the Clarigester sludge such as size, form, and the mechanical strength, density and porosity of sludge flocs/aggregates. Despite all the efforts made to develop systems with high sludge retention, nobody apparently noticed that the major part of the sludge consisted of a granular type of sludge.

When studying the start-up and feasibility of anaerobic upflow filters, Young and McCarty [18] quickly recognized the ability of anaerobic sludge to form very well settleable aggregates. These granules were as large as 3.1 mm in diameter and settled readily. In AF experiments with potato starch wastewater and methanol solutions conducted in the Netherlands, similar observations were made [24, 25]. Whereas the interest in anaerobic wastewater treatment in the USA and South Africa diminished, great emphasis was put on developing industrial scale systems in the Netherlands, where the introduction of new surface water protection acts coincided with the world energy crisis of the 1970s as outlined above. As a result, increasing emphasis could be given to applied and fundamental research in this field, particularly also to the phenomenon of sludge granulation [26]. A worldwide growing interest occurred from both the engineering and the microbiological fields. As a result, sufficient insight into the mechanism of the sludge granulation process for anaerobic treatment has been achieved, at least for practical application (e.g., [21, 27–40]). Granulation can proceed under mesophilic, thermophilic, and psychrophilic conditions. It is considered of great practical importance to unravel further the fundamentals concerning the growth of mixed balanced granular aggregates, not only from the microbial but also from the process engineering points of view.

A variety of process operational and external factors have an effect on granule stability, e.g., HRT, VLR, temperature, pH, upflow velocity, presence of divalent cations and heavy metals, salinity, and nutrient availability [27, 32, 41, 42]. The seed sludge and the chemical composition of industrial wastewater have significant impact on the chemical composition of the granular sludge [43]. In addition, macro- and micronutrients, e.g., iron, copper, calcium, magnesium, cobalt, and aluminum, are vital for the aggregation of the cells [37].

The morphological and spatial structure of granules in a UASB reactor was examined by MacLeod et al. [44]. They found that the granular aggregates were three-layered structures. Whereas the exterior layer of the granule contained a heterogeneous microbial population, the middle layer consisted of more homogeneous biomass. Moreover, the internal core consisted of a "single species," such as *Methanothrix*-like cells, later renamed *Methanosaeta* spec. [45]. Similar findings have been reported in the study by Baloch et al. [46], in which anaerobic granules

were found to possess a multi-layered structure with complex microbial ecology and dominating methanogenic subpopulations. Apparently, *Methanosaeta* plays an important role in sludge granulation [31]. The structured characteristics and layered 'ecological zones' of the granules were defined as a stable metabolic arrangement that creates optimal nutritional and environmental conditions for all microorganisms included in it [47]. The carbon source or substrate was considered the most important factor affecting the microstructure of the UASB granules [31, 43, 48]. The extent of required acidification and the acidogenesis rate of the substrate affect the concentration profiles of the substrate, metabolites in the granule and its structure. For example, granules in a UASB reactor treating sucrose and brewery wastewaters had a three-layered structure; however, those in a UASB reactor treating glutamate exhibited a rather uniform structure. McHugh et al. [49] reported that, in a granule, a central core of acetoclastic methanogens is surrounded by a layer of hydrogen- and/or formate-producing acetogens, and hydrogen- and/or formate-consuming methanogens. The outside layer of this granule structure consists of microorganisms that hydrolyze and acidify the complex organic matter [35]. *Methanosaeta* spp. populations have been found abundant in stable granules in various studies. Apparently, these organisms are necessary for the successful operation of anaerobic sludge bed reactors. Methanogens related to *Methanosaeta* spp. have a filamentous morphology, are more or less hydrophobic, have an electrophoretic mobility of about 0, and are considered the most important component of the granule structure, providing support for other microorganisms in the granule [36, 41, 50]. It is hypothesized that, after the formation of such methanogenic nucleus, acetogenic bacteria adhere, followed by the formation of biofilm layers consisting of hydrogenotrophic methanogens [27]. On the other hand, the bacteriophage in the granular sludge may cause the breakdown of the granules [37].

Molecular techniques are increasingly used to study the microbial community structure of environmental ecosystems such as anaerobic granular sludge without cultivation [43]. By using molecular techniques, Sekiguchi et al. [51] localized the methanogens in anaerobic granular sludge systems. They showed that a significant fraction of the granule is inactive and this probably consists of cellular fragments. Satoh et al. [52] combined 16S rRNA gene-based molecular techniques with microsensors to provide direct information about the phylogenetic diversities, spatial distributions, and activities of bacteria and archaea in anaerobic granules. They found that acid and H_2 production occurred in the outer part of the granule, below which H_2 consumption and CH_4 production were found.

In essence, sludge granulation finds its ground in the fact that bacterial retention is imperative when dilution rates exceed the bacterial growth rates [53]. Immobilization further requires the presence of support material and/or specific growth nuclei [54], as well as the presence of exopolymeric substances (EPS) acting as a kind of glue creating a microbial matrix [55]. The occurrence of granulation can be explained as follows:

- Proper growth nuclei, i.e., inert organic and inorganic bacterial carrier materials as well as bacterial aggregates, are already present in the seed sludge.
- Finely dispersed matter, including viable bacterial matter, becomes decreasingly retained once the superficial liquid and gas velocities increase, applying dilution rates higher than the bacterial growth rates under the prevailing environmental conditions. As a result, film and/or aggregate formation automatically occurs.
- The size of the aggregates and/or biofilm thickness are limited, viz. it depends on the intrinsic strength (binding forces and the degree of bacterial intertwinement) and the external forces exerted on the particles/films (shear stress). Therefore, in due course, particles/films fall apart, evolving a next generation. The first generation(s) of aggregates, indicated by Hulshoff Pol et al. [54] as "filamentous" granules, are quite voluminous and in fact more a flock than a granule.
- Retained secondary growth nuclei grow in size again, but also in bacterial density. Growth is not restricted to the outskirts, but also proceeds inside the aggregates. In due course, they fall apart again, evolving a third generation, etc.

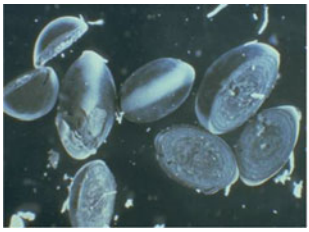
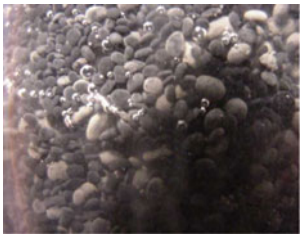
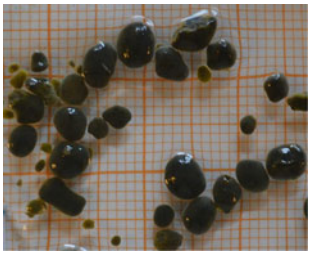
During the selection process described above, both organic and hydraulic loading rates gradually increase, increasing the shear stress inside the system. Granular sludge is easily cultivated for treatment of partially acidified non-particulate wastewaters. Table 2 lists some common characteristics of methanogenic granular sludge.

5 Upflow Anaerobic Sludge Blanket (UASB) Reactors

One of the most remarkable and significant developments in high-rate anaerobic treatment technology is the invention of UASB reactor by Lettinga and his co-workers in the Netherlands [11, 24]. The sludge retention in such a reactor is based on the formation of well settleable sludge aggregates (flocs or granules), and on the application of a reverse funnel-shaped internal gas–liquid–solids separation (GLSS) device. Many successful performance results have been reported in laboratory- and pilot-scale applications using anaerobic granular sludge bed processes, which resulted in the establishment of thousands of full-scale reactors worldwide [1, 56, 57]. Anaerobic sludge bed reactors are, undoubtedly, by far the most popular anaerobic wastewater treatment systems so far, having a wide application potential in industrial wastewater treatment. In view of its prospects, and the fact that almost 90% of the newly installed high-rate reactors are sludge bed systems [1], the UASB process is elaborated in more detail than the other systems.

The first UASB reactors were installed for the treatment of food, beverage, and agro-based wastewaters, rapidly followed by applications for paper and board mill effluents in 1983 [58]. Most of the full-scale reactors are used for treating agro-industrial wastewater, but applications for the treatment of wastewaters from

Table 2 Proposal for definition and characteristics of good quality granular sludge (photos: Paques B.V.)

Granular sludge examples	"Good quality granule" characteristics
 Potato wastewater-grown granules	Metabolic activity: Specific methanogenic activity range of granular sludge: 0.1–2.0 kg COD-CH_4 kg^{-1} VSS day (amount of CH_4 expressed in COD equivalents per amount of biomass per time unit) Typical values for industrial wastewater : 0.3–1.0 kg COD-CH_4 kg^{-1} VSS day
	
 Paper mill wastewater grown granules	Settleability and other physical properties: Settling velocities: 2–100 m h^{-1}; typically: 30–75 m h^{-1} Density: 1.00–1.05 g L^{-1} Diameter: 0.1–8 mm; typically: 0.15–4 mm Shape: spherical formed and well defined surface Color: black/gray/white

chemical industries are increasing, as discussed below [1, 59]. Similar to the AF system, the wastewater moves in an upward flow through the UASB reactor. However, in contrast to the AF system, no packing material is present in the UASB reactor. Good settleability, low HRTs, elimination of the packing material cost, high biomass concentrations (up to 80 g L^{-1}), effective solids/liquid separation, and operation at high VLRs can be achieved by UASB reactor systems [60]. The design VLR is typically in the range of 4 to 15 kg m^{-3} day COD [9]. One of the major limitations of this process is related to wastewaters having a high suspended solids content, which hampers the development of dense granular sludge [61]. The sludge bed reactor concept is based on the following ideas:

- Anaerobic sludge has or acquires good sedimentation properties, provided the process is operated correctly. Small particles or slowly settleable sludge are washed out from the system.
- The required good contact between the sludge and wastewater in UASB reactors is generally accomplished by feeding the wastewater as uniformly as possible over the bottom of the reactor. The increased up-flow velocity also results in a better contact between the sludge particles and the pollutants. At VLRs exceeding 5 kg COD m^{-3} day^{-1}, mixing of sludge and wastewater is brought about by biogas turbulence. Mechanical mixing is not applied in UASB reactors.
- With wastewaters containing biodegradable inhibitory compounds, the hydrodynamic mixing is additionally achieved by applying a liquid recirculation flow. As a result, a more completely mixed flow pattern is acquired and stratification of the substrate and intermediate products over the height of the reactor is minimized, thereby minimizing potential inhibition.
- The wash-out of the active sludge aggregates is prevented by separating the produced biogas using a gas collection dome installed at the top of the reactor. In this way, a zone with relatively little turbulence is created in the uppermost part of the reactor, in fact functioning as an in-built secondary clarifier.
- The GLSS-device constitutes an essential part of a UASB reactor and serves the following functions:

    1. To collect, separate, and discharge the produced biogas. For a satisfactory performance the gas-liquid surface area within the device should be sufficiently large, so that gas can escape easily. This is particularly important if scum layers were to develop.
    2. To reduce liquid turbulences in the settler compartment (resulting from bio-gas production), enhancing sludge settling.
    3. To retain sludge particles by a mechanism of sedimentation, flocculation.
    4. To limit the expansion of the sludge bed towards the settler compartment.
    5. To reduce or prevent the buoying sludge particles underneath the gas dome washing out from the system.

Some researchers and practitioners suggest replacing the GLSS-device by a packed bed in the upper part of the reactor. This so-called up-flow hybrid reactor combines a UASB reactor in the lower part with an AF in the upper part and promotes the advantages of both reactor types. Anaerobic hybrid reactors have been applied for treatment of various kinds of industrial wastewaters and domestic wastewaters (e.g., [62, 63]). The first study on the performance of a hybrid reactor was reported by Guiot and van den Berg [64] who obtained high efficiency in retaining biomass by using packing material in a hybrid reactor (UASB + AF). It is reported that performance of high rate anaerobic sludge bed reactors has significantly increased by locating the packing material to the top 25–30% of the reactor [65]. Kennedy and Guiot [66] reported that hybrid reactor systems were able to withstand severe organic shock loads and recover within a reasonable period of time. They achieved a COD removal rate of 95% at an OLR of 33 kg m^{-3} day COD

in an anaerobic hybrid system treating municipal landfill leachate. Similarly, an anaerobic hybrid reactor was successfully used with a COD removal efficiency of 97% for the treatment of dairy effluents [67].

The performance of hybrid up-flow anaerobic filters depends on the contact of the wastewater with both the attached biofilm in the media and suspended growth in the sludge bed part [68]. In some designs the packing material is mounted only in the settling compartment, leaving the GLSS at its original position. About 2–3% of all anaerobic reactors installed are hybrid reactors. In most applications the majority of organic matter conversion is located in the sludge bed section, whereas the removal of a specific fraction of pollutants is located in the filter area at the top. Specific chemical wastewaters show better treatment efficiencies for all compounds using hybrid systems compared to UASB reactor. Ramakrishnan and Gupta [69] investigated the biodegradation of complex phenolic mixture in an anaerobic hybrid reactor that was a combination of UASB reactor and AF. They found that the optimum COD/NO_3-N ratio for maximum COD and phenolics removal was about 6.4. At this ratio, the removal of COD and phenolics were 96% and 99%, respectively. Kleerebezem et al. [70, 71] performed laboratory research on the treatment of purified terephthalic acid (TA) wastewater. Their results showed that the conversion of terephthalic acid to benzoate is only possible at low concentrations of acetate and benzoate. By applying a hybrid system, the latter two are converted in the sludge bed area, whereas terephthalic acid and other refractory compounds are converted in the hybrid section, where specific flora is retained. Despite these laboratory findings, full-scale anaerobic plants treating TA wastewater merely consist of a single stage sludge bed system. Because these reactors are generally followed by an activated sludge post-treatment system, any non-degraded aromatic is subsequently aerobically converted. Full-scale anaerobic reactors treating TA wastewaters are generally characterized by good treatment efficiencies. In addition to TA, several other chemical wastewaters are typically treated by anaerobic reactor systems as reviewed by Macarie [72] and Kleerebezem and Macarie [73]. Although some full-scale reactors consist of hybrid systems, single sludge bed systems seem to be preferred; after prolonged periods of operation the filter sections at the top part of the reactor often deteriorate.

6 Fluidized and Expanded Bed Systems (FB, EGSB, IC Reactors)

Fluidized bed and expanded bed systems are regarded as the second generation of anaerobic sludge bed reactors achieving extreme VLRs (at laboratory scale: 30–60 kg m^{-3} day^{-1} COD, at full-scale: 20–40 kg m^{-3} day^{-1} COD). The FB process is based on the occurrence of bacterial attachment to non-fixed or mobile carrier particles, which consist of, for example, fine sand (0.1–0.3 mm), basalt, pumice, or plastic. The FB system can be regarded as an advanced anaerobic

technology [74, 75], which may reach loading rates exceeding 40 kg m^{-3} day^{-1} COD, when operated under defined conditions [76]. Good mass transfer resulting from liquid turbulence and high flow rate around the particles, less clogging and less short-circuiting because of the occurrence of large pores through bed expansion, and high specific surface area of the carriers because of their small size makes FB reactors highly efficient. However, long-term stable operation appears to be problematic. The system relies on the formation of a more or less uniform (in thickness, density, and strength) attached biofilm and/or particles. To maintain a stable situation with respect to the biofilm development, a high degree of pre-acidification is considered necessary and dispersed matter should be absent in the feed [77]. Despite that, an even film thickness is very difficult to control and in many situations a segregation of different types of biofilms over the height of the reactor occurs. In full-scale reactors, bare carrier particles may segregate from the biofilms, leading to operational problems. To keep the biofilm particles in the reactor, flow adjustments are necessary, after which the support material starts to accumulate in the lower part of the reactor as a kind of stationary bed, whereas light fluffy aggregates (detached biofilms) are present in the upper part. Retention of these fluffy aggregates can only be accomplished when the superficial velocity remains relatively low, which in fact is not the objective of an FB system.

Modern FB reactors such as the Anaflux system [78] rely on bed expansion rather than bed fluidization applying upflow velocities < 10 m h^{-1}. As bed expansion allows a much wider distribution of prevailing biofilms, the system is much easier to operate. As in the conventional AF systems, an inert porous carrier material (particles <0.5 mm, density about 2) is used for bacterial attachment in the Anaflux system. The Anaflux reactor uses a triple phase separator at top of the reactor, more or less similar to the GLSS device in UASB reactors. When the biofilm layer attached to the media becomes excessively overdeveloped and the concerning (lighter) aggregates subsequently accumulate in the separator device, the material is periodically extracted from the reactor by an external pump, in which it is subjected to sufficient shear to remove part of the biofilm. Then both the media and detached biomass are returned to the reactor, and the free biomass is allowed to be washed out from the system. In this way the density of the media is controlled and a more homogeneous reactor bed is created. Up to 30–90 kg m^{-3} volatile suspended solids (VSS), reactor can be retained in this way, and because of the applied high liquid upflow velocities, i.e., up to 10 m h^{-1}, an excellent liquid-biomass contact is accomplished. The system is applicable to wastewaters with a suspended solids (SS) concentration <500 mg L^{-1}. Most of the full-scale anaerobic FB reactors are installed as Anaflux processes. Nonetheless, at present, the EGSB reactors are much more of commercial interest for full-scale applications than the more expensive FB systems.

The EGSB reactor can be considered an upgrade of the conventional UASB reactor. The EGSB system employs granular sludge, which is characterized by good settling characteristics and a high methanogenic activity. As a consequence, the applied VLR and upward flow velocities are distinctly higher in EGSB reactors compared to UASBs. Sludge bed expansion is achieved by prevailing process

conditions. When applying extreme sludge loading rates, the settleability reduces because of the biogas hold-up in the granules. Nonetheless, because of the high sludge settleability, high superficial liquid velocities, i.e. exceeding 6 m h^{-1}, can also be applied. These high liquid velocities, together with the lifting action of gas evolved in the bed, lead to a (modest) expansion of the sludge bed. As a result, an excellent contact between sludge and wastewater prevails in the system, leading to significantly higher loading potentials compared to conventional UASB installations. In some expanded bed systems, e.g., the Biopaq®IC-reactor, the superficial flow velocities, resulting from both hydraulic and gas flows, may reach 25–30 m h^{-1}, causing an almost complete mixing of the reactor medium with the available biomass.

Excellent results have been obtained with modern full-scale EGSB installations, such as the Biobed EGSB and Biopaq®IC reactors, using various kinds of wastewaters and applying VLRs of 25–35 kg m^{-3} day^{-1} COD. The extreme COD loading rates of EGSB-type systems result in extreme biogas loading rates:

$$V_{\text{biogas}} = \text{COD}_{\text{conc}} \times \frac{E_{\text{ff-meth}}}{100} \times \frac{0.35}{F_{\text{meth-biogas}}} \times \frac{(T + 273)}{273} \times V_{\text{upw,liquid}}$$

in which, $E_{\text{ff-meth}}$ = amount of COD converted to CH_4 or COD efficiency based on CH_4 production, $F_{\text{meth-biogas}}$ = fraction of methane in biogas (e.g., 0.6 for 60% CH_4), T = operational temperature of UASB reactor in °C, $V_{\text{upw, liquid}}$ = upward liquid velocity in UASB reactor. Generally, a biogas loading rate of no more than 2–3 m^3 m^{-2} h^{-1} are applied for conventionally designed GLSS devices in UASB reactors. For biogas loading rates exceeding these values, more advanced gas separators are required. EGSB reactors have a high height-diameter ratio, with reactor heights reaching up to 25 m. Consequently, biogas turbulence accumulates from bottom to top. Because the EGSB systems rely on a complete retention of the granular sludge, efficient sludge separation at the top part of the system is of the utmost importance. The various contractors supplying EGSB reactors have their own typical features for separating actively the sludge from the liquid and gas flow, applying specifically designed GLSS units. It may be clear that, under EGSB conditions, conventionally designed GLSS devices are of no use. Interestingly, by applying an EGSB reactor system, several other types of wastewaters can be treated that cannot be treated using conventional UASB systems. Examples are:

- Wastewaters containing highly toxic but anaerobically degradable components. Treatment of these wastewaters requires that external or internal dilution keeps the toxicant concentration to which the biomass is exposed sufficiently low. For example, full-scale reactors have shown stable performance over many years treating wastewaters with high influent formaldehyde concentrations, reaching values up to about 10 g L^{-1} [79, 80]. By applying recirculation ratios of 1:30, inlet formaldehyde concentrations are always below IC_{50} values, i.e., 350 mg L^{-1}.

- Wastewater containing dyes and other toxic textile auxiliary compounds which can be successfully converted into biogas without inhibitory effects on the biomass [81].
- Cold (<10 °C) and dilute (COD < 1 g L^{-1}) wastewaters, i.e., when specific gas production is very low and biogas mixing is absent [82]. EGSB reactors are characterized by an improved hydraulic mixing, independent from the biogas production. As a consequence, and in contrast to UASB systems, all retained sludge is optimally mixed with the incoming wastewater and small inactive particles are washed out from the system.

A special version of the EGSB concept is the so-called Internal Circulation (Biopaq®IC) reactor, depicted in Fig. 2 [83]. In this type of reactor, the produced biogas is separated from the liquid halfway the reactor by means of an in-built GLSS device and conveyed upwards through a pipe to a degasifier unit or expansion

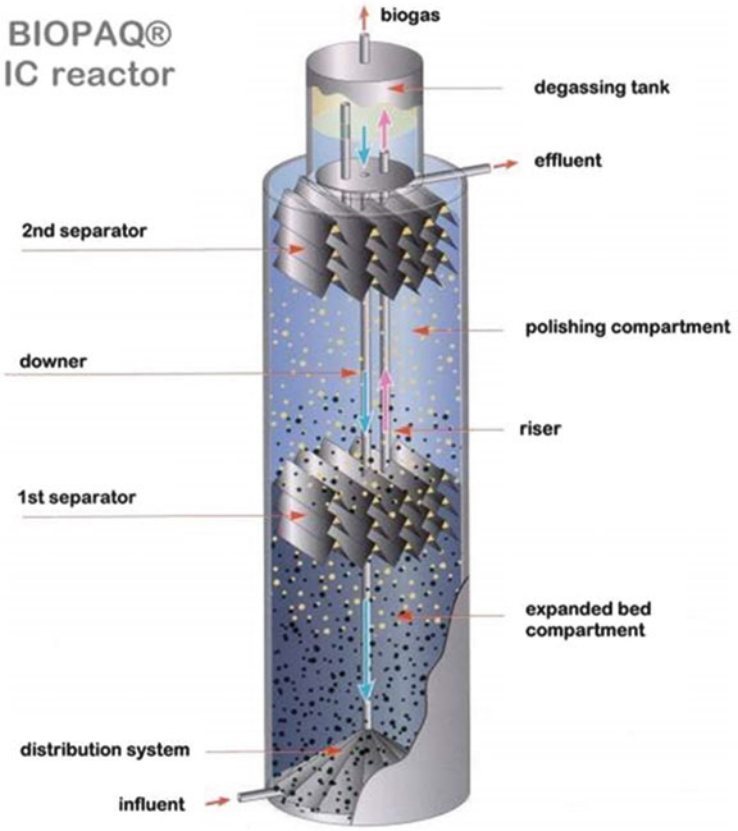


Fig. 2 Schematic representation of the Biopaq® Internal circulation (IC) reactor. Reactor height reaches 24 m, with sludge bed heights between 6 and 14 m. Applied liquid upward velocities are between 4 and 8 m h^{-1}. Liquid recirculation is brought forward by the biogas production

device. Here, the separated biogas is removed from the system, whereas the sludge–water mixture drops back to the bottom of the reactor via another pipe. In fact, the lifting forces of the collected biogas are used to bring about a recirculation of liquid (and granular sludge) over the lower part of the reactor, which results in an improved contact between sludge and wastewater. The extent of liquid/sludge recirculation depends on the gas production [83–85].

7 Anaerobic Baffled (Staged) Reactors (ABR)

Although ACP, UASB, and EGSB reactors are based on a mixed to completely mixed reactor content, various designs have been tested that employ staging of the various phases of anaerobic treatment, creating a plugflow in the waterline [86]. An extreme example is the two-stage process where the acidification step is completely separated from the methanogenic step. Although a complete separation of these steps initially showed good results in laboratory scale research, in practice, pre-acidification is generally combined with influent buffering [86, 87]. In fact, a too high degree of pre-acidification negatively impacts stable granule formation. On one hand, the suspended solids formed during acidification and subsequently carried over to the methanogenic reactor deteriorate the granular sludge bed stability [61]. On the other hand, the occurrence or presence of fermentative substrate conversion by acidifying organisms is indispensable for the production of sufficient exopolymeric substances (EPS) which are required for the formation of a stable granular structure with high granule strength [55]. Various authors suggested that the EPS are particularly produced by acidifying organisms, creating the matrix in which all bacteria and archaea are embedded [88–90]. At present, in most full-scale applications, a pre-acidification of maximally 40% is pursued.

Horizontal staging is obtained in ABRs, which is best characterized as a series of serially operated upflow units without GLSS devices [91, 92]. Although some larger scale applications were made on domestic sewage, the reactor has not been developed further than the pilot scale [93]. A problem of concern is the hydrodynamic limitation giving constraints to the achievable SRT in the system, because the superficial liquid velocity in a baffled system is substantially higher than in a single step sludge bed reactor. As a result, the sludge mass may slowly move with the liquid flow through the various compartments. Vertically staged reactors such as the upflow staged sludge bed system [38, 86, 94, 95] overcome this problem and were specifically developed for high temperature treatment. Although the staged reactor concept showed very promising results on a pilot scale, full-scale reactors are very scarce for this type of high-rate reactor.

8 High-Rate Reactors with Advanced Sludge-Liquid Separation

8.1 Reactors with Advanced Settling or Flotation for Sludge Retention

Most, if not all, research on anaerobic sludge granulation, and factors impacting granule growth, has been conducted under laboratory-scale conditions [21, 37]. However, the predictive value of the extensive laboratory-scale research might be questioned, realizing the completely different hydrodynamic conditions prevailing in the full-scale high rate reactors. In fact, the prevailing shear forces are of another order, meaning that full-scale experiences on a similar wastewater can be very different from the carefully conducted laboratory tests. Disappointing granule formation restricts contractors in offering proper anaerobic high-rate reactors to industries for treating their wastewater. In those situations, conventional sludge bed reactors might then be offered, such as the UASB, of which the treatment efficiency is not dependent on the presence of granular sludge. For the more complex types of wastewaters, such as those characterized by a high SS content, expanded bed reactors are not very appropriate. Under the prevailing flow conditions the SS are washed out from the system, and/or the heavier SS may negatively impact granule formation and granule growth [61]. During the treatment of wastewaters that are characterized by COD concentrations exceeding 50 g L^{-1}, e.g., distillery slops or vinasse, the cultivation of granular sludge is extremely difficult, if possible at all. Because of the high influent COD concentrations, resulting HRTs are very long, drastically diminishing the hydraulic selective pressure inside the reactor, which is regarded as crucial for sludge granulation [21]. In the increasingly competitive market, however, contractors are forced to develop anaerobic high rate systems that are as robust as UASB reactors, whereas the COD loading potentials should reach the levels of EGSB systems, although the presence of granular sludge cannot be guaranteed. This calls for more enhanced sludge-solids separation devices that can operate under high hydraulic flow conditions, but which are not dependent on discrete particle settling as is more or less the case with granular sludge. Enhanced flocculent sludge-liquid separation can be established by physically enhanced settling, flotation, filtration. The novel reactor systems making use of this principle are explained below.

Physically enhanced settling can be achieved by mounting a tilted plate settling device for sludge liquid separation into the bioreactor. In fact, Biothane Systems International is already incorporating a tilted plate settler into the GLSS device in their BioBed®EGSB system [79, 80]. In the past few years, the Dutch contractor Paques applied this idea to an upflow sludge bed reactor with a high height-diameter ratio, in a system denominated as the Biopaq®UASBplus (Fig. 3). Although the UASBplus sludge separator device can also be employed for the retention of anaerobic granules, it is very well suitable for anaerobic flocculent sludge, which is prevalent in case of more concentrated wastewater, such as

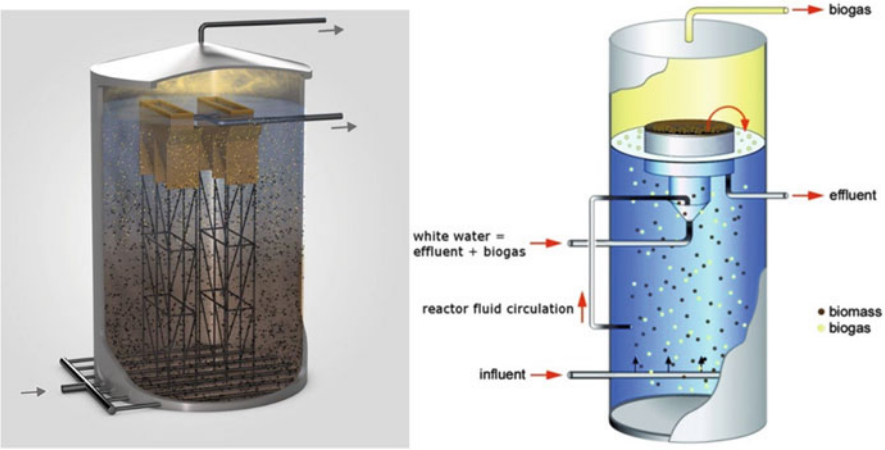


Fig. 3 Schematic representation of (*left*) the Biopaq®UASBplus reactor for the treatment of concentrated waste waters and (*right*) the Biopaq®AFR reactor for the treatment of fats, oil, and grease (FOG)-rich wastewater, in which sludge separation is based on sludge flotation. Reactors operate with either granular or flocculent sludge

bioethanol waste(water), e.g. vinasse. At present 25 full-scale UASBplus systems are operational of which approximately one-third of the reactors contain flocs or small aggregates; most UASBplus reactors are installed in China.

Sludge separation by *flotation* is a well-known pre- and post-treatment technique to separate small particles, low-density floating mass, and/or hydrophobic compounds such as fats, oil, and grease (FOG) from the liquid. Particularly the presence of FOG in wastewaters may cause problems with sludge flotation and sludge washout in both UASB and EGSB reactors [96, 97]. However, the buoyant force of entrapping biogas in FOG-loaded anaerobic sludge can also be used to separate the reactor sludge from the discharging effluent by mounting the flotation device inside the anaerobic reactor. In this way, the effluent is clarified and, meanwhile, the active methanogenic sludge is retained in the bioreactor. The Dutch contractor Paques developed this so-called anaerobic flotation reactor, denominated as the Biopaq® AFR, to convert high concentrations of fats and oils into methane (Fig. 3). The AFR system is successfully applied in three full-scale projects, two at dairy/food industries in the Netherlands treating each 4,000 kg day^{-1} COD with a reactor volume of 500 m^3 [98]. There are two AFR reactors in China, one has a volume of 28,000 m^3 and the other 9,000 m^3. The former AFR reactor treats 1,63,000 kg day^{-1} COD wastewater from a bio-ethanol industry in China.

The separation of active methanogenic sludge from the bulk liquid by *filtration* is applied in anaerobic MBR systems. In AnMBR systems (see next section) the biomass is present in very small flocs, or even as single cells [99].

8.2 Membrane Coupled Anaerobic Reactors

In recent years, with growing application experiences from aerobic MBRs, AnMBRs have received much attention, particularly for those applications where the commonly applied sludge bed systems are less successful. AnMBRs combine the advantages of both MBR and anaerobic technology [13, 100]. Operational costs related to energy requirements for gas/liquid recirculation for membrane fouling control and chemical costs required for membrane cleaning are still heavy burdens on the economic feasibility of AnMBRs. However, membrane acquisition and/or replacement costs have decreased significantly because of a decline in membrane module costs [15]. Despite those constraints, AnMBRs offer high quality effluents free of solids and complete retention of biomass, regardless of their settling and/or granulation properties. Furthermore, AnMBRs can be used to retain special microbial communities that can degrade specific pollutants in the wastewater. Therefore, AnMBR technology may present an attractive option for treating industrial wastewaters at extreme conditions, such as high salinity [101], high temperature [102], high SS concentrations [103], and presence of toxicity [104], which hamper granulation and biomass retention or reduce the biological activity [13]. Industrial wastewaters with extreme physicochemical characteristics are likely to occur more often in the future as cleaner industrial production processes require reduction of water consumption, water reuse, and resource recovery [1, 13]. Both organic and inorganic membranes can be used in AnMBRs [15]. Membrane material characteristics may affect the degree of fouling in AnMBRs; e.g., organic and inorganic membranes may show different fouling behaviors. Kang et al. [105] reported that cake layer formation was the main mechanism for fouling of organic membranes, whereas inorganic precipitation, mainly struvite, played the key role in the fouling of inorganic membranes. Futselaar et al. [106] claim it is possible to obtain higher COD to methane conversion efficiencies in AnMBRs in comparison to conventional UASB reactors for the treatment of industrial wastewaters. Both physical and chemical methods can be used for membrane cleaning depending on membrane operation, fouling degree and type, and membrane configuration. Detailed information about the operation of AnMBRs for industrial wastewater treatment and cleaning of membranes in AnMBRs can be found in different studies [13, 15, 107].

Combinations of membranes with different types of high-rate anaerobic reactor configurations such as CSTR, ACP, UASB, EGSB, FB, and hybrid reactors seem possible alternatives for treatment of industrial wastewaters [15]. However, membrane integration eliminates the hydraulic selection pressure required for granulation whereas flocculent biomass with poor immobilization characteristics is retained instead of washed out. Moreover, by applying cross flow filtration, the prevailing shear forces minimize the particle's diameter. Therefore, no granulation is expected in sludge bed reactors coupled to membrane filtration, which would decrease the settleability of the biomass in the long-term operation. Nonetheless, a sequenced approach of a UASB reactor followed by separate membrane modules offers interesting perspectives for full treatment. The preceding UASB provides a

Table 3 Memthane AnMBR references as of February 2015

Year	Region	Industry	Membrane configuration	Reactor volume (m^3)	Load (kg day^{-1} COD)	Flow (m^3 h^{-1})
2015	Africa	Dairy	Parallel	2,900	16,500	83
2013	Europe	Food + Pet food	Parallel	2,400	20,000	39
2013	Europe	Food	Parallel	1,200	7,500	15
2012	Europe	Dairy	Serial	675	5,500	23
2012	Americas	Bioethanol	Serial	20,000	63,800	174
2012	Americas	Food	Serial	1,250	7,500	18
2012	Americas	Food	Serial	1,700	10,200	21

pre-elimination of SS by entrapment and biodegradation in the sludge bed, which reduces the SS load to the membrane and thus minimizes membrane fouling related to cake layer formation [15]. Most researched AnMBR systems consist of a CSTR bioreactor coupled to cross-flow membrane skids or a CSTR bioreactor equipped with submerged membrane modules.

Successful commercial implementation of AnMBR technology started in the early 2000s. In Japan, Kubota realized 13 rather small-scale plants with flow rates up to 2.5 m^3 h^{-1} using flat-sheet submerged membranes. The same configuration was picked up at larger scale in the USA by ADI, where three full-scale systems have so far been realized from 2008 onward [107 108]. The year 2008 also saw the construction of the first multi-tube demonstration scale AnMBR for treating whey from a cottage cheese producer in the USA. This system utilized Pentair's (formerly Norit) ultrafiltration membranes. Based on this success, Biothane Systems International and Pentair co-developed a low-energy AnMBR system called Memthane. There are now seven full-scale Memthane plants (see Table 3).

9 Types of Anaerobic High-Rate Reactors Currently Installed

Although various high-rate reactors are available in the market, sludge bed systems are by far the most used. Van Lier [1] presented a survey taken from various international contractors regarding their sales and concluded that, of all reactors installed between 1981 and 2007, about 77% consisted of sludge bed systems, mainly UASB and EGSB/IC reactors. However, focusing on the period 2002–2007, the contribution of sludge bed reactors to total sales was almost 90%. These numbers illustrate the popularity of anaerobic sludge bed systems for wastewater treatment. In that survey [1] it was also recognized that the sales of conventional UASB reactors were declining, whereas the EGSB type of reactors were becoming more popular. This trend has continued and currently the sales of conventional UASB reactors dropped to low levels for both Dutch contractors Paques BV and Biothane-Veolia as depicted in Figs. 4 and 5, respectively.

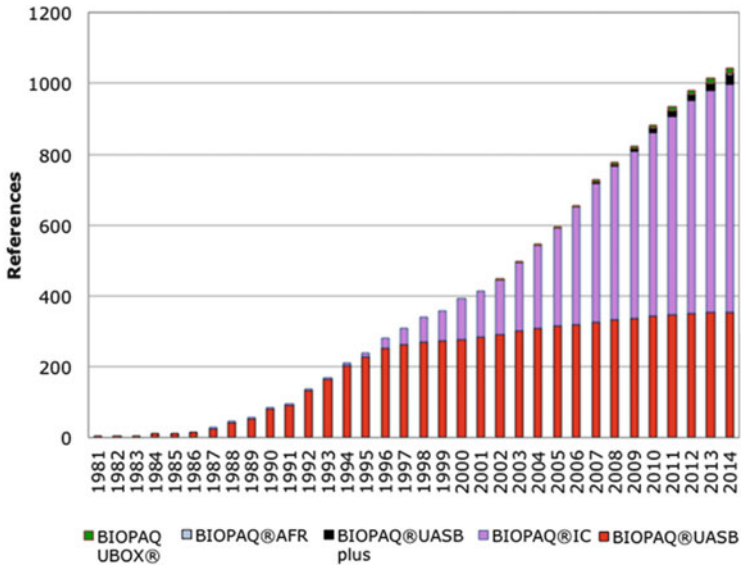


Fig. 4 Sales of anaerobic high rate reactors by Paques BV since the company's start-up (1981)

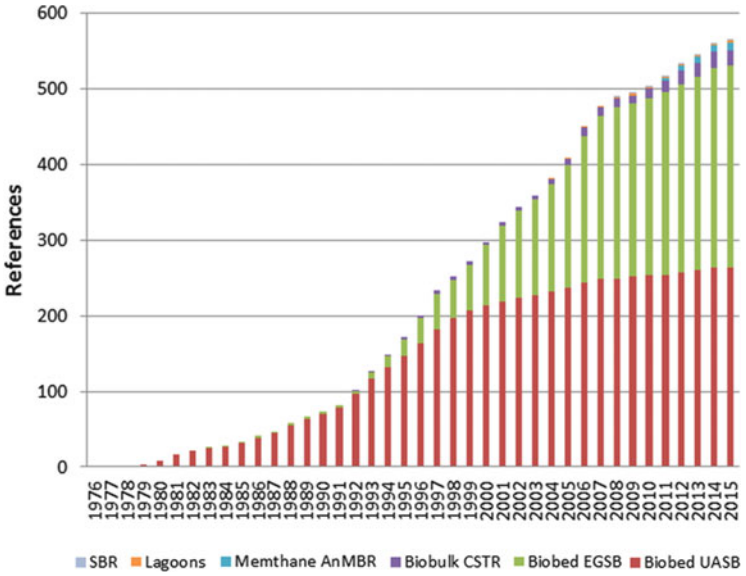


Fig. 5 Sales of anaerobic high rate reactors by Biothane-Veolia since the company's start-up (1976)

Table 4 Energy output and CO_2 emission reduction applying anaerobic high-rate wastewater treatment systems [10]

Parameter	Values[a]
Loading capacity (kg m^{-3} day^{-1} COD)	5–35
Energy output (MJ m^{-3} day^{-1} reactor installed)	55–390
Electric power output (kW-e m^{-3} reactor installed)	0.25–1.7
CO_2 emission reduction (ton m^{-3} $year^{-1}$ CO_2, based on coal-driven power plant)	1.9–13

[a]Assumptions: 80% CH_4 recovery relative to influent COD load and 40% electric conversion efficiency using a modern combined heat power generator. Intermediate values are obtained by linear interpolation

In addition to the conventional UASB and EGSB reactor sales, it is of interest to see that the new technologies are also beginning to be accepted by the market. For Paques this concerns the Biopaq®UASBplus and Biopaq®AFR reactors (depicted in Fig. 3) as well as the Biopaq®UBOX reactor, which is a sequential anaerobic/aerobic single-reactor system for the treatment of municipal wastewater. In this system, the activated sludge compartment is vertically mounted on top of the UASB compartment [109]. For Biothane-Veolia the new technologies particularly concern the BioBulk CSTR and the Memthane AnMBR.

At present, because of the concerns related to depleting fossil fuels and thus increasing energy prices, as well as to ongoing concerns related to greenhouse gas emissions linked to fossil fuel consumption, anaerobic high-rate treatment is receiving renewed interest worldwide. Depending on the loading potentials of the various high-rate reactors and the anaerobic treatability of the wastewater, the energy potential of anaerobic reactor can be easily estimated. Table 4 lists the expected energy output and CO_2 emission reduction when anaerobic high-rate treatment is applied; meanwhile, the generated CH_4 is used inside the industry instead of fossil fuel-derived electricity. Any intermediate value can be derived by linear interpolation.

10 Non-traditional Applications of Anaerobic High-Rate Reactors

High-rate anaerobic biotechnology has a significant potential for the recovery of bio-energy by the treatment of medium and/or high strength wastewaters, particularly from those produced in agro-industries. High COD removal efficiencies with a bio-methane production of about 250–350 m^3 per ton of removed COD can be recovered depending on the inert COD content of the substrate. At present, most applications of anaerobic wastewater treatment can be found as end-of-the-pipe treatment technology for food processing wastewaters and agro-industrial wastewaters. In some recycled paper-based paper mills, mainly those

which are producing corrugated or massive cardboard, inline treatment is applied consisting of sequenced anaerobic-aerobic treatment. These paper mills have no effluent and evaporated water in the paper drying section is replenished by fresh water [110, 111]

The number of anaerobic applications in the non-food sector is rapidly growing. Common examples are the paper mills and the chemical wastewaters, such as those containing formaldehyde, benzaldehydes, terephthalates, etc. [112]. The latter is surprising, as the chemical industry usually has prejudices against biological treatment and anaerobic treatment in particular. Although various industrial wastewaters can be treated by anaerobic technology, various organic and inorganic materials in industrial wastewaters may be toxic to the anaerobic biomass. For example, some of the main problems encountered in the UASB reactors treating wastewaters from baker's yeast industries are the accumulation of the inorganic matter, i.e., struvite ($MgNH_4PO_4$), but also ammonia toxicity produced by high pH values, and high hydrogen sulfide content in the biogas. Another example is the biodegradable cyanide, which is present in some food processing wastewaters, and which is known to be inhibitory to acetoclastic methanogens [113]. Alkaloid wastewaters can be given as another example of refractory and inhibitory wastewaters, which contains some toxic organic chemicals such as *N*,*N*-dimethylaniline and toluene that are inhibitory for anaerobic biomass [114]. However, many organic toxicants can be anaerobically biodegraded if precautions are taken, e.g., gradual increase in toxicant concentration during start-up period and prevention of biomass wash-out until acclimation is completed. With regard to the chemical compounds, it is of interest to mention that certain compounds, such as poly-chloro-aromatics and poly-nitro-aromatics as well as the azo-dye linkages can only be degraded when a reducing (anaerobic) step is introduced in the treatment line [86, 115]. Anaerobics are then complementary to aerobics for achieving full treatment. For textile wastewater this is shown at full scale: the wastewater can be decolorized and detoxified in a serial full-scale anaerobic-aerobic treatment system [81]. At full scale, the application of a sequenced anaerobic–aerobic wastewater treatment system is commonly applied for the treatment of industrial wastewaters. In such a system, which may consist of an anaerobic high-rate process followed by an activated sludge process, the energy required for aeration and the amount of excess sludge in the aerobic second stage is significantly reduced when using an anaerobic first stage. In addition, with a net energy production in the first anaerobic stage, the total energy efficiency of the treatment plant can be increased, even becoming a net energy producer. Moreover, when industries are hampered by a limited aerobic wastewater treatment capacity, the implementation of an anaerobic first stage significantly relieves this pressure, even giving potentials to increase the industrial production capacity without the need to enlarge the aerobic treatment step.

The treatment of cold and very low-strength wastewaters can be achieved by applying optimized hydraulic mixing conditions in sludge bed reactors [82, 116]. In addition to municipal sewage, many industrial wastewaters are discharged at low

temperatures, e.g., beer and maltery wastewaters. A more recent example shows the successful long-term treatment of medium strength cereal-processing wastewaters under low temperature (17 °C) conditions at an HRT of 5.2 h using a pilot-scale UASB reactor [117]. Full-scale results so far show that all the cited wastewaters are anaerobically treated using common seed materials, illustrating the robustness and flexibility of the anaerobic process.

The application of high-rate anaerobic treatment to novel types of industrial wastewaters is generally preceded by pilot plant testing and extensive laboratory-scale research. However, in the past 15 years, considerable progress has been achieved in developing mathematical models and simulation programs, which can partly overcome the extensive laboratory tests, provided all wastewater characteristics are known. Mathematical modeling can also be used as a valuable tool to determine the effects of different operation alternatives or to assess the optimum conditions for the maximization of the biogas production capacity in anaerobic systems. By using mathematical modeling, it is possible to obtain insights into dynamic responses to changes in influent flow characteristics [118]. Although various kinetic models have been developed in the past 50 years, the Anaerobic Digestion Model No. 1 (ADM1), developed by the IWA Task Group for Mathematical Modeling on Anaerobic Digestion, is one of the most popular models used for simulation of sludge bed reactors in the past 15 years. In fact, ADM1 aggregates various existing models, whereas the structure of ADM1 is similar to that of the IWA activated sludge models, using similar notations for parameters, state variables, and constants [119]. Although ADM1 was initially used to describe the anaerobic digestion of excess waste activated sludge, its generic structure also allows modeling of high-rate anaerobic processes for industrial wastewater treatment [119, 120]. The effect of different process alternatives and shock loadings on the system can be investigated by using a verified model. At present, ADM1 is being successfully applied to modeling of full-scale anaerobic sludge bed reactors treating different kinds of industrial wastewaters [43, 118, 120–124].

11 Final Remarks and Conclusions

Sludge bed systems played a key role in the acceptance of high-rate anaerobic reactor systems for the treatment of industrial wastewater. UASB reactors and expanded bed related systems are applied at a wide variety of industrial sites, offering cost-effective solutions to comply with legislative constraints in combination with complementary technology. Reduced costs for treatment and bio-energy recovery lower the threshold to implement industrial wastewater treatment on industrial premises. On-site treatment of these wastewaters opens perspectives for resource recovery (bio-energy, process water) and reuse in the industrial process. Such development is regarded as important for developing the so-called ‘green industrial approach’. Decades of development of high-rate anaerobic reactor

systems expanded the application potential enormously, currently also including the more extreme types of wastewater. For conditions where sludge immobilization or granulation cannot be guaranteed, novel high-rate reactors equipped with advanced sludge retentions systems may offer the appropriate solution. Following this development, the authors feel that any industrial wastewater containing biodegradable organic pollutants should be treatable with a high-rate anaerobic reactor system. In the meantime, the upflow sludgebed technology remains the working horse of anaerobic high-rate treatment. Only a few decades ago, reactor systems treating 10 tons day^{-1} COD were regarded as considerable projects for the various contractors. At present, anaerobic sludge bed systems are treating more than 100 tons day^{-1} COD, generating an electric energy potential of about 5 MW. To realize such projects, technical developments should coincide with process engineering developments. In the end, the anaerobic high-rate reactor should sustain its lifetime, treating organically polluted wastewater, meanwhile converting the wasted organics into a valuable fuel.

An extensive assessment of 40 years anaerobic sludge bed technology for industrial wastewater treatment reveals the following:

- Anaerobic sludge bed treatment technology has been successfully applied to a wide spectrum of industrial wastewaters at full-scale as a consolidated technology.
- Anaerobic high-rate treatment technology is a cost-effective alternative, providing energy-saving, reduction in sludge production, operation at high organic loadings, compact footprints, and net energy production. These characteristics make anaerobic sludge bed technology feasible and sustainable for the treatment of virtually all organically polluted industrial wastewaters.
- Although the key mechanism of sludge bed technology is immobilization of microorganisms, various modern anaerobic high-rate reactors employ flocculent biomass which is retained in the system by advanced (gas-)liquid-sludge separation devices. In such reactors, sludge separation is brought about by in-built flotation units or advanced tilted plate settlers. Alternatively, membrane separation is employed, ensuring complete biomass retention without any necessity for granulation.
- The intensive research conducted on anaerobic sludge-bed systems using laboratory-scale reactor systems and which include molecular techniques and mathematical modeling resulted in the development of new reactor configurations, and applications of full-scale sludge bed systems, enabling the treatment of very complex wastewaters from chemical industries.
- As a waste-to-energy technology, high-rate anaerobic sludge (bed) systems enable renewable energy production and nutrient-rich effluent production for irrigation purposes in agricultural fields. Therefore, this technology significantly contributes to achieve the so-called “environmentally friendly” industrial production concept.

References

1. van Lier JB (2008) High-rate anaerobic wastewater treatment: diversifying from end-of-the-pipe treatment to resource-oriented techniques. Water Sci Technol 57(8):1137–1148
2. Lettinga, G (2014) My anaerobic sustainability story. LeAF, Wageningen, 200 pp. http://www.leaf-wageningen.nl/en/leaf.htm
3. Ersahin ME, Ozgun H, Dereli RK, Ozturk I (2011) Anaerobic treatment of industrial effluents: an overview of applications. In: Einschlag FSG (ed) Waste water-treatment and reutilization. InTech, India, pp 415–456
4. McCarty PL (2001) The development of anaerobic treatment and its future. Water Sci Technol 44(8):149–156
5. Imhoff K (1916) Separate sludge digestion improves Imhoff tank operation by keeping sewage fresh. Eng Record 74:101–102
6. Buswell AM (1957) Fundamentals of anaerobic treatment of organic wastes. Sewage Ind Waste 29:717–721
7. Buswell AM, Boruff CS, Wiesman CK (1932) Anaerobic stabilization of milk waste. Ind Eng Chem 24:1423–1425
8. Buswell AM, Sollo FW (1948) The mechanism of the methane fermentation. J Am Chem Soc 70:1778
9. Rittmann BE, McCarty PL (2001) Environmental biotechnology: principles and applications. McGraw-Hill, New York
10. van Lier JB, Mahmoud N, Zeeman G (2008) Anaerobic biological wastewater treatment. In: Henze M, van Loosdrecht MCM, Ekama GA, Brdjamovic D (eds) Biological wastewater treatment: principles, modeling and design. IWA, London
11. Lettinga G, van Velsen AFM, Hobma SW, de Zeeuw W, Klapwijk A (1980) Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol Bioeng 22(4):699–734
12. Rajeshwari KV, Balakrishnan M, Kansal A, Lata K, Kishore VVN (2000) State-of-the-art of anaerobic digestion technology for industrial wastewater treatment. Renew Sustain Energy Rev 4:135–156
13. Dereli RK, Ersahin ME, Ozgun H, Ozturk I, Jeison D, van der Zee F, van Lier JB (2012) Potentials of anaerobic membrane bioreactors to overcome treatment limitations induced by industrial wastewaters. Bioresour Technol 122:160–170
14. Ozgun H, Gimenez JB, Ersahin ME, Tao Y, Spanjers H, van Lier JB (2015) Impact of membrane addition for effluent extraction on the performance and sludge characteristics of upflow anaerobic sludge blanket reactors treating municipal wastewater. J Membr Sci 479:95–104
15. Ozgun H, Dereli RK, Ersahin ME, Kinaci C, Spanjers H, van Lier JB (2013) A review of anaerobic membrane bioreactors for municipal wastewater treatment: integration options, limitations and expectations. Sep Purif Technol 118:89–104
16. Schroepfer GJ, Fullen WJ, Johnson AS, Ziemke NR, Anderson JJ (1955) The anaerobic contact process as applied to packinghouse wastes. Sewage Ind Waste 27(4):460–486
17. Young JC (1991) Factors affecting the design and performance of upflow anaerobic filters. Water Sci Technol 24(8):133–155
18. Young JC, McCarty PL (1969) The anaerobic filter for waste treatment. J Water Pollut Control Fed 41:160–173
19. Young JC, Yang BS (1989) Design considerations for full-scale anaerobic filters. J Water Pollut Control Fed 61(9):1576–1587
20. Seyfried CF (1988) Reprints verfahrenstechnik abwasserreiningung, GVC-Diskussionstagung. October 17–19, Baden-Baden, Germany
21. Hulshoff Pol LW, de Castro Lopes SI, Lettinga G, Lens PNL (2004) Anaerobic sludge granulation. Water Res 38(6):1376–1389

22. Sung S, Dague RR (1995) Laboratory studies on the anaerobic sequencing batch reactor. Water Environ Res 67(3):294–301
23. Wirtz RA, Dague RR (1995) Enhancement of granulation and start-up in the anaerobic sequencing batch reactor. Water Environ Res 68(5):883–892
24. Lettinga G, van der Sar J, van der Ben J (1976) Anaerobe zuivering van het afvalwater van de bietsuikerindustrie (2). H2O 9:38–43
25. Lettinga G, van Velsen L, de Zeeuw W, Hobma SW (1979) The application of anaerobic digestion to industrial pollution traetment. In: 1st International symposium on anaerobic digestion, Cardiff, 17–21 September 1979
26. Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW (eds) (1987) GASMAT: international workshop on granular anaerobic sludge, microbiology and technology, Lunteren, PUDOC, Wageningen, 25–27 October 1987
27. Abbasi T, Abbasi SA (2012) Formation and impact of granules in fostering clean energy production and wastewater treatment in upflow anaerobic sludge blanket (UASB) reactors. Renew Sustain Energy Rev 16(3):1696–1708
28. De Zeeuw WJ (1982) Korrelslibbvorming Bij De Anaerobe Zuivering Van Destruktieafvalwater. Intern rapport, Vakgroep Waterzuivering, Landbouwhogeschool Wageningen
29. De Zeeuw WJ (1987) Granular sludge in UASB-reactors. Granular anaerobic sludge, microbiology and technology workshop, Lunteren, 25–27 October 1987
30. Dolfing J (1987) Microbiological aspects of granular methanogenic sludge, Ph.D. thesis. Agricultural University, Wageningen
31. Fang HHP, Chui HK, Li YY (1994) Microbial structure and activity of UASB granules treating different wastewaters. Water Sci Technol 30(12):87–96
32. Habeeb SA, Aziz Bin Abdul Latiff AB, Bin Daud Z, Bin Ahmad Z (2011) A review on granules initiation and development inside UASB reactor and the main factors affecting granules formation process. IJEE 2(2):311–320
33. Hulshoff Pol LW, Lettinga G (1986) Advanced reactor design, operation and economy. Water Sci Technol 18(12):99–108
34. Hulshoff Pol LW, Heijnekamp K, Lettinga G (1987) The selection pressure as driving force behind the granulation of anaerobic sludge. Granular anaerobic sludge; microbiology and technology workshop, Lunteren, 25–27 October 1987
35. Liu Y, Xu HL, Yang SF, Tay JH (2003) Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. Water Res 37:661–673
36. Song M, Shin SG, Hwang S (2010) Methanogenic population dynamics assessed by real-time quantitative PCR in sludge granule in upflow anaerobic sludge blanket treating swine wastewater. Bioresour Technol 10(Suppl 1):S23–S28
37. Subramanyam R (2013) Physicochemical and morphological characteristics of granular sludge in upflow anaerobic sludge blanket reactors. Environ Eng Sci 30(5):201–212
38. van Lier JB, Boersma F, Debets MMWH, Lettinga G (1994) High rate thermophilic wastewater treatment in compartmentalized upflow reactors. Water Sci Technol 30(12):251–261
39. Wiegant WM, de Man AWA (1986) Granulation of biomass in thermophilic anaerobic sludge blanket reactors treating acidified wastewaters. Biotechnol Bioeng 28:718–727
40. Wu WM, Hickey RF, Zeikus JG (1991) Characterisation of metabolic performance of methanogenic granules treating brewery wastewater: role of sulfate-reducing bacteria. Appl Environ Microbiol 57:3438–3449
41. Calderon K, Gonzales-Martinez A, Gomez-Silvan C, Osorio F, Rodelas B, Gonzales-Lopez J (2013) Archaeal diversity in biofilm technologies applied to treat urban and industrial wastewater: recent advances and future prospects. Int J Mol Sci 14(9):18572–18598
42. Ismail SB, Gonzalez P, Jeison D, van Lier JB (2008) Effects of high salinity wastewater on methanogenic sludge bed systems. Water Sci Technol 58(10):1963–1970
43. Batstone DJ, Keller J, Blackall LL (2004) The influence of substrate kinetics on the microbial community structure in granular anaerobic biomass. Water Res 38:1390–1404

44. MacLeod FA, Guiot SR, Costerton JW (1990) Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. Appl Environ Microbiol 56 (6):1598–1607
45. Patel GB, Sprott GD (1990) *Methanosaeta concilii* gen. nov., sp. nov. ("*Methanothrix concilii*") and *Methanosaeta thermoacetophila* nom. rev., comb. nov. Int J Syst Evol Microbiol 40:79–82
46. Baloch MI, Akunna JC, Kierans M, Collier PJ (2008) Structural analysis of anaerobic granules in a phase separated reactor by electron microscopy. Bioresour Technol 99:922–929
47. Guiot SR, Pauss A, Costerton JW (1992) A structured model of the anaerobic granule consortium. Water Sci Technol 25(7):1–10
48. Grotenhuis JTC, Smit M, Plugge CM, Xu Y, Van Lammeren AAM, Stams AJM, Zehnder AJB (1991) Bacteriological composition and structure of granular sludge adapted to different substrates. Appl Environ Microbiol 57:1942–1949
49. McHugh S, Carton M, Mahony T, O'Flaherty V (2003) Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiol Lett 219(2):297–304
50. Grotenhuis JTC, Stams AJM, Zehnder AJB (1992) Hydrophobicity and electrophoretic mobility of anaerobic isolates from methanogenic granular sludge. Appl Environ Microbiol 58:1054–1056
51. Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H (1999) Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic granules. Appl Environ Microbiol 65(3):1280–1288
52. Satoh H, Miura Y, Tsushima I, Okabe S (2007) Layered structure of bacterial and archaeal communities and their in situ activities in anaerobic granules. Appl Environ Microbiol 73 (22):7300–7307
53. van Loosdrecht MCM, de Kreuk MK, Heijnen JJ (2002) Aerobic granular sludge formation. In: van Lier JB, Lexmond M, de Vos H (eds) Proceedings of "Granulation and Auto-immobilisation Processes in Wastewater Treatment", farewell seminar Hulshoff Pol, Wageningen, 28 June 2002
54. Hulshoff Pol LW, de Zeeuw WJ, Velzeboer CTM, Lettinga G (1983) Granulation in UASB-reactors. Water Sci Technol 15(8/9):291–304
55. Vanderhaegen B, Ysebaert E, Favere K, Van Wambeke M, Peeters T, Panic V, Vandenlangenbergh V, Verstraete W (1992) Acidogenesis in relation to in-reactor granule yield. Water Sci Technol 25:75–81
56. Lim SJ, Kim TH (2014) Applicability and trends of anaerobic granular sludge treatment processes. Biomass Bioenergy 60:189–202
57. Nnaji CC (2013) A review of the upflow anaerobic sludge blanket reactor. Desalination Water Treat 52:4122–4143
58. Habets LHA, Knelissen JH (1985) Application of the UASB reactor for anaerobic treatment of paper and board mill effluent. Water Sci Technol 17(1):61–75
59. Rajagopal R, Saady NMC, Torrijos M, Thanikal JV, Hung YT (2013) Sustainable agro-food industrial wastewater treatment using high rate anaerobic process. Water 5:292–311
60. Speece RE (1996) Anaerobic biotechnology for industrial wastewaters. Archae, Nashville
61. Alphenaar PA (1994) Anaerobic granular sludge: characterization and factors affecting its functioning. Ph.D. thesis, G. Lettinga (promotor), Department of Environmental Technology, Agricultural University, Wageningen
62. Banu JR, Kaliappan S, Yeom IT (2007) Treatment of domestic wastewater using upflow anaerobic sludge blanket reactor. Int J Environ Sci Technol 4(3):363–370
63. Monroy O, Fama G, Meraz M, Montoya L, Macarie H (2000) Anaerobic digestion for wastewater treatment in Mexico: state of the technology. Water Res 34(6):1803–1816
64. Guiot SR, van den Berg L (1984) Performance and biomass retention of an upflow anaerobic reactor combining a sludge blanket and a filter. Biotechnol Lett 6(3):161–164

65. Guiot SR, Van den Berg L (1985) Performance of an upflow anaerobic reactor combining a sludge blanket and a filter treating sugar waste. Biotechnol Bioeng 27:800–806
66. Kennedy KJ, Guiot SR (1986) Anaerobic upflow bed-filter-development and application. Water Sci Technol 18(12):71–86
67. Strydom JP, Britz TJ, Mostert JF (1997) Two-phase anaerobic digestion of three different dairy effluents using a hybrid bioreactor. Water SA 23(2):151–156
68. Buyukkamaci N, Filibeli A (2002) Concentrated wastewater treatment studies using an anaerobic hybrid reactor. Process Biochem 38:771–775
69. Ramakrishnan A, Gupta SK (2008) Effect of COD/NO_3-N ratio on the performance of a hybrid UASB reactor treating phenolic wastewater. Desalination 232:128–138
70. Kleerebezem R, Hulshoff Pol LW, Lettinga G (1999) The role of benzoate in anaerobic degradation in terephthalate. Appl Environ Microbiol 65(3):1161–1167
71. Kleerebezem R, Hulshoff Pol LW, Lettinga G (1999) Anaerobic degradation of phthalate isomers by methanogenic consortia. Appl Environ Microbiol 65(3):1152–1160
72. Macarie H (1999) Overview of the application of anaerobic treatment to chemical and petrochemical wastewaters. Water Sci Technol 42(5-6):201–214
73. Kleerebezem R, Macarie H (2003) Treating industrial wastewater: anaerobic digestion comes at age. Chem Eng 56–64
74. Heijnen SJ, Mulder A, Weltevrede R, Hols PH, van Leeuwen HLJM (1990) Large-scale anaerobic/aerobic treatment of complex industrial wastewater using immobilized biomass in fluidized bed and air-lift suspension reactors. Chem Eng Technol 13(1):202–208
75. Li A, Sutton PM (1981) Dorr Oliver Anitron system, Fluidized Bed technology for methane production from dairy wastes. Whey Products Institute Annual Meeting. Chicago, USA
76. Moletta R, Escoffier Y, Frédéric Ehlinger F, Coudert JP, Leyris JP (1994) On-line automatic control system for monitoring an anaerobic fluidized-bed reactor: response to organic overload. Water Sci Technol 30(12):11–20
77. Ehlinger F (1994) Anaerobic biological fluidized beds: operating experiences in France. In: 7th International symposium on anaerobic digestion, Cape Town, 23–27 January 1994
78. Holst TC, Truc A, Pujol R (1997) Anaerobic fluidised beds: ten years of industrial experience. Water Sci Technol 36(6–7):415–422
79. Zoutberg GR, Frankin R (1996) Anaerobic treatment of chemical and brewery waste water with a new type of anaerobic reactor; the biobed® EGSB reactor. Water Sci Technol 34 (5-6):375–381
80. Zoutberg GR, De Been P (1997) The Biobed® EGSB (Expanded Granular Sludge Bed) system covers shortcomings of the Upflow Anaerobic Sludge Blanket reactor in the chemical industry. Water Sci Technol 35(10):183–188
81. Frijters CTMJ, Vos RH, Scheffer G, Mulder R (2006) Decolorizing and detoxifying textile wastewater, containing both soluble and insoluble dyes, in a full scale combined anaerobic/aerobic system. Water Res 40(6):1249–1257
82. Rebac S, van Lier JB, Lens P, van Cappellen J, Vermeulen M, Stams AJM, Swinkels KTM, Lettinga G (1998) Psychrophilic (6–15°C) high rate anaerobic treatment of malting wastewater in a two-module expanded granular sludge bed system. Biotechnol Prog 14:856–864
83. Vellinga SHJ, Hack PJFM, van der Vlugt AJ (1986) New type "high rate" anaerobic reactor; first experience on semi-technical scale with a revolutionary and high loaded anaerobic system. Anaerobic treatment: a grown-up technology, aquatech water treatment conference, Amsterdam, 15–19 September 1986
84. Habets LHA, Engelaar AJHH, Groeneveld N (1997) Anaerobic treatment of inuline effluent in an internal circulation reactor. Water Sci Technol 35(10):189–197
85. Pereboom JHF, Vereijken TLFM (1994) Methanogenic granule development in full scale internal circulation reactors. Water Sci Technol 30(8):9–21
86. van Lier JB, van der Zee F, Tan FP, Rebac S, Kleerebezem R (2001) Advances in high-rate anaerobic treatment: staging of reactor systems. Water Sci Technol 44(8):15–25

87. Lettinga G, Hulshoff Pol LW (1991) UASB process design for various types of wastewater. Water Sci Technol 24(8):87–107
88. Batstone DJ, Keller J (2001) Variation of bulk properties of anaerobic granules with wastewater type. Water Res 35(7):1723–1729
89. Fukuzaki S, Nishio N, Nagai S (1995) High rate performance and characterization of granular methanogenic sludges in upflow anaerobic sludge blanket reactors fed with various defined substrates. J Ferment Bioeng 79(4):354–359
90. Puñal A, Brauchi S, Reyes JG, Chamy R (2003) Dynamics of extracellular polymeric substances in UASB and EGSB reactors treating medium and low concentrated wastewaters. Water Sci Technol 48(6):41–49
91. Bachmann A, Beard VL, McCarty PL (1985) Performance-characteristics of the anaerobic baffled reactor. Water Res 19:99–106
92. Barber WP, Stuckey DC (1999) The use of the anaerobic baffled reactor (ABR) for wastewater treatment: a review. Water Res 33:1559–1578
93. Zhu G, Zou R, Jha AK, Huang X, Liu L, Liu C (2015) Recent developments and future perspectives of anaerobic baffled bioreactor for wastewater treatment and energy recovery. Crit Rev Environ Sci Technol 45(12):1243–1276
94. Guiot SR, Safi B, Frignon JC, Mercier P, Mullignan C, Tremblay R (1995) Performances of a full-scale novel multiplate anaerobic reactor treating cheese whey effluent. Biotechnol Bioeng 45:398–405
95. Tagawa T, Takahashi H, Sekiguchi Y, Ohashi A, Harada H (2002) Pilot-plant study on anaerobic treatment of a lipid- and protein-rich food industrial wastewater by a thermophilic multi-staged UASB reactor. Water Sci Technol 45(10):225–230
96. Hwu CS, Molenaar G, Garthoff J, van Lier JB, Lettinga G (1997) Thermophilic high-rate anaerobic treatment of wastewater containing long-chain fatty acids: impact of reactor hydrodynamics. Biotechnol Lett 19:447–451
97. Hwu CS, van Beek B, van Lier JB, Lettinga G (1997) Thermophilic high-rate anaerobic treatment of wastewater containing long-chain fatty acids: effect of washed out biomass recirculation. Biotechnol Lett 19:453–456
98. Frijters CTMJ, Jorna T, Hesselink G, Kruit J, van Schaick D, van der Arend R (2014) Experiences with anaerobic treatment of fat-containing food waste liquids: two full scale studies with a novel anaerobic flotation reactor. Water Sci Technol 69(7):1386–1394
99. Jeison D, van Lier JB (2007) Thermophilic treatment of acidified and partially acidified wastewater using an anaerobic submerged MBR: factors affecting long-term operational flux. Water Res 41:3868–3879
100. Ersahin ME, Ozgun H, Tao Y, van Lier JB (2014) Applicability of dynamic membrane technology in anaerobic membrane bioreactors. Water Res 48:420–429
101. Yang J, Spanjers H, Jeison D, van Lier JB (2013) Impact of Na^+ on biological wastewater treatment and the potential of anaerobic membrane bioreactors: a review. Crit Rev Environ Sci Technol 43(24):2722–2746
102. Jeison D, Telkamp P, van Lier JB (2009) Thermophilic sidestream anaerobic membrane bioreactors: the shear rate dilemma. Water Environ Res 81(11):2372–2380
103. Jeison D, van Betuw W, van Lier JB (2008) Feasibility of anaerobic membrane bioreactors for the treatment of wastewaters with particulate organic matter. Sep Sci Technol 43:3417–3431
104. Muñoz Sierra JD, Spanjers H, van Lier JB (2014) Biomass acclimatisation during start-up of AnMBR reactors treating saline phenolic wastewater. In: Proceedings of the 11th Latin American workshop and symposium on anaerobic digestion, IWA conference, La Habana, 25–28 November 2014
105. Kang IJ, Yoon SH, Lee CH (2002) Comparison of the filtration characteristics of organic and inorganic membranes in a membrane-coupled anaerobic bioreactor. Water Res 36:1803–1813
106. Futselaar H, Rosink R, Smith G, Koens L (2013) The anaerobic MBR for sustainable industrial wastewater management. Desalination Water Treat 51:4–6

107. Christian S, Grant S, McCarty P, Wilson D, Mills D (2011) The first two years of full-scale anaerobic membrane bioreactor (AnMBR) operation treating high-strength industrial wastewater. Water Practice Technol 6(2). doi:10.2166/wpt.2011.032
108. Allison M, Grant S, Christian S, Wilson D (2013) Full-scale operating experience with USA-based ADI-AnMBR systems for food wastes. Proc Water Environ Fed 2013 (10):5255–5270
109. van Lier JB, Vashi A, van der Lubbe J, Heffernan B (2010) Anaerobic sewage treatment using UASB reactors: engineering and operational aspects, Chapter 4. In: Fang HHP (ed) Environmental anaerobic technology; applications and new developments. World Scientific, Imperial College Press, London, pp 59–89, ISBN 978-1-84816-542-7
110. Habets LHA, Knelissen HJ (1997) In line biological water regeneration in a zero discharge recycle paper mill. Water Sci Technol 35(2–3):41–48
111. Van Lier JB, Boncz MA (2002) Controlling calcium precipitation in an integrated anaerobic aerobic treatment system of a 'zero-discharge' paper mill. Water Sci Technol 45 (10):341–348
112. Razo-Flores E, Macarie H, Morier F (2006) Application of biological treatment systems for chemical and petrochemical wastewaters. In: Cervantes FJ, Pavlostathis SP, van Haandel AC (eds) Advanced biological treatment processes for industrial wastewaters. IWA, London
113. Zaher U, Moussa MS, Widyatmika IN, van Der Steen P, Gijzen HJ, Vanrolleghem PA (2006) Modelling anaerobic digestion acclimatisation to a biodegradable toxicant: application to cyanide. Water Sci Technol 54(4):129–137
114. Aydin AF, Ersahin ME, Dereli RK, Sarikaya HZ, Ozturk I (2010) Long-term anaerobic treatability studies on opium alkaloids industry effluents. J Environ Sci Health A Tox Hazard Subst Environ Eng 45(2):192–200
115. Dos Santos AB, Cervantes FJ, van Lier JB (2007) Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. Bioresour Technol 98(12):2369–2385
116. Rebac S, van Lier JB, Lens PNL, Stams AJM, Dekkers F, Swinkels KTM, Lettinga G (1999) Psychrophilic anaerobic treatment of low strength wastewaters. Water Sci Technol 39 (5):203–210
117. Esparza Soto M, Solis Morelos C, Herna JJ (2011) Anaerobic treatment of a medium strength industrial wastewater at low-temperature and short hydraulic retention time: a pilot-scale experience. Water Sci Technol 64(8):1629–1635
118. Ersahin ME, Dereli RK, Insel G, Ozturk İ, Kinaci C (2007) Model based evaluation for the anaerobic treatment of corn processing wastewaters. Clean (Weinh) 35(6):576–581
119. Batstone DJ, Keller J, Angelidaki I, Kalyuzhnyi SV, Pavlostathis SG, Rozzi A, Sanders WTM, Siegrist H, Vavilin VA (2002) Anaerobic Digestion Model No. 1. Scientific and Technical Report No. 13, IWA, London
120. Dereli RK, Ersahin ME, Ozgun H, Ozturk I, Aydin AF (2010) Applicability of anaerobic digestion model no.1 (ADM1) for a specific industrial wastewater: opium alkaloid effluents. Chem Eng J 165(1):89–94
121. Barrera EL, Spanjers H, Solon K, Amerlinck Y, Nopens I, Dewulf J (2015) Modeling the anaerobic digestion of cane-molasses vinasse: extension of the anaerobic digestion model no. 1 (ADM1) with sulfate reduction for a very high strength and sulfate rich wastewater. Water Res 71:42–54
122. Batstone DJ, Keller J (2003) Industrial application of the IWA anaerobic digestion model no.1 (ADM1). Water Sci Technol 47(12):199–206
123. Chen Z, Hu D, Zhang Z, Ren N, Zhu H (2009) Modeling of two-phase anaerobic process treating traditional Chinese medicine wastewater with the IWA anaerobic digestion model no. 1. Bioresour Technol 100:4623–4631
124. Hinken L, Huber M, Weichgrebe D, Rosenwinkel KH (2014) Modified ADM1 for modelling an UASB reactor laboratory plant treating starch wastewater and synthetic substrate load tests. Water Res 64:82–93

Adv Biochem Eng Biotechnol (2016) 156: 397–432
DOI: 10.1007/10_2015_5008

Published online: 24 February 2016

Anaerobic Probiotics: The Key Microbes for Human Health

Hesham El Enshasy, Khairuddin Malik, Roslinda Abd Malek, Nor Zalina Othman, Elsayed Ahmed Elsayed, and Mohammad Wadaan

Abstract Human gastrointestinal microbiota (HGIM) incorporate a large number of microbes from different species. Anaerobic bacteria are the dominant organisms in this microbial consortium and play a crucial role in human health. In addition to their functional role as the main source of many essential metabolites for human health, they are considered as biotherapeutic agents in the regulation of different human metabolites. They are also important in the prevention and in the treatment of different physical and mental diseases. Bifidobacteria are the dominant anaerobic bacteria in HGIM and are widely used in the development of probiotic products for infants, children and adults. To develop bifidobacteria-based bioproducts, therefore, it is necessary to develop a large-scale biomass production platform based on a good understanding of the ideal medium and bioprocessing parameters for their growth and viability. In addition, high cell viability should be maintained during downstream processing and storage of probiotic cell powder or the final formulated product. In this work we review the latest information about the biology, therapeutic activities, cultivation and industrial production of bifidobacteria.

H. El Enshasy (✉)
Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru, Malaysia

City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria, Egypt
e-mail: henshasy@ibd.utm.my

K. Malik, R.A. Malek, and N.Z. Othman
Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru, Malaysia

E.A. Elsayed
Bioproducts Research Chair, Faculty of Science, King Saud University, Riyadh, Saudi Arabia

Natural and Microbial Products Department, National Research Centre, Dokki, Cairo, Egypt

M. Wadaan
Bioproducts Research Chair, Faculty of Science, King Saud University, Riyadh, Saudi Arabia

Keywords Anaerobic probiotics, *Bifidobacteria* spp, Biomass production, Functional food, Gastrointestinal microbiota, Therapeutic activities of bifidobacteria

Contents

Abbreviations

ASD	Autism spectrum disorders
ATCC	American type culture collection
ATP	Adenosine triphosphate
BSH	Bile salt hydrolase
CAGR	Compound annual growth rate
CFU	Colony forming unit
CLB	Liver cysteine lactose
FDA	Food and Drug Administration
FOS	Fructooligosaccharides
GIT	Gastro intestinal tract
GRAS	Generally regarded as safe
HGIM	Human gastrointestinal microbiota
IBS	Irritable bowel syndrome
LAB	Lactic acid bacteria
MERCOSUR	Mercado Común del Sur (Common Market of South)
MRS	Man–Rogosa–Sharp medium
NYA	National Yoghurt Association
NRSP	Natural rubber serum powder
RCM	Reinforced clostridia medium
sEPS	Surface exopolysaccharides

SMF	Submerged fermentation
SSF	Solid state fermentation
TPY	Trypticase-phytone-yeast extract
WHO	World Health Organization

1 Introduction

The human body can be considered as a mixed culture system composed not only of different types of human cells but also of large numbers of highly diversified microbes. It has been estimated that the healthy adult harbours about 10 trillion microbial cells in the gastrointestinal tract (GIT) alone or, in other words, the number of microbial cells in the human body is almost ten times greater than the number of human cells in our bodies [1, 2]. These microbes are highly diversified, and based on molecular and phylogenetic studies, it is estimated that microbiota of the GIT is composed of over 35,000 species [2, 3]. These microbes play a crucial role in human health and are now of great interest to both the scientific and industrial communities. The microbes in the human body which exhibit health benefits directly or indirectly are widely known as probiotics. The term 'probiotic' is derived from Latin, meaning pro-life. This term was historically used to describe 'organisms and substances' that contribute to intestinal microbial balance, although a new definition proposed by Fuller [4] puts more emphasis on the importance of supplements that are composed of viable microorganisms and that have beneficial effects on the host animal by improving its intestinal microbial balance [4, 5]. The formal definition of probiotic, which is widely used nowadays, is that given by World Health Organization (WHO) as follows: "Probiotics are live microorganisms which, when administered in sufficient numbers, confer a health benefit to the host" [6]. Moreover, for any particular strain to be considered as probiotic, it should have Generally Regarded as Safe (GRAS) status [7].

Probiotic microorganisms are highly diversified in their nature and belong to different eukaryotic and prokaryotic groups, including yeast, fungi and bacteria [8]. These also include aerobic/anaerobic, spore-forming/non-spore-forming microbes. Among the beneficial bacteria in the human intestine, probiotic microorganisms mostly belong to *Lactobacillus*, *Bifidobacterium* and *Streptococcus* [9, 10]. These play an important role in the production of vitamins, organic acids and antimicrobial factors to inhibit pathogenic organisms. In addition, probiotic bacteria engage in various metabolic activities in the intestine, thereby influencing the host's health, including nutrition, physiological function, immunological responses and resistance to infection [11–14]. In the human gastrointestinal system there is a constant interaction between the endogenous microflora and potentially pathogenic microorganisms, and probiotics seem to play a significant role in the maintenance of intestinal homeostasis and prevention of diseases [5, 13]. Any disturbance in this homeostasis, however, rapidly causes many diseases, with side-effects including chronic inflammation and diarrhoea. In addition, recent

research has shown clear evidence for the positive relationship between the presence of certain types of GIT flora and the human mood. This appears to be based on the ability of probiotics to release important regulatory signalling molecules which influence brain activity and, subsequently, human behaviour [16, 17]. Bifidobacteria have also potential applications in the future beyond their current use in the GIT. Some studies have demonstrated the potential use of *B. longum* in skin care products, based on its ability to produce antimicrobial compounds against skin pathogens. Bifidobacteria have demonstrated the ability to produce hyaluronic acid and peptidoglycan, which are important compounds in skin protection and regeneration [18].

2 Probiotics as Functional Foods

Increasing popular concern with health and a healthy lifestyle has encouraged a rapidly growing market for lactic acid bacteria as functional food probiotics. As reported in some market studies, the global probiotic annual market reached about US$26.1 billion in 2012 and increased to US$32.6 billion by 2014 [19, 20]. Europe is leading the international market based on dairy products, especially yoghurt, which accounts for 42% of the total revenues, followed by the Asian market which contributes to about 30% of the world market with a compound annual growth rate of almost 30%. In Europe, retail sales have reached a market volume of more than 1 billion kg and account for over 1.2 billion Euros annually [21]. As shown by Raja and Arunachalam [19], probiotic yoghurts in Denmark, Germany, United Kingdom and France have the largest demand in Europe, accounting for 20, 13, 13, and 11% of the yogurt markets, respectively, followed by the Netherlands and Belgium (both at 6%) and then Finland and Sweden (both at 5%). In Asia, the production of probiotics in India alone is reported to have quadrupled from 2010 to 2015, and the market value is estimated at US$2 billion, dominated by international companies such as Nestle, Amul, Yakut, Danone and Mother Dairy [19]. The US contributed up to about 17% of the global probiotic market with growth at 14% because of the increased awareness and demand for functional foods.

Nowadays, different types of *Bifidobacterium* sp. are widely used as concentrated microbial cell formulations in powder, granule, liquid, capsule and sachet forms, or as supplements to functional foods in either dairy or non-dairy products (Table 1). Formerly, probiotics were widely formulated into milk-based products, but the use of probiotics in non-milk based products has recently increased in line with the demand for probiotic-based products as a component of a healthy lifestyle [22–24]. This is reflected in the growing trend to include pro- and prebiotics in new functional foods such as symbiotic drinking, yoghurt, cheese, ice cream and chocolate [23].

Table 1 Commercially available *Bifidobacterium* cultures used as probiotics

Bifidobacteria sp.	Company/country	Reference
B. longum BB536	Morinaga Milk Industry Co., Ltd., Japan	[25]
*B. lactis*HN011(DR20)	Danisco, USA	[25]
B. longum BB536	Morinaga Milk Industry, Japan	[19]
B. infantis 35264	Protect and Gamble, USA	[25]
B. lactis	Nestlé Research Centre, Switzerland	[26]
B. lactis Bb12®	Chr. Hansen, Denmark	[27, 28]
B. lactis LAFTI B94 a	DSM Food Specialties, Australia	[29]
B. lactis	Bioferme, Finland	[23, 30]
B. bifidum	Biohorma, The Netherland	[23]
Bifidus actiregularis	Danone, France	[23]
B. bifidum	Eko-Bio, The Netherlands	
Bifidobacterium sp.	Chefaro, Belgium	
Bifidobacteriumsp, 420 Wisby, *B. lactis*, HOWARU™, *Bifidobacterium* HN019, DR10™	Danisco, Niebüll, Germany	[31]
B. lactis, DELVO-PRO™LAFTI™B94	DSM Food Specialist, Delft, The Netherlands	[31]
Bifidus actiregularus	Danone, Italy	[32]

3 *Bifidobacterium* spp.

The human digestive system contains large and complex groups of microbiota, which consist mostly of normal microflora bacteria. There are approximately 400 different species of microbiota, mainly obligate anaerobes (95%) and facultative anaerobes (1–2%). It is estimated that more than 1,500 different microbes can be isolated from the human intestinal tract [10]. Strains belonging to *Bifidobacterium* species are the major microflora that inhabit the human and animal intestines, and are considered as obligate anaerobic bacteria [33]. This type of bacteria exhibits different ecological adaptations dependent on the species. In addition to their wide existence in mammals, some studies have also reported the presence of bifidobacteria in the gut and intestine of social insects, fish and reptiles [34]. Bifidobacteria were first reported by the French paediatrician, Henri Tissier in 1899 who isolated and described a Y shaped (bifid) bacterium from the faeces of breast-fed infants and named it at that time as *Bacillus bifidus communis*. This genus was traditionally grouped within lactic acid bacteria and was initially included in the genus *Lactobacillus*. It bears little phylogenetic relationship to lactic acid bacteria, however, and bifidobacteria were reclassified as a separate genus in the 8th Edition of Bergey's manual of determinative bacteriology [35]. Bifidobacteria are Gram-positive, non-motile, non-sporulating rod- to Y-shaped. Most of the isolated strains grow anaerobically and are able effectively

to colonize the gut of humans, other warm-blooded animals, fish and insects [36]. This type of bacteria occurs in single-cell form or in multicellular chains or clumps in the form of branched or pleomorphic rods, which gives the name of this genus. Bifidobacteria are also non-filamentous, non-motile, non-capsulated and non-spore forming [37]. Bifidobacteria belong to the high GC content Gram positive bacteria, because their genomic GC content varies from 42 to 67 mol%. The genome size of bifidobacteria is highly type- and strain-dependent, and ranges between 1.93 Mbp (for *Bifidobacterium animalis* subsp. *lactis*) and 2.83 Mbp (for *B. longum* subsp. *infantis*) [38].

The *Bifodobacterium* genus is now known to include 48 species from highly diversified sources. The initial molecular taxonomic research clustered bifidobacterial species into six main phylogenetic clusters: *B. borum*, *B. asteroids*, *B. adolescentis*, *B. pullorum*, *B. longum* and *B. pseudolongum* groups. Based on the new 16S rDNA-sequence-based neighbour-joining tree given by Bottacini and his group, an additional three phylogenetic clusters (*B. crudilactis*, *B. bohemicum* and *B. scardovii*) have been added recently [38].

The species belonging to *Bifidobacterium* use a specific pathway for degradation of hexoses (bifid shunt) which differs from that of facultative anaerobic lactic acid bacteria [39, 40]. This involves the key characteristic enzyme fructose-6-phosphoketolase (EC 4.1.2.2), which is considered to be one of the taxonomic characteristics for this type of bacteria. Based on their therapeutic effects, bifidobacteria have been widely used in cultured milk, beverages, cheese products and cookies [41, 42]. The probiotic activities of bifidobacteria species were first demonstrated in 1958 and since then bifidobacteria have been established as probiotics because they promote desirable changes in the colon [43]. As probiotics, bifidobacteria provide a beneficial effect to the body by adhesion to and colonization of the lower intestinal mucosal membrane. They provide a good protective barrier by preventing the adherence of pathogenic bacteria and concurrently providing necessary metabolites and vitamins to the host's body [44].

3.1 Bifidobacterium *spp.*: *Going from Mother to Infant*

During delivery and passage through the mother's birth canal, the newborn is exposed to large numbers of microbial cells that are immediately ingested and start to colonize in the gut. This has been proven by some researchers, who have found high similarity between the infant intestinal microbes and the vaginal microbiota of the mother in the case of normal delivery [45]. Other studies have also shown that in the case of Caesarean delivery, the infant gut microbiota is different from the vaginal microbes [46, 47]. Breast milk is considered to be the second source of probiotics for newborns, providing balanced nutrients necessary for infant growth, prebiotic compounds to support probiotic growth and colonization, and a well-balanced consortium of microbiota belonging to different species of bifidobacteria and lactic acid bacteria [48–52].

Bifidobacteria live in the colon during the early stages of life as the predominant microorganisms, making this species a suitable indicator for infant faecal contamination [53, 54]. The variation in the composition of the microbial consortium of bifidobacteria in the human gut depends on the stages of the host's life, because the population of this microflora is different between infants and adults. It has been reported that, in humans, the predominant strains are *B. breve*, *B. parvulorum* and *B. infantis* in infants, and *B. adolescentis* and *B. longum* in adults [43, 55, 56]. During probiotic product design, therefore, it is necessary to understand fully the microbiota of the targeted customer group in order to deliver suitable microbes for their age.

3.2 *Criteria and Characteristics Necessary for the Use of* Bifidobacterium *spp. as Probiotics*

To select a probiotic strain for human use, different basic requirements are usually considered. These include safety for human use, with no previous pathogenic activities having been reported, sourced from healthy individuals, biological efficiency in humans, high adhesion potential to human intestinal epithelial cells, the ability to interact and inhibit the growth of enteropathogenic microorganisms, and their potential medicinal properties. In addition, probiotics are selected based on their resistance to a variety of stresses during product formulation and packaging and during their passage through the intestinal tract. These stresses, in fact, make bifidobacteria species difficult to process, especially with respect to maintaining their viability during storage. As shown in Fig. 1, to have therapeutic effects as probiotic bacteria, the selected *Bifidobacterium* sp. should be able not only to tolerate the processing but also to withstand the gastrointestinal environment. The viability of probiotics is a priority in developing probiotic products. The actual suitable amount of the probiotic cells is not defined and may vary depending on the strain of bacteria, health effect and the matrix. The following sections explore in more detail the criteria that need to be fulfilled by a bacterium before it can be selected for probiotic use [33, 57].

3.2.1 Oxygen Tolerance

Bifidobacteria are generally considered to be anaerobic bacteria because of their metabolism that is devoid of a respiratory chain or catalase; this makes the presence of oxygen a major problem in the cultivation of bifidobacteria for industrial applications. The level of oxygen may, in general, have some effect on the carbohydrate metabolism and growth of these strains, although it has been reported that several bifidobacteria strains are able to consume oxygen [58]. These strains (including *B. breve*, *B. infantis*, *B. longum* and *B. adolescentis*) achieve tolerance

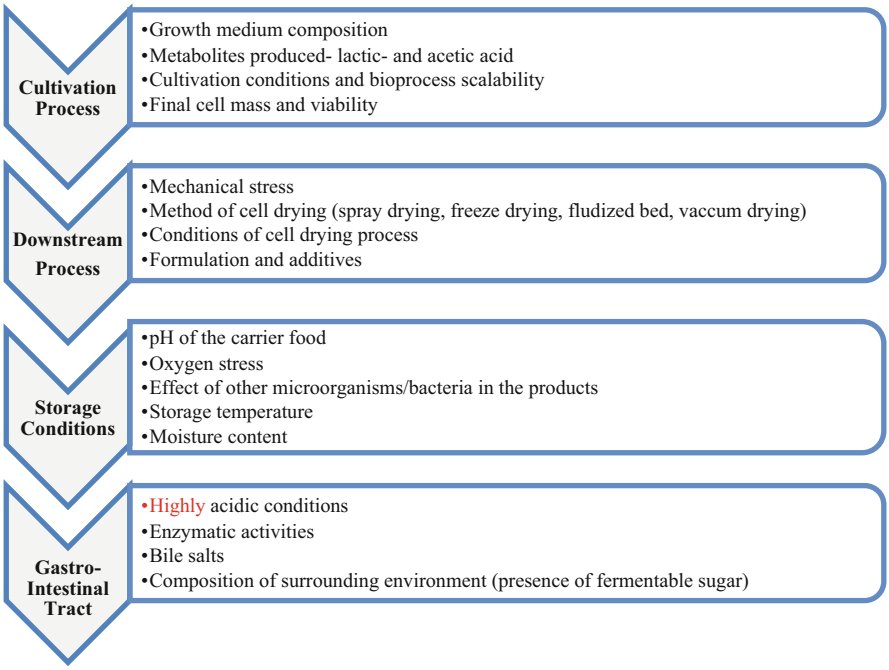


Fig. 1 Main factors having strong impact on probiotics production and application. (Modified from Lacroix and Yildirim [57])

for oxygen by using NADH peroxidase, NADH oxidase and low superoxidase dismutase activities to minimize the toxicity of oxygen compounds. Among the bifidobacteria, only *B. minimum*, *B. pyschraerophilum* [59], *B. longum*, *B. breve* and *B. bifidum* [60] show high tolerance to oxygen; others such as *B. choerinum*, *B. animalis* subsp. *lactis*, *B. animalis* subsp. animalis, B. magnum, *B. pseudolongum* subsp. *globosum*, *B. pseudolongum* subsp. *pseudolongum*, *B. suis* and *B. thermacidophilm* exhibit moderate tolerance to oxygen [59, 61]. *B. ruminantium*, *B. catenulatum*, *B. pseudocatenulatum*, *B. angulatum*, *B. merycicum*, *B. dentium*, *B. adolescentis* and *B. ruminantium*, however, have a low tolerance [56, 59] and *B. bifidum* and *B. adolescentis* require strict anaerobic and fastidious conditions for cultivation. Understanding the oxygen tolerance of the strains used is very important in industrial applications because of the high cost in maintaining anaerobic conditions. Different oxygen-free gases, such as pure nitrogen (N_2), pure carbon dioxide (CO_2), and gas mixtures consisting of 85% N_2 + 10% CO_2 + 5% H_2 or 95% N_2 + 5% H_2, have been used to maintain anaerobic conditions to support bifidobacteria growth [62].

3.2.2 Bile Acid/Salts Tolerance

Bile acids act as signalling molecules, and are important in the immune system. Bile acids are produced in the liver and excreted in the intestinal tract in the toxic forms of glycine or taurine conjugates [63]. The resistance to bile acid shows biological variability between species and even between independent strains within a species. Bile acid tolerance is thought to help probiotic bacteria survive during their journey along the duodenum before colonization via adhesion to enterocyte cells. Indeed, recent studies have stated that almost all bifidobacteria possess metabolic activity that can counter the toxicity of bile acid by deconjugating this salt using bile salt hydrolase (BSH). This enzyme functions by catalysing the hydrolysis of the toxic compound into amino acid compounds and deconjugated bile salt [64]. Comparisons between *B. bifidum* and *L. casei*, including *L. acidophilus*, have shown no major differences in cell survival after 120 min exposure to 0.6% bile salt [65]. In conclusion, moderate tolerance to low pH after 60 min of exposure was observed for *B. longum*, *B. breve* or *B. dentium* strains, whereas *B. adolescentis*, *B. bifidum* and *B. pseudocatenulatum* strains showed acid tolerance for only a short time [60]. It is well known that no probiotic strains show high tolerance to prolonged exposure to acidic conditions. In fact, the viability of bifidobacteria at pH values of gastric juices is considered to be generally low [22, 66].

3.2.3 Adhesion to Intestinal Cells

The ability of bifidobacteria to adhere to intestinal epithelial cells is one of the crucial factors for considering any group of microorganisms as potential probiotics. Colonization of these bacteria by adhesion to intestinal epithelial cells contributes to their ability to resist pathogenic microorganisms through the production of antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocin and bacteriocin-like substances [67, 68]. Sustained host–microbe interactions, therefore, play a pivotal role in intestinal homeostasis [69, 70]. Investigation of the adhesive abilities of infant gut commensal *B. bifidum* to human intestinal mucosa by inoculating the bacterial strain onto Caco-2 and HT-29 cell monolayers showed great cell adhesion capacity, and thereby inhibition of the adhesion of pathogenic microorganisms such as *Escherichia coli* and *Cronobacter sakazakii* [71]. The ability of cells to adhere in this in vitro model was reduced by almost 75% after 42 h exposure to oxgall [70, 71]. The external features of bifidobacteria, such as presence of pili, play a significant role in their successful adhesion and colonization of the host gut. The auto-aggregation of the bifidobacteria and their adhesion capacity to the mucosal membrane of the intestine are governed by the hydrophobicity of the cell surface. This is usually strain specific and is also affected by environmental factors, such as pH and temperature [72]. Recent functional genomic analysis of *B. brevis* has revealed the important role of type IVb tight adherence (Tad) pili in the host colonization process [73], although the mechanism of cell

interaction with mucosal cells has still not been fully studied because adhesion of cells to the intestinal surface is complicated and involves many factors. Recent research has also revealed the involvement of the external features of cells on the adhesion process. For example, bifidobacteria species such as *B. breve*, *B. animalis*, *B. bifidium*, *B. longum* and many others are characterized by their ability to produce extracellular and capsular surface exopolysaccharide (sEPS), which play a significant role in cell adhesion, supporting long term persistence, colonization and stable biofilm formation on the intestinal mucosa [74–76]. In addition to the role of sEPS in the colonization process, these species can also modulate the immune system of the host to protect against pathogens. The biosynthesis and biological functions of exopolysaccharides produced by different strains of *Bifidobacterium* spp. have recently been reviewed in detail by Hidalgo-Cantabrana et al. [76].

3.2.4 Antimicrobial Activity

Successful colonization of probiotic bacteria depends significantly on their antibacterial activity because it is this that provides a barrier effect and defence against pathogens. Recent research showed that, of the pathogenic bacteria, *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Bacillus subtilis*, all except *P. aeruginosa* were inhibited by *B. longum* [67]. It was also reported that the presence of *B. longum* inhibits the growth of *E. coli* and *S. aureus* in food substances, and can therefore help to extend the shelf life of food products [77]. The extracellular metabolites produced by *B. longum*, such as lactic and acetic acids, are thought to be among the main mechanisms by which it inhibits the growth of other microbes through a pH-lowering effect and also by interfering with the colonization of intestinal pathogens. In addition to the production of acid, the antimicrobial activity of bifidobacteria is also mediated through the production of a group of antibiotic peptides (bacteriocins). Different strains are able to produce different types of bacteriocins such as: Bifid 1 produced by *B. infantis* and exhibiting inhibitory activity against *Staphylococcus*, *Bacillus*, *Salmonella* and *Shigella* [78]; Bifidocin B produced by *B. bifidum* with antimicrobial activity against *Bacillus cereus*, *Listeria monocytogenes* and *Streptococcus faecalis* [79]; Bisin produced by *B. longum* which was active against *Streptococcus thermophiles*, *Bacullus subtilis* and *Serratia marcescens* [80]; and other peptide antibiotics such as Bifilact Bb-12, Bifilact Bb-46 and Thermophilicin B67 [81].

3.3 Requirements for Large Scale Production and Application of Bifidobacteria

When growing bacteria on a large scale, both upstream and downstream processing techniques have to be handled to achieve good production. Because Bifidobacteria are known as highly oxygen sensitive and fastidious microbes, the growth media needs to be specific and rich in nutrients. Furthermore, isolates that give high growth at laboratory scale are not guaranteed to replicate this when grown on an industrial scale. Optimization of growth conditions for the probiotic is also necessary, with the right oxygen tension, pH value and a suitable temperature. The pH of the media needs to be controlled constantly because the pH decreases because of accumulation of metabolite products during the fermentation process, such as organic acids. The fermentation process should result in a highly concentrated biomass without harmful effects on the probiotic cells [10]. It is thus imperative that the probiotic bacteria is metabolically stable during processing and active in the product, so as to be able to survive passage through the upper digestive tract in large numbers, and thus have beneficial effects when present in the host intestine [65].

In general, based on many regulatory guidelines, any probiotic food product should have a minimum number of active cells to show functional probiotic activities, which is estimated to be 10^6 viable cells per millilitre or gram of food [22, 57, 82]. A lower limit of 10^9 colony-forming units (CFU) is often used for probiotic-based product formulation, however, taking into account the storage conditions [10]. Food and nutraceutical regulatory bodies in many countries have recently approved a number of probiotic bacteria in fermented products. For example, bifidobacteria are usually added to fermented milks in Japan, Australia and the MERCOSUR region in Latin America (Argentina, Paraguay, Brazil, Uruguay, etc.). The National Yoghurt Association (NYA) of the United States specifies that to use the NYA "Live and Active Culture" logo on the container of their products, there should be 10^8 CFU of live probiotics per gram of product at the time of manufacturing [83, 84]. Because probiotics are not drugs but living organisms that are trying to transplant into digestive tract, it is necessary for them to be taken regularly to achieve the purpose of their use. To obtain the positive health benefits of probiotics, it is necessary to consume between 10^9 and 10^{11} CFU per day. However, this number is also dependent on the type of strain used and the required effect [85]. At each probiotic intake, the beneficial bacterial colony in the body is reinforced, and this may gradually push out harmful bacteria and yeasts from the GIT.

4 Adaptation of Bifidobacteria to Human GIT Environment

The gastrointestinal tract of humans, starting in the stomach and ending at the rectum, is an extremely complex living system with a length ranging between 500 and 700 cm. As a result, the probiotic bacteria do not always reach the colon in the right dosage. Hence, encapsulated probiotic bacteria must be formulated taking into account the gastrointestinal conditions, which can be affected by factors such as age, gastrointestinal diseases, administration of drugs and fermentation of food residues [86] which sometimes releases toxic metabolites. In addition, the pH range of the GIT varies from highly acidic in the stomach (pH 1–3) to about pH 6 in the duodenum, before increasing again gradually in the small intestine up to pH 7.4 in the terminal ileum, dropping to pH 5.7 in the caecum and increasing gradually up to pH 6.7 in the rectum area [87].

Before reaching the colon, therefore, the probiotic bacteria must successfully pass through a range of pH regions, being exposed to enzymes and other metabolites within the gastrointestinal tract. Most bacterial cells are unable to survive transition through the stomach because of its high acidity. This acidic environment, in addition to acid production (lactic and acetic acid) by the probiotic bacteria itself, stresses the cells to adapt to this highly acidic environment. It has been reported that *Bifidobacterium* spp. possess a proton-pump mechanism that can help to prevent damage to the cells [88]. In the small intestine, the ingested probiotic bacteria are subject to attack by the digestive enzymes, which are secreted by the pancreas, and bile secreted by the gall bladder [86, 89]. The bile concentrations in the small intestine can reach 2% during the first hour of food digestion. Because of the short transit time (4–6 h) in the small intestine, however, encapsulated probiotic bacteria can resist the digestive enzymes before significantly increasing in number during their more lengthy transit through the colon (54–56 h). They are able to proliferate in the colon because the pH is nearly neutral (pH 7) and the environment is rich with nutrients. These nutrients include fibre, digestible sugars, material from the host (mucus and dead cells) and products of bacterial enzyme activity, particularly with respect to carbohydrate digestion. Through these combinations, probiotic bacteria are able to reach population levels between 10^{11} and 10^{12} CFU/g [13].

5 Therapeutic Effects of Bifidobacteria

The application of probiotics, especially the consortium of lactobacilli and bifidobacteria, in functional food industries has been shown to contribute to improvements in human health. The consumption of probiotic bacteria is helpful in maintaining good health, restoring body vigour, and combating intestinal and other diseases. Through the bacteria's active enzymes, foods exposed to probiotic bacteria are broken down and pre-digested. These nutrients are therefore more

readily available for absorption and often improve the biological value of foods. Researchers have found that most probiotic bacteria can inhibit intestinal pathogens through various anti-microbial mechanisms such as competitive colonization and production of organic acids such as lactic acid, bacteriocins, hydrogen peroxide, deconjugated bile salts, carbon dioxide and diacetyl [90]. The probiotic effect is not limited to gastrointestinal disorders but also includes immunomodulating and anticancer activities [67, 91–95]. Different studies have reported that live indigenous bacteria, or the chemicals they make, can penetrate the intestinal wall and stimulate immune cells [17, 96]. Administration of the probiotic bacteria can reduce the activity of certain undesired bacterial enzymes such as β-glucuronidase, azoreductase, urease, nitroreductase and glycocholic acid reductase and thus inhibit the conversion from a pro-carcinogenic form to a carcinogenic substance, in turn reducing the incidence of bowel cancer and perhaps other cancers in these areas [15, 97].

There are several studies showing that bifidobacteria can exhibit preventive and therapeutic effects for a wide range of diseases and symptoms (Table 2). Recent research has provided strong evidence to support the relationship between gut microbiota and human mood, based on the microbiome–gut–brain axis [98]. This is based on the fact that the intestine and the brain are bidirectionally connected and communicate through neural, endocrine and immune pathways [17, 99, 100]. Modification of gut microbiota via exogenous supplementation of probiotic consortium has been put to therapeutic use to modify stress response and symptoms of anxiety and depression [98, 101]. The positive effect of probiotics on reducing depression has been shown in vivo using a rat model fed with *B. infantis* [102]. Studies using adult rat models have also shown how feeding with *B. infantis* results in a significant reduction of depressive-like behaviour in the fed animals compared to the control group. The results were almost comparable with those treated with antidepressant drugs such as citalopram [102]. Other interesting research has also confirmed the advantage of using *B. bifidum* to reduce academic-related stress among students [103]. This effect was found to be mediated through the ability of the probiotic *B. infantis* to produce some neuroactive substances and their precursors, such as tryptophan, which reach the brain through endocrine and afferent autonomic pathways. Recent research has further shown clear evidence of a relationship between imbalances in the GIT microbiota and autism, based on the role of the gut–brain connection in this condition [104]. Clinical studies have also shown that the GI symptoms associated with autism spectrum disorders (ASD) such as abdominal pain and discomfort, and diarrhoea, are almost the same symptoms as those associated with irritable bowel syndrome (IBS). These symptoms could be reduced significantly by treatment with *B. infantis* alone or in combination with other lactobacilli, such as *Lactobacillus salivarius*, and thus help in the management of the symptoms of this disease [93, 105].

Besides these known benefits of the oral application of bifidobacteria in the treatment of many diseases, a recent new trend is their use in skin care products for regenerating and protecting the skin and for the treatment of diseases such as atopic dermatitis. This function is based on the ability of strains such as *B. breve* and

Table 2 The potential therapeutic effects of *Bifidobacterium* spp.

Probiotic properties	Actions	Reference
Antimutagenic and anticarcinogenic	Inhibition of the development of azoxymethane (AOM) (inducing various colon tumours in ~77% of treated animals) and prevention of colon and liver tumours by avoiding food mutagens such as 2-amino-3-methylimidazo [4,5-*f*] quinolone (IQ), which induces cancer	[91, 92, 108]
	Reduction of the number of tumour lesions and suppression of growth of different types of tumours	
Immune system stimulation (immunomodulators)	Stimulation of the production of several immunomodulatory molecules by various effector cells in intestine, e.g. cytokines and chemokines	[67, 94, 109]
	B. longum culture tested on peripheral blood mononuclear cells (PBMCs) produces interferons (IFN) and interleukins (IL-12) (chemokines)	
	Interaction of probiotic bacteria with immunomodulatory cells of the mucosal immune system such as enhanced leucocyte, where it exerts phagocytic activities upon adherence to the intestinal epithelial cells	
Effectiveness against diarrhea	Participation in competitive exclusion against acute diarrhoea caused by rotavirus infection among infants in hospitals	[110–112]
	Reduction of antibiotic-associated diarrhoea	
Reduction of serum cholesterol	Production of metabolites such as propionate can affect the hydroxymethylglutaryl-CoA reductase, which is involved in the cholesterol biosynthesis	[109, 113]
Acting against *Helicobacter pylori* infections	*Helicobacter pylori* is the main causative agent of gastritis and gastric ulcer and might increase the risk of gastric cancer. Suppression of *H. pylori* colonization by regular consumption of probiotic products as well as reduction of stomach inflammation	[6, 21]
Reduce Inflammatory bowel disease	Fermentation of poorly digestible carbohydrates by probiotic bacteria produces high levels of butyrate (short chain fatty acid)	[114, 115]
Production of vitamins and improved minerals absorption	Probiotic bacteria produce some necessary vitamins in the host gut such as B-complex vitamins, riboflavin and folate	[27, 116]
Antimicrobial activity	Production of antimicrobial compounds such as bacteriocins	[115, 117]
	Reduction of the pH in the colon, thus inhibiting the growth of many pathogenic bacteria such as *Clostridium* sp., *Shigella* sp., and *E. coli* and increasing intestinal peristalsis	

(continued)

Table 2 (continued)

Probiotic properties	Actions	Reference
Anti-inflammatory activity	Induction of intestinal IL-10 producing Tr1 cells	[93, 118]
	Anti-inflammatory activities through activation of pro-inflammatory transcription factor and modulation of pro-inflammatory cytokine production in mucosa	
Decrease stress and depression symptoms, autism management	Production of neuroactive substances and their precursors such as tryptophan	[17, 105, 119]
Skin regeneration and protection, treatment of atopic dermatitis	Regeneration and protection of skin, and improvement of adult atopic dermatitis through the production of lipoteichoic acid, hyaluronic acid, sphingomyelinase, antimicrobial peptides, peptidoglycan and organic acids	[18, 106, 107]
Reducing lactose intolerance	Increasing the lactose digestion because of beta-galactosidase activity	[110, 120]

B. longum to produce skin-regenerating metabolites such as peptidoglycans and hyaluronic acid, in addition to other compounds of antimicrobial properties such as bacteriocins and organic acids (lactic and acetic) [18, 106, 107].

6 Bifidobacteria Growth and Metabolism

Studies on cultivation of bifidobacteria are mainly carried out in submerged cultivation systems. Recent research has, however, suggested that solid state fermentation (SSF) is also suitable for the cultivation of some strains such as *B. bifidum* and *B. longum*, using a medium composed of substrates such as wheat bran and soybean meal [121–123]. However, submerged fermentations using both batch and fed-batch cultivation strategies remain the preferred methods for biomass production of bifidobacteria on an industrial scale [7]. Other cultivation systems, such as continuous culture, immobilized cell and co-cultivation systems with other bacteria, have recently been investigated for their suitability for cultivation of bifidobacteria, with improvements in cell yield and cell stability [57, 124, 125]. Compared with other organisms, however, information about the cultivation of bifidobacteria on a large scale, and the development of optimal cultural conditions for their growth, is still limited [126].

6.1 Growth Media

Bifidobacteria have strict nutritional requirements, but are not fastidious as are other probiotic strains, and are able to grow in a semi-synthetic medium composed of a simple carbon source, cysteine, glycine and tryptophan, vitamins, nucleotides

and minerals [90]. In many studies of bifidobacteria isolation, enumeration and laboratory scale cultivation, however, various common media for the cultivation of lactic acid bacteria are widely used, such as De Man-Rogosa Sharpe (MRS), Reinforced Clostridia Medium (RCM) and Liver Cysteine Lactose (LCL) [128]. Some research has also shown that MRS complex media supplemented with Whey Permeate gives a higher biomass production of *B. longum* up to 1.7×10^{10} CFU mL^{-1} [127]. Other additives such as L-cysteine HCl and human blood have also been applied to increase cell growth [128].

Many commercial media that have been used for bifidobacteria cultivation contain glucose as carbon source. The low efficiency of the transport system of monosaccharides compared to oligosaccharides means that media containing glucose alone cannot support the growth of many bifidobacteria strains. Better growth was achieved for some strains by using a medium consisting of a mixture of mono- and oligosaccharides [129]. It has also been reported that the addition of raffinose (oligosaccharides) during cultivations can promote growth rates of bifidobacteria. Oliveira et al. [125], meanwhile, suggest that the addition of inulin as a prebiotic can aid the growth of probiotic bacteria. A previous study by Mlobeli et al. [130] also showed that the utilization of media containing both glucose and lactose promotes growth rates of *B. bifidum*, at 0.84 h^{-1} which compared well with media containing only one sugar. Most bacteria favour monosaccharides compared to other polymeric forms of carbon. Nevertheless, bifidobacteria prefer to utilize di- and oligosaccharides as their carbon source [129]. A study done by Parche et al. [131] shows that *B. longum*, when grown in media containing lactose and glucose, prefers lactose to glucose as the primary carbon source. They observed, however, that this was also dependent on the origin of the isolated strain. It has also been reported that *B. longum* isolated from infants fed with milk prefer lactose to glucose during continuous culture, even though the specific consumption rate of glucose was higher than that of lactose [132].

The tolerance of bifidobacteria to different types of carbohydrates can be studied by their growth on semi-synthetic media rather than complex media (TPY and MRS) which consist of several carbohydrates. Besides carbon sources, addition of phosphate and mineral sources helps to improve the growth of bifidobacteria. Etoh et al. [133] proved that a medium composed of ammonium sulphate and yeast extract may also increase production of cell mass.

6.2 Carbohydrate Metabolism of Bifidobacteria

Bifidobacteria are able to utilize a wide range of mono-, di- and oligosaccharides, meaning that they are able to take advantage of the abundance of energy sources and metabolic intermediates that are produced by other microbiota in the human intestine. Bifidobacteria, however, are unable to produce either aldolase or glucose-6-phosphate $NADP^+$ oxidoreductase [134], and are therefore unable to make use of the usual glycolytic pathway. In 1967, it was found that bifidobacteria can use

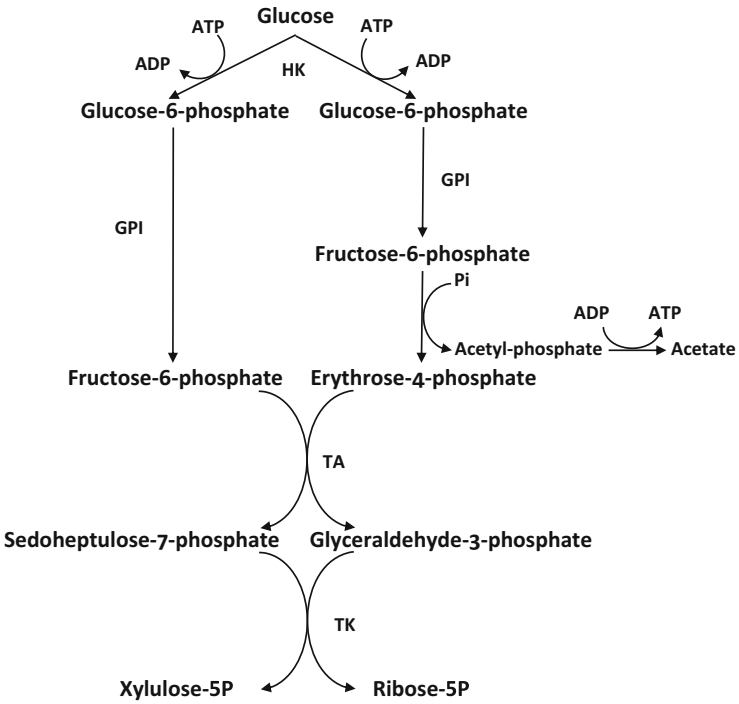


Fig. 2 Fructose-6-phosphate phosphoketolase Pathway (F6PPK) for D-glucose catabolism in bifidobacteria: HK, hexokinase (EC 2.7.1.1); GPI, glucose-phosphate isomerase (EC 5.3.1.9); F6PPK, F6P phosphoketolase (EC 4.1.2. 9.); AK, acetokinase (EC 2.7.2.1); TA, transaldolase (EC 2.2.1.2); TK, transketolase (EC 2.2.1.1). Adapted from: Caescu et al. [144]

glucose through a new specific pathway (bifido shunt) [43]. In particular, the bifidus pathway, which is characterized by the presence of fructose-6-phosphate phosphoketolase [135], is specifically found in this group of organisms (Fig. 2). This enzyme is considered as a taxonomic marker for the family of *Bifidobacteriaceae* which can differentiate them from other bacterial groups such as actinomycetes, lactobacilli and anaerobic cyanobacteria [136]. It functions by splitting the hexose phosphate into erythrose-4-phosphate and acetyl phosphate [137, 138]. As shown in Fig. 2, subsequent action of transaldolase and transketolase, which catalyse the transfer of 3 and 2 C molecular fragments from a ketose donor to an aldose acceptor, leads to an increase in the formation of pentose phosphates and eventually the amount of acetic and lactic acids. This allows bifidobacteria to produce more ATP from carbohydrates (2.5 mol ATP/mol glucose) than that produced through the conventional hetero- and homofermentative pathways [139]. Bifidobacteria are known as intrinsically heterofermentative microorganisms, meaning that they can produce lactic and acetic acid, ethanol and formic acid, as well as small amounts of succinic acid. However, the ratio between acetate and lactate production is specific to the strain

and the type of carbon source [140]. For example, the molar ratios between acetic and lactic acids were 1.18, 1.21 and 0.83 when *B. longum* was cultivated on glucose, fructose and FOS mixtures, respectively [141]. *B. animalis* subsp. *lactis* Bb 12, however, exhibited a significant increase in the acetic acid to lactic acid molar ratio, from 0.8 to 1.55, when cultivated in a skimmed milk-based medium [27].

The availability of less lactic acid for regeneration of NAD^+ (ATP production) can actually shift the metabolic pathways to acetic, succinic and formic acids [27]. Formation of lactic acid and acetic acid are in a molar ratio of 2:3 [137], although the additional production of formic acid and ethanol can alter the fermentation balance [142]. Bifidobacteria can produce lactic acid in the form of L(+) and this is advantageous for the host because it can be utilized directly. On the other hand, acetic acid, which has a sour taste, is considered to be one of the disadvantages of fermenting milk using bifidobacteria, because it acts as an inhibitory metabolite retarding the further growth of bifidobacteria and other lactic acid bacteria in the fermentation [143].

6.3 Culture Supplements for Cell Growth

It has been shown that cultivation using an oligosaccharide-based medium supplemented with mupirocin and glacial acetic acid at 50 μg mL^{-1} and 1 vol.%, respectively, is helpful for selective isolation of bifidobacteria and also supports high cell numbers [145]. Other studies have reported that Trypticase-Phytone-Yeast extract (TPY) medium is the optimal medium for bifidobacteria growth [138]. Furthermore, it was also proposed that addition of growth promoters along with the complex synthetic media promotes high biomass production. The growth of bifidobacteria in synthetic media supplemented with growth promoters such as bovine casein digest and yeast extract at 20 g L^{-1} showed optimum growth similar to that with TPY media [146]. Another study has demonstrated the positive effect of the addition of natural rubber serum powder (NRSP) to the culture medium on the growth of *B. bifidum* in submerged cultures. NRSP is considered as natural rubber waste and is rich in many kinds of amino acids, peptides and inorganic salts [135].

7 Biomass Production of Bifidobacteria

Compared to other probiotic bacteria and yeasts, literature on the biomass production of bifidobacteria on an industrial scale is very limited. This may be because of the growth limitations of this type of organisms, related to their anaerobic growth behaviour, or the high industrial potential of this group of microbes, with research being protected under intellectual property rights or as trade secrets.

Despite a few reports on the cultivation of bifidobacteria using solid state fermentation (SSF), industrial biomass production of this type of bacteria still typically uses submerged fermentation systems (SMF). Batch cultivation is widely used for many types of microorganisms because it is the simplest cultivation system which requires minimal set-up, measurement and control. This process has been applied successfully for many years to the cultivation and cell mass production of all types of probiotics. The main drawback of this process, however, is low biomass yields, as a function of substrate limitations that inhibit accumulation in the cultivation medium. To improve biomass production, fed-batch cultivation has also been successfully applied to bifidobacteria cultivation. This fermentation technique has been used to reduce or prevent substrate inhibition or nutrient depletion, which can have a negative effect on both cell growth and bacteriocin production. Nutrient exhaustion can be overcome by the addition of a limiting substrate during the fermentation, which can serve to increase the bacterial concentration. Using fed-batch strategies to produce probiotic cultures has some advantages, and by applying thermal stress to the bacteria during cultivation, the cells are able to cope with the subsequent downstream processing steps. Moreover, probiotic cultures that undergo these protocols form lower amounts of exopolysaccharides and thus reduce the medium viscosity during the cultivation process [7]. Another study has also reported that fed-batch fermentation is one of the most effective processes for obtaining maximum specific growth rates during cultivation leading to faster oxidation of substrate [147]. This was further supported by a study on the successful use of fed batch cultivation for economical production of large amounts of probiotic biomass and bacteriocins using culture media from food waste [148, 149]. Table 3 shows the different media applied for Bifidobactia cultivation in batch and fed-batch cultures using different types of bioreactors and cultivation systems.

8 Downstream Processing and Stabilization

In most probiotic production processes, cells are separated from the cultivation broth immediately after termination of the fermentation. The separated cells are then washed to remove the remaining traces of the medium components before going further in downstream processing. In most cases, probiotic bacteria should be kept and transported in dry form to reduce the risk of contamination and to extend the shelf life of the bacteria. Thus, probiotics should be kept as latent living cells either before direct application as probiotic microbes in powder form of different formulations or by addition to dairy or non-dairy food for functional food production. The selection of the drying process is therefore very critical to maintaining cell viability during storage [156]. Nowadays, freeze drying (lyophilization) and spray drying are the most widely used techniques in industry for the drying of probiotic microorganisms [157]. Some researchers, however, have also reported on the

Table 3 Biomass production of *Bifidobacterium* spp. using different cultivation strategies in pure and in mixed culture systems

Strains	Cultivation mode	Carbon source	Biomass (g L^{-1})	Cell count (CFU mL^{-1})	Reference
Free cell cultivation – stirred tank bioreactor					
B. longum CCRC 14634	Batch				[92]
	Controlled at pH 6.0	Glucose	4.1	1.3×10^9	
		Lactose	6.1	3.5×10^9	
	Repeated batch for 6 cycles	Glucose	3.94	1.9×10^9	
	Controlled at pH 6.0	Lactose	6.58	3.4×10^9	
	Fed-batch				
	Controlled pH	Glucose	5.57	5.2×10^9	
	Continuous feeding				
B. longum ATCC 15707	Batch				[141]
	Controlled at pH 6.5	Glucose	1.6	–	
		Fructose	2.7		
		FOS	4		
	Fed-batch				
	Uncontrolled pH				
	Continuous feeding				
	Glucose ($D_{max} = 0.2\ h^{-1}$)	Glucose	3	–	
	Fructose ($D_{max} = 0.2\ h^{-1}$)	Fructose	3.9		
	FOS ($D_{max} = 0.15\ h^{-1}$)	FOS	2.54		
B. longum	Fed-batch				[150]
	Controlled at pH 6.0	Glucose and fructose	2.62	1.8×10^{12}	
	Continuous feeding				
	$D = 0.38\ h^{-1}$				
B. infantis	Batch				[140]
	Controlled at pH 6.0	Glucose	2.1	–	
B. longum SH2	Batch			–	[132]
	Controlled pH 5.0	Glucose	4.37		
		Lactose	3.42		
	Fed-batch			–	
	Controlled pH 5.0	Glucose	2.74		
	Continuous feeding with dilution rate from 0.066 to 0.396 h^{-1}	Lactose	2.99		

(continued)

Table 3 (continued)

Strains	Cultivation mode	Carbon source	Biomass (g L^{-1})	Cell count (CFU mL^{-1})	Reference
Free cell cultivation – membrane bioreactor					
B. bifidum BGN 4	Batch				[113]
	Controlled at pH 6.0	Sucrose	4.5	3.0×10^9	
	Fed-batch				
	Controlled at pH 6.0	Sucrose	12	2.2×10^{10}	
	Continuous feeding				
	$D = 0.06\ h^{-1}$				
B. longum ATCC 15707	Fed-batch	Glucose and fructose	22.18	1.15×10^{15}	[150]
	Controlled at pH 6.0				
	Continuous feeding				
	$D = 0.3–0.45\ h^{-1}$				
B. longum SH-2	Batch				[151]
	Controlled at pH 5.0				
	Cultivation as free cells	Lactose	13	2.9×10^9	
	Immobilized cells – calcium carbonate (2.0%, w/v) mixed with alginate (2.0%, w/v) beads		16.8	5.0×10^{10}	
B. animalis subsp. *lactis* Bb 12	Batch				[152, 153]
	Uncontrolled pH 5.0	Whey and lactose	4.1–4.8		
	Alginate-chitosan			–	
	k-carrageenan-locust bean gums			–	
	Gellan xanthan				
B. longum	Fed-batch				[37]
	Immobilized cells – gelrite, gellan mixed with xanthan (0.25%, w/v) + sodium citrate (0.2%, w/v)	Glucose	4.6	8.6×10^9	
	Controlled at pH 6.0				
	Continuous feeding without glucose limitation, $D = 0.2\ h^{-1}$				

(continued)

Table 3 (continued)

Strains	Cultivation mode	Carbon source	Biomass (g L^{-1})	Cell count (CFU mL^{-1})	Reference
B. longum NCC 2705	Batch				
	Immobilized cells – gelrite, gellan mixed with xanthan (0.25%, w/v)	Glucose	–	2.9×10^9	[88]
	Controlled at pH 6.0				
	Continuous feeding at rate of 2.6 mL/min				
Free cells-mixed culture					
B. animalis	Batch (mixed culture)	Goat milk	–	6.3×10^7	[154]
L. acidophilus				7.1×10^8	
B. lactis	Batch				[125]
Streptococcus thermophillus	Single culture				
	S. thermophillus	Milk	2.7	-	
	B. lactis		1.3		
	Mixed culture				
	S. thermophillus		3.1		
	B. lactis		1.7		
	Single culture				
	S. thermophillus	Milk + inulin	3.1		
	B. lactis		1.8		
	Mixed culture				
	S. thermophillus		4.1		
	B. lactis		2.6		
	Batch				
B. longum	Controlled pH 6.5	Glucose			[124]
B. breve	Single culture			–	
Propionibacterium freudenreichii	*B. longum*		4.1		
	B. breve		8.2		
	Mixed culture				
	B. longum		8.6		
	P. freudenreichii		2		
	B. breve		19.3		
	P. freudenreichii		2		

(continued)

Table 3 (continued)

Strains	Cultivation mode	Carbon source	Biomass ($g\ L^{-1}$)	Cell count ($CFU\ mL^{-1}$)	Reference
B. thermophilum RBL67	Batch	Glucose			[155]
Pediococcus acidilactici UVA1	Controlled pH 6.0				
	Single culture				
	B. thermophilum RBL67		1.1		
	Mixed culture				
	B. thermophilum RBL67				
	Pediococcus acidilactici UVA1		1.4–2.0		

D = dilution rate (h^{-1})

suitability of other methods for probiotic cell drying such as low temperature vacuum and fluidized beds [158–161].

In general, freeze drying is a mild process and supports long-term stability and preservation of the microbial cells without significant loss in viability. The process of freeze drying involves three main steps: freezing, primary drying and secondary drying. During the freezing step, bacteria are frozen to reach temperatures as low as −196°C using liquid nitrogen. This step is most critical for cell viability, and if the cells survive the freezing process they most probably survive the subsequent dehydration/drying process [162]. The drying phase involves ice sublimation under high vacuum by increasing the temperature (primary drying); this is considered to be a transition phase to convert water from a solid to a gaseous form at a temperature and pressure below water's triple point. In this step, almost 95% of the water is removed. The secondary drying process involves the removal of the water bound by hydrogen bonds by a desorption process. Once the water content reaches less than 4%, the temperature is gradually increased up to the ambient temperature [7].

To increase cell viability during freeze drying and storage, and their resistance to pH/bile salts during application, some cell-protecting agents such as skimmed milk powder, milk whey, butter milk, glycerol, low molecular weight carbohydrates (trehalose, glucose, sucrose, lactose), dextran, polyethylene glycol and pepsin are usually added [22, 157, 163, 164]. It has recently been reported that using trehalose as a cryoprotectant during freeze drying protects the stress-sensitive cells of bifidobacteria such as *B. longum* and *B. animalis* subsp. *lactis*. This research also shows the importance of keeping the storage temperature as low as −80°C during storage in order to retain the viability of the freeze-dried cells for up to 10 months [165]. Other research has shown that using a mixture of human-like collagen (HLC), trehalose and glycerol during the freeze drying process can increase the cell viability of *B. longum* [166]. The effect of storage temperature on cell viability

during long-term storage of freeze-dried bifidobacteria is highly strain-dependent [165, 167, 168]. In general, freeze drying is a batch process with a low yield, characterized by long drying times and high energy consumption. Even though freeze drying supports high cell viability, it carries significant disadvantages for industrial applications because of the high capital and running costs.

To overcome the cost and time limitations of the freeze drying method, spray drying has been considered as a potential alternative. This method is characterized by its high yield, shorter time, continuous operation and lower capital and operating costs compared to freeze drying. In this process, the cell suspension (usually with additives) is pumped through a heated nozzle and atomized into small droplets between 10 and 200 μm in diameter using compressed air. The atomization temperature is usually varied between 130 and 200°C. The liquid droplets are sprayed into the drying chamber with a co- or counter-current flow of hot air that dries the droplets. The dried solid particles are then collected at the bottom of the spray drier. During this process, the cells are exposed to high shear, high pressure and high temperature, which affect the cell viability for many probiotic heat-sensitive strains and are the main drawbacks compared to freeze drying. In addition, other stress factors such as dehydration, osmotic pressure and oxygen exposure have a negative influence on cell viability, especially for bifidobacteria [7, 169]. To minimize cell death during spray drying, the influence of different drying parameters such as inlet and outlet temperature, flow rate of the feed suspension, cell concentration in the feed suspension, flow rate of drying air, type of nozzle used, relative humidity, residence time and protective agents should be well understood [170–172]. In addition to the effect of drying parameters, the time of cell harvest after fermentation has been reported as a critical factor affecting the cells' ability to withstand the harsh conditions in the downstream process. As shown in some studies, the cells harvested in the stationary phase showed higher stability during the drying process than those harvested in the exponential phase [173, 174].

Besides freeze drying and spray drying, some other methods have also been reported in the literature as primary or secondary drying processes for probiotics, such as fluidized bed and vacuum drying. Fluidized bed drying is usually considered to be the most cost effective and mild drying process because cells are not exposed to either ultra-low temperatures, as in freeze drying, or high temperatures, as in spray drying. In this process, the bacterial cells are first granulated and then encapsulated using supporting materials before drying. In practice, therefore, this method is usually used as a second drying process after spray drying. This allows the use of a lower spray drying outlet temperature and thus increases cell viability [7]. Vacuum drying is usually considered for drying materials sensitive to freezing, because the drying temperature used is higher than for freeze drying. The advantage of this process is the minimization of oxidation reactions during the drying of oxygen-sensitive microorganisms such as bifidobacteria, but the main disadvantages are the long drying time and low yield [175].

Different approaches have been applied during the last few years to increase cell viability during downstream processes, the shelf life of the probiotic cells, and cell resistance against the harsh pH and chemicals present during their passage through

the GIT before reaching the colonizing site in the small intestine. These include (micro/nano)-encapsulation, spray coating and cell immobilization [176–181]. Cell encapsulation is widely used to protect cells during the spray drying process. Different materials, such as starch, skimmed milk, alginate, k-carrageenan, casein and many others, have been used as potential cell-encapsulating agents. A mixture of two materials such as casein and alginate can also be used [182]. Another study has shown that using a mixture of calcium alginate and mannitol for *B. animalis* subsp. *lactis* cell microencapsulation was effective in protecting cell envelopes and proteins during and after freeze drying, and in long-term storage at room temperature, especially when stored at low water activity [183, 184]. Microencapsulation using a mixture of calcium alginate, probiotic and glycerol also resulted in a significant increase in cell viability during spray drying and storage, and stability under the harsh environmental conditions of the GIT [176, 185]. Improved cell viability was also evident when alginate/pullulan-microencapsulated *B. lactis* cells were further coated with alginate, chitosan or gelatin using the dip coating method and crosslinking [180].

It is also worth noting that cell tolerance to downstream stresses, storage conditions and the harsh conditions of the GIT can be increased by provoking stress adaptations in cells by exposure to sub-lethal doses of acid or heat during different phases of the fermentation process. For example, the tolerance of *B. longum* and *B. animalis* to bile salt and low pH was increased to a certain extent when cells were exposed to short term thermal treatment at 47°C and pH 3.5 during the stationary phase [186]. The observed increase in thermotolerance of heat treated bifidobacteria is mediated through the expression and production of heat stress proteins, as has been confirmed by proteomic studies of *B. longum* and *B. breve* [187–189]. Recent research has also shown that heat shock by short time exposure to sub-lethal temperatures enhances the production and excretion of exopolysaccharides, leading to a significant increase in *B. bifidum* cell robustness and survival during freeze drying [190].

9 Conclusions and Future Perspectives

The type and number of microbial cells in the human body can influence its health status. Thus, supplementation with specific functional microbial systems, and enhancement of the growth and colonization of specific groups of beneficial microbes, could be one of the future strategies to control disease without exogenous extensive use of antibiotics. In addition, microbial cells in the human body have other functions beyond their antimicrobial effect: they provide a natural prophylactic mechanism in body homeostasis, and protection against many non-microbial-related diseases. In the future, therefore, a biotic approach to the treatment of different diseases may be a safer way to treat many human ailments. The potential future application of bifidobacteria as prophylactic/biotherapeutic agents is not limited to human use, but also has a wide scope for the control and treatment of

diseases in economically important animals, especially because the ban on the use of antibiotics in many countries has been implemented to reduce the risk of microbial antibiotic resistance. Recent research has also shown the potential application of bifidobacteria in the development of safe skin protection and regeneration products, which open the way for a new trend in probiotic-based cosmetic products. In addition, the approach of using bifidobacteria in the treatment of the symptoms of mental diseases can lead to the development of a new class of probiotic-based psychotherapeutics. Further research is needed, however, to study in depth the mechanism of action of microbiota bioecosystems in the human body and its health status in an omics approach to understand how bifidobacteria help in homeostasis and in adjusting the body's ecosystem. The study of the relationship between the human microbiome and diet to support the growth, colonization and functionality of bacteria in the human body also needs further investigation. This may help to develop new and safer treatment approaches which can shape the future of human and animal health industries.

References

1. Ley RE, Peterson DA, Gordon JL (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848
2. Sekirov I, Russell SL, Antunes LCM, Finlay BB (2010) Gut microbiota in health and disease. Physiol Rev 90:859–904
3. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci USA 104:13780–13785
4. Fuller R (1989) A review: probiotics in man and animals. J Appl Bacteriol 66:365–378
5. Fooks LJ, Gibson GR (2002) Probiotics as modulators of the gut flora. Brit J Nutr 88:S39–S49
6. Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D (2005) Getting better with bifidobacteria. J Appl Microbiol 98:1303–1315
7. Muller JA, Ross RP, Fitzgerald GF, Stanton C (2009) Manufacture of probiotic bacteria. In: Charalampopulos D, Rastall R (eds) Prebiotics and probiotics science and technology. Springer, Heidelberg, pp 725–759
8. Sarmidi MR, El Enshasy HA (2012) Biotechnology for wellness industry: concepts and biofactories. Int J Biotechnol Well Ind 1:3–28
9. Shah NP (2007) Functional cultures and health benefits. Int Dairy J 17:1262–1277
10. Forssten SD, Sindelar CW, Ouwehand AC (2011) Probiotics from an industrial perspective. J Clin Microbiol 17:410–413
11. Salminen S, Isolauri E, Salminen E (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie van Leeuwenhoek 70:347–358
12. Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau M-C, Roberfroid M, Rowland I (1998) Functional food science and gastrointestinal physiology and function. Brit J Nutr 80(Suppl 1):S147–S171
13. BourliouxP KB, Guarner F, Braesco B (2003) The intestine and its microflora are partners for the protection of the host. Am J Clin Nutr 78:675–683
14. Villena J, Salva S, Nuñez M, Corzo J, Tolaba R, Faedda J, Font G, Alvarez S (2012) Probiotics for everyone! The novel immunobiotic *Lactobacillus rhamnosus* CRL1505 and the beginning of social probiotic programs in Argentina. Int J Biotechnol Well Ind 1:189–198

15. Heyman M, Menard S (2002) Probiotic microorganisms: how they affect intestinal pathophysiology. Cell Mol Life Sci 59:1–15
16. Foster JA, McVey Neufeld KA (2013) Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci 36:305–312
17. Steenbergen L, Sellaro R, van Hemert S, Bosch JA, Colzato LS (2015) A randomized controlled trial to test the effect of multi species probiotics on cognitive reactivity to sad mood. Brain Behav Immunity 48:258–264
18. Lew L-C, Gan C-Y, Liong M-T (2013) Dermal bioactives from lactobacilli and bifidobacteria. Ann Microbiol 63:1047–1055
19. Raja BR, Arunachalam KD (2011) Market potential for probiotic nutritional supplements in India. Afr J Business Manag 5:5418–5423
20. Markets and Markets (2014) Global probiotics market worth US$32.6 billion by 2014. http://www.prnewswire.com/news-releases/marketsandmarkets-global-probiotics-market-worth-us326-billion-by-2014-62565667.html. Accessed 10 June 2015
21. Saxelin M, Tynkkynen S, Mattila-Sandholom T, de Vos WM (2005) Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 16:204–211
22. Mättö J, Alakomi H-L, Vaari A, Virkajärvi I, Saarela M (2006) Influence of processing conditions on *Bifidobacterium animalis* subsp. *lactis* functionality with a special focus on acid tolerance and factors affecting it. Int Dairy J 16:1029–1037
23. Vergari F, Tibuzzi A, Basile G (2010) An overview of the functional food market: from marketing issues and commercial players to future demand from life in space, "Bio-farms for nutraceuticals". In: Giardi MT, Rea G, Berra B (eds) Advances in experimental medicine and biology. Springer, Berlin, pp 308–321
24. Meena GS, Gupta S, Majumdar GC, Banerjee R (2011) Growth characteristics modelling of *Bifidobacterium bifidum* using RSM and ANN. Braz Arch Biol Technol 54:1357–1366
25. Bhadoria PBS, Mahapatra SC (2011) Prospects, technological aspects and limitations of probiotics – a worldwide review. Eur J Food Res Rev 1:23–42
26. Philippe D, Heupel E, Blum-Sperisen S, Riedel CU (2011) Treatment with *Bifidobacterium bifidum* 17 partially protects mice from Th1-driven inflammation in a chemically induced model of colitis. Int J Food Microbiol 149:45–49
27. Jalili H, Razavi SH, Safari M, Malcata FX (2009) Enhancement of growth rate and β-galactosidase activity, and variation in organic acid profile of *Bifidobacterium animalis* subsp. *lactis* Bb 12. Enz Microb Technol 45:469–476
28. Kabeerdoss J, Devi RS, Mary RR, Prabhavathi D, Vidya R, Mechenro J, Mahendri NV, Pugazhendhi S, Ramakrishna BS (2011) Effect of yoghurt containing *Bifidobacterium lactis* Bb12® on faecal excretion of secretory immunoglobulin A and human beta-defensin 2 in healthy adult volunteers. Nutr J 23; 10:138.
29. Su P, Henriksson A, Mitchell H (2007) Selected prebiotics support the growth of prebiotic mono-cultures in vitro. Anaerobe 13:134–139
30. Siro I, Kápolna E, Kápolna B, Lugasi A (2008) Functional food: product development, marketing and consumer acceptance – a review. Appetite 51:456–467
31. Mayer HK, Amtmann E, Philippi E, Steinegger G, Mayrhofer S, Kneifel W (2007) Molecular discrimination of new isolates of *Bifidobacterium animalis* subsp *lactis* from reference strains and commercial probiotic strains. Int Dairy J 17:565–573
32. Bonannoxs A (2012) Some like it healthy: demand for functional and conventional yogurts in the Italian market. Agribusiness 28:67–85
33. Grmanová M, Vlková E, Rada V, Homutová I (2010) Survival of Bifidobacteria in adult intestinal tract. Folia Microbiol 55:281–285
34. Kopećnỳ J, Mrázek J, Killer J (2010) The presence of Bifidobacteria in social insects, fish and reptiles. Folia Microbiol 55:336–339
35. Buchanan RE (1974) Bergey's manual of determinative bacteriology, 8th edn. NEE Gibbons, Williams and Wilkins, Baltimore

36. Mitsuoka T (1992) The human gastrointestinal tract. In: Wood BJB (ed) The lactic acid bacteria in health and disease. Elsevier, Amsterdam, pp 69–114
37. Reimann S, Grattepanche F, Benz R, Mozetti V, Rezzonico E, Berger B, Lacroix C (2011) Improved tolerance to bile salts aggregated *Bifidobacterium longum* produced during continuous culture with immobilized cells. Bioresour Technol 102:4559–4567
38. Bottacini F, Ventura M, van Sinderen D, Motherway MO (2014) Diversity, ecology and intestinal function of bifidobacteria. Microb Cell Fact 13(Suppl 1):S4
39. Ventura M, van Sinderen D, Fitzgerald GF, Zink R (2004) Insights into the taxonomy, genetics and physiology of bifidobacteria. Antoine Van Leeuwenhoek 86:205–223
40. Ventura M, Canchaya C, Dei Casale A, Dellaglio F, Neviani E, Fitzgerald GF, van Sinderen D (2006) Analysis of bifidobacterial evolution using a multilocus approach. Int J Syst Evol Microbiol 56:2783–2792
41. Hughes D, Hoover DG (1991) *Bifidobacteria* their potential for use in American dairy products. J Food Technol 45:74–80
42. Kim HS (1988) Characterization of lactobacilli and bifidobacteria as applied to dietary adjuncts. Cult Dair Prod J 23:6–9
43. Ballongue J (1998) Bifidobacteria and probiotic action. In: Salminen S, von Wright A (eds) Lactic Acid Bacteria. Marcel Dekker, New York, pp 519–587
44. Chen B, Wang X, Zhang L (2010) Culture medium for *Bifidobacterium longum*, composition comprising the same and preparation method. USP, US 2010/0098667 A1
45. Mandar R, Mikelsaar M (1996) Transmission of mother's microflora to the newborn at birth. Biol Neonate 96:30–35
46. Huurre A, Kalliomaki M, Rautava S, Rinne M, Salminen S, Isolauri E (2008) Mode of delivery on gut microbiota and humoral immunity. Neonatol 93:236–240
47. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. PNAS 107:11971–11975
48. Othman NZ, El Enshasy HA, Abdel Malek R, Sarmidi MR, Aziz RA (2009) Kinetics of cell growth and functional characterization of probiotic strains *Lactobacillus delbrueckii* and *Lactobacillus paracasei* isolated from breast milk. Deut Lebensmittel Rund 105:444–450
49. Elsayed EA, Othman NZ, Malek R, Tang T, El Enshasy HA (2014) Improvement of cell mass production of *Lactobacillus delbrueckii* sp. *bulgaricus* WICC-B-02: a newly isolated probiotic strain from mother's milk. J Appl Pharmceut Sci 4:8–14
50. Solís G, de los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M (2010) Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe 16:307–310
51. Gueimonde M, Laitinen K, Salminen S, Isolauri E (2007) Breast milk: a source of bifidobacteria for infant gut development and maturation? Neonatology 92:64–66
52. Putignani L, Del Chierico F, Petrucca A, Vernocchi P, Dallapiccola B (2014) The human gut microbiota: a dynamic interplay with the host from birth to senescence settled during childhood. Pediat Res 76:2–10
53. Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–1402
54. Harmsen HJM, Wildeboer-Veloo ACM, Raangs GC, Wagendorp AA, Klijn N, Bindels JG (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 30:61–67
55. Hopkins MJ, Cummings JH, Macfarlane GT (1998) Inter-species differences in maximum specific growth rates and cell yields of Bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. J Appl Microbiol 85:381–386
56. Reuter G (2001) The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol 2:43–53
57. Lacroix C, Yildirim S (2007) Fermentation technologies for the production of probiotics with high viability and functionality. J Biotechnol 18:176–183

58. Shimamura S, Abe F, Ishibashi N, Miyakawa H, Yaeshima T, Tomita M (1990) Endogenous oxygen uptake and polysaccharide accumulation in *Bifidobacterium*. Agric Biol Chem 54:2869–2874
59. Simpson PJ, Stanton C, Fitzgerald OF, Ross RP (2005) Intrinsic tolerance of *Bifidobacterium longum* species to heat and following spray drying and storage. J Appl Microbiol 99:493–501
60. Andriantsoanirina V, Allano S, Butel MJ, Aries J (2013) Tolerance of *Bifidobacterium* human isolates to bile, acid and oxygen. Anaerobe 21:39–42
61. Meile L, Ludwig W, Rueger U, Gut C, Kaufmann P, Dasen G, Wenger S, Teuber M (1997) *Bifidobacterium lactis* sp. nov., a moderately oxygen tolerant species isolated from fermented milk. Syst Appl Microbiol 20:57–64
62. Ninomiya K, Matsuda K, Kawahat T, Kanaya T, Kohno M, Katakura Y, Masanori A, Shioya S (2009) Effect of CO_2 concentration on the growth and exopolysaccharide production of *Bifidobacterium longum* cultivated under anaerobic conditions. J Biosci Bioeng 107:535–537
63. Kleerebezem M, Vaughan EE (2009) Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. Annu Rev Microbiol 63:269–290
64. Tanaka H (2000) Bile salts hydrolase of *Bifidobacterium longum*-biochemical and genetic characterization. Appl Environ Microbiol 66:2502–2512
65. Krasaekoopt W, Bhandari B, Deeth H (2004) The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. Int Dairy J 14:737–743
66. Takahashi N, Xiao JZ, Miyaji K, Yaeshiima T, Hiramatsu A, Iwatsuki K, Kokubo S, Hosono A (2004) Selection of acid tolerant bifidobacteria and evidence for a low-pH-inducible acid tolerance response in *Bifidobacterium longum*. J Dairy Res 71:340–345
67. Tham CSC, Peh KK, Bhat R, Liong MT (2011) Probiotic properties of bifidobacteria and lactobacilli isolated from local dairy products. Ann Microbiol 62:1079–1087
68. Sonnenburg ED, Sonnenburg JL, Manchester JK, Hansen EE, Chiang HC, Gordon JI (2006) A hybrid two-component system protein of a prominent human gut symbiont couples glycan sensing in vivo to carbohydrate metabolism. Proc Natl Acad Sci USA 103:8834–8839
69. Rautava S, Walker WA (2007) Commensal bacteria and epithelial cross talk in the developing intestine. Curr Gastroenterol Rep 9:385–392
70. Guglielmetti S, De Noni I, Caracciolo F, Molinari F, Parini C, Mora D (2008) Bacterial cinnamoyl esterase activity screening for the production of a novel functional food product. Appl Environ Microbiol 74:1284–1288
71. Serafini F, StratiF R-MP, Turroni F, Foroni E, Duranti S, Milano F, Perotti A, Viappiani A, Guglielmetti S, Buschini A, Margolles A, van Sinderen D, Ventura M (2013) Evaluation of adhesion properties and antibacterial activities of the infant gut commensal *Bifidobacterium bifidum* PRL2010. Anaerobe 21:9–17
72. Rahman MM, Kim W-S, Kumura H, Shimazaki K-I (2008) Auto aggregation and surface hydrophobicity of bifidobacteria. World J Microbiol Biotechnol 24:1593–1598
73. O'Connell Motherway M, Zomer A, Leathy SC, Reunanen J, Bottacini F, Claesson MJ, O'Brien F, Flynn K, Casey PG, Munoz JAM, Bearney B, Houston AM, O'Mahony C, Higgins DG, Shanahan F, Palva A, de Vos WM, Fitzgerald GF, Ventura M, O'Toole PW, van Sinderen D (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proc Natl Acad Sci USA 108:11217–11222
74. Fanning S, Hall IJ, Cronin M, Zomer A, MacSharry J, Goulding D, Motherway MO, Shanahan F, Nally K, Dougan K, Dougan G, van Sinderen D (2012) Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Natl Acad Sci USA 109:2108–2113
75. Fanning S, Hall LJ, van Sinderen D (2012) *Bifidobacterium breve* UCC2003 surface exopolysaccharide production is a beneficial trait mediating commensal-host interaction through immune modulation and pathogen protection. Gut Microbes 3:420–425

76. Hidalgo-Cantabrana C, Sánchez B, Milani C, Ventura M, Margolles A, Ruas-Madiedo P (2014) Genomic overview and biological function of exopolysaccahride biosynthesis in *Bifodobacterium* spp. Appl Env Microbiol 80:9–18
77. Ebhodaghe SO, Abiose SH, Adeniran HA (2012) Assessment of physico-chemical characteristics, viability and inhibitory effect of Bifidobacteria in soymilk. J Food Res 1:159–170
78. Cheikhyoussef A, Cheikhyoussef N, Chen H, Zhao J, Tang J, Zhang H, Chen W (2010) Bifidin 1 - a new bacteriocin produced by *Bifidobacterium infantis* BCRC 14602: purification and partial amino acid sequence. Food Control 21:746–753
79. Yildirim Z, Winters D, Johnson M (1999) Purification, amino acid sequence and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. J Appl Microbiol 86:45–54
80. Lee JH, Li X, O'Sullivan DJ (2011) Transcription analysis of a lantibiotic gene cluster from *Bifidobacterium longum* DJO10A. Appl Environ Microbiol 77:5879–5887
81. Martinez FAC, Balciunas EM, Converti A, Cotter PD, de Souza Oliveira RP (2013) Bacteriocin production by *Bifidobacterium* spp. A review. Biotechnol Adv 31:482–488
82. Arora M, Sharma S, Baldi A (2013) Comparative insight of regulatory guidelines for probiotics in USA, India and Malaysia: a critical review. Int J Biotechnol Well Ind 2:51–64
83. Talwalkar A, Kailasapathy K (2004) A review of oxygen toxicity in probiotic yogurts: influence on the survival of probiotic bacteria and protective technique. Comp Rev Food Sci Food Safety 13:117–124
84. Guarner F, Malagelada JR (2003) Gut flora in health and disease. Lancet 361:512–519
85. Jose NM, Bunt CR, Hussain MA (2015) Comparison of microbiological and probiotic characteristics of *Lactobacilli* isolates from dairy food products and animal rumen contents. Microorganisms 3:198–212
86. Vandamme TF, Lenourry A, Charrueau C, Chaumeil J-C (2002) The use of polysaccharides to target drugs to the colon. Carbohyd Polym 48:219–231
87. Fallingborg J (1999) Intraluminal pH of the human gastrointestinal tract. Dan Med Bull 46:183–196
88. Mozzetti V, Grattepanche F, Moine D, Berger B, Rezzonico E (2010) New method for selection of hydrogen peroxide adapted bifidobacteria cells using continuous culture and immobilized cell technology. Microb Cell Fact 9:60
89. Lui LS, Fishman ML, Kost J, Hicks KB (2003) Pectin-based systems for colon specific drug delivery via oral route. Biomaterials 24:3333–3343
90. Gomes AMP, Malcata FX (1999) *Bifidobacterium* sp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends Food Sci Technol 10:139–157
91. Singh J (1997) *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. Carcinogenesis 18:833–841
92. Her SL, Duan KJ, Sheu DC, Lin CT (2004) A repeated batch process for cultivation of *Bifidobacterium longum*. J Ind Microbiol Biotechnol 31:427–432
93. O'Mahony D, Murphy S, Boileau T, Park JS, O'Brien F, Groeger D, Konileczna P, Ziegler M, Scully P, Shanahan F, Kiely B, O'Mahony L (2010) *Bifidobacterium animalis* AHC7 protects against pathogen-induced NF-κB activation in vivo. BMC Immunol 11:63
94. Donkor ON, Vasiljevic T, Gill HS (2010) Probiotics and immunomodulation. In: Watson RR, Zibadi S, Preedy VR (eds) Dietary components and immune function, Nutrition and Health Series. Springer, New York, pp 625–655
95. Ashraf R, Shah NP (2014) Immune system stimulation by probiotic microorganisms. Crit Rev Food Sci Nutr 54:938–956
96. Tannock GW (2004) A special fondness for *Lactobacilli*. Appl Environ Microbiol 70:3189–3194
97. Reid G (1999) The scientific basis for probiotic strains of *Lactobacillus*. Appl Environ Microbiol 65:3763–3766

98. Cryan JF, O'Mahony SM (2011) The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 23:187–192
99. Mayer EA (2011) Gut feelings: the emerging biology of gut-brain communication. Nat Rec Neurosci 12:453–466
100. Mayer EA, Knight R, Mazmanian SK, Gryan JF, Tillisch K (2014) Gut microbes and the brain: paradigm shift in neuroscience. J Neurosci 34:15490–15496
101. Bruce-Keller AJ, Salbaum JM, Luo M, Blanchard E IV, Taylor CM, Welsh DA, Berthoud HR (2015) Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. Biol Psychiatry 77:607–615
102. Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG (2008) The probiotic *Bifidobacterium infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 43:164–174
103. Langkamp-Henken B, Rowe CC, Ford AL, Christman MC, Nieves C Jr, Khouri L, Specht GJ, Girard SA, Spaiser SJ, Dahl WJ (2015) *Bifidobacterium bifidum* R0071 results in a greater proportion of healthy days and a lower percentage of academically stressed students reporting a day of cold/flu: a randomized, double-blind, placebo-controlled study. Brit J Nutr 113:426–434
104. Hsiao EY (2014) Gastrointestinal issues in autism spectrum disorder. Harvard Rev Psych 22:104–111
105. Critchfield JW, van Hemert S, Ash M, Mulder L, Ashwood P (2011) The potential role of probiotics in the management of childhood autism spectrum disorders. Gastroenterol Res Pract 2011:161358
106. Iemoli E, Trabattoni D, Parisotto S, Borgonovo L, Toscano M, RizzaridiniG CM, Ricci E, Fusi A, De Vecchi E, Piconi S, Drago L (2012) Probiotics reduce gut microbial translocation and improve adult atopic dermatitis. J Clin Gastroenterol 46(Suppl):S33–S40
107. Yoshida Y, Seki T, Matsunaka H, Watanabe T, Shindo M, Yamada N, Yamamoto O (2010) Clinical effects of probiotic *Bifidobacterium breve* supplementation in adult patients with atopic dermatitis. Yonago Acta Medica 53:37–45
108. De Vrese M, Schrezenmeir J (2008) Probiotics, prebiotics, and synbiotics. Adv Biochem Eng/Biotechnol 111:1–66
109. Wang Y-C, Yu R-C, Chou C-C (2002) Growth and survival of bifidobacteria and lactic acid bacteria during the fermentation and storage of cultured soymilk drinks. Food Microbiol 19:501–508
110. Lin DC (2003) Probiotics as functional foods. Nutr Clin Pract 18:497–506
111. Gill H, Guarner F (2004) Probiotics and human health: a clinical perspective. Postgrad Med J 80:516–526
112. De Vrese M (2003) Effects of probiotic bacteria on gastrointestinal symptoms, *Helicobacter pylori* activity and antibiotics-induced diarrhoea. Gastroenterol 124:A560
113. Kwon SG, Son JW, Kim HJ, Park CS, Lee JK, Ji GE, Oh DK (2006) High concentration cultivation of *Bifidobacterium bifidum* in a submerged membrane bioreactor. Biotechnol Prog 22:1591–1597
114. Zampa A, Silvi S, Fabiani R, Morozzi G, Orpianesi C, Cresci A (2004) Effects of different digestible carbohydrates on bile acid metabolism and SCFA production by human gut micro-flora grown in an in vitro semi-continuous culture. Anaerobe 10:19–26
115. Martin R, Miquel S, Ulmer J, Kechaou N, Langella P, Bermúdez-Humaran LG (2013) Role of commensal and probiotic bacteria in human health: a focus on inflammatory bowel disease. Microb Cell Fact 12:71
116. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M (2013) Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol 24:160–168
117. Gibson GR, Wang X (2008) Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J Appl Bacteriol 77:412–420

118. Jeon SG, Kayama H, Ueda Y, Takahashi T, Asahara T, Tsuji H, Tsuji NM, Kiyono H, Ma JS, Kusu T, Okumura R, Hara H, Yoshida H, Yamamoto M, Momoto T (2012) Probiotic *Bifidobacterium breve* induces IL-10-producing Tr1 cells in the colon. PLOS Pathog 8(5), e1002714
119. Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG (2010) Effects of the probiotic *Bifodobacterium infantis* in the materials separation model of depression. Neuroscience 170:1179–1188
120. Kaplan H, Hutkins RW (2000) Fermentation of fructooligosaccharides by lactic acid bacteria and Bifidobacteria. Appl Environ Microbiol 66:2682–2684
121. Han R, Ebert EC, Zhao Z, Li L, Zhang H, Ian R (2005) Novel characteristics of *Bifidobacterium bifidum* in solid state fermentation system. World J Micrbiol Biotechnol 21:1245–1248
122. Yu Z, Dong B, Lu W (2009) Dynamics of bacterial community in solid state fermented feed revealed by 16S rRNA. Lett Appl Microbiol 49:166–172
123. Rodriguez de Olmos A, Bru E, Garro MS (2015) Optimization of fermentation parameters to study the behavior of selected lactic cultures on soy solid state fermentation. Int J Food Microbiol 196:16–23
124. Kouya T, Ishiyama Y, Tanaka T, Taniguchi M (2013) Evaluation of positive interaction for cell growth between *Bifidobacterium adolescentis* and *Propionibacterium freudenreichii* using a co-cultivation system with two microfiltration modules. J Biosci Bioeng 115:189–192
125. Oliveira RPS, Perego P, Oliveira MN, Converti A (2012) Growth, organic acids profile and sugar metabolism of *Bifidobacterium lactis* in co-culture with *Streptococcus thermophilus*: the inulin effect. Food Res Int 48:21–27
126. Hsu CA, Yu RC, Lee SL, Chou CC (2007) Cultural condition affecting the growth and production of β-galactosidase by *Bifidobacterium longum* CCRC 15708 in a jar fermenter. Int J Food Microbiol 116:186–189
127. Doleyres Y (2002) *Bifidobacterium longum* ATCC 15707 Cell Production during free and immobilized cell cultures in MRS-whey permeate medium. Appl Microbiol Biotechnol 60:168–173
128. Roy D (2001) Media for the isolation and enumeration of bifidobacteria in dairy products. Int J Food Microbiol 69:167–182
129. Amaretti A, Bernardi T, Tamburini E, Zanoni S, Lomma M, Matteuzzi D, Rossi M (2007) Kinetics and metabolism of *Bifidobacterium adolescentis* MB 239 growing on glucose, galactose, lactose and galactooligosaccharides. Appl Environ Microbiol 73:3637–3644
130. Mlobeli NT, Gutierrez NA, Maddox IS (1998) Physiology and kinetics of *Bifidobacterium bifidum* during growth on different sugars. Appl Microbiol Biotechnol 50:125–128
131. Parche S, Amon J, Jankovic I, Rezzonico E, Beleut M, Barutçu H, Schendel I, Eddy MP, Burkovski A, Arigoni F, Titgemeyer F (2007) Sugar transport system of *Bifidobacterium longum* NCC2705. J Mol Microbiol Biotechnol 12:9–19
132. Kim TB, Song SH, Kang SC, Oh DK (2003) Quantitative comparison of lactose and glucose utilization in *Bifidobacterium longum* cultures. Biotechnol Prog 19:672–675
133. Etoh S, Sonomoto K, Ishizaki A (1999) Complementary effects of bifidogenic growth stimulators and ammonium sulfate in natural rubber serum powder on *Bifidobacterium bifidum*. J Biosci Biotechnol Biochem 63:627–631
134. Mayo B, Aleksandrzak-Piekarczyk T, Fernández M, Kowalczyk M, Pablo Álvarez-Martín P, Bardowski J (2010) Updates in the metabolism of lactic acid bacteria. In: Mozzi F, Raya RR, Vignolo GM (eds) Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Iowa, pp 3–33
135. Fandi KG, Ghazali HM, YazidAM RAR (2001) Purification and N-terminal amino acid sequence of fructose-6-phosphate phosphoketolase from *Bifidobacterium longum* BB536. Lett Appl Microbiol 32:235–239

136. Pokusaeva K, Motherway MO, Zomer A, MacSharry J, Fitzgerald GF, Sinderen DV (2011) Cellodextrin utilization by *Bifidobacterium breve* UCC2003. Appl Environ Microbiol 77:1681–1690
137. Scardovi V (1986) Bifidobacterium. In: Sneath PH, Mair NS, Sharpe ME, Holt JG (eds) Bergey's Manual of Systematic Bacteriology, vol 2, 9th edn. Williams and Wilkins, Baltimore, p 1418
138. de Vries W, Stouthamer AH (1967) Pathway of glucose fermentation in relation to the taxonomy of *Bifidobacteria*. J Bacteriol 93:574–576
139. Cronin M, Ventura M, Fitzgerald GF, Sinderen DV (2011) Progress in genomics, metabolism, and biotechnology of bifidobacteria. Int J Food Microbiol 149:4–18
140. Gonzalez R, Blancas A, Santillana R, Azaola A, Wacher C (2004) Growth and final product formation by *Bifidobacterium infantis* in aerated fermentations. Appl Microbiol Biotechnol 65:606–610
141. Shene C, Mardones M, Zamora P, Bravo S (2005) Kinetics of *Bifidobacterium longum*ATCC 15707 fermentations: effect of the dilution rate and carbon source. Appl Microbiol Biotechnol 67:623–630
142. Biavati B, Vescovo M, Torriani S, Bottazzi V (2000) *Bifidobacteria*: history, ecology, physiology and applications. Annals Microbiol 50:117–132
143. Nguyen TMP, Lee YK, Zhou W (2012) Effect of high intensity ultrasound on carbohydrate metabolism of bifidobacteria in milk fermentation. J food Chem 130:866–874
144. Caescu CI, Vidal O, Krzewinski F, Artenie V, Bouquelet S (2004) *Bifidobacterium longum* requires a fructokinase (Frk; ATP:D-fructorse 6-phosphotransferase, EC 2.7.1.4) for fructose catabolism. J Bacteriol 186:6515–6525
145. Thitaram SN, Siragusa GR, Hinton A Jr (2005) *Bifidobacterium*-selective isolation and enumeration from chicken caeca by a modified oligosaccharide antibiotic-selective agar medium. Lett Appl Microbiol 41:355–360
146. Poch M, Bezkorovainy A (1988) Growth-enhancing supplements for various species of the genus *Bifidobacterium*. J Dairy Sci 71:3214–3221
147. Giridhar R, Srivastava AK (2000) Fed-batch sorbose fermentation using pulse and multiple feeding strategies for productivity improvement. Biotechnol Bioprocess Eng 5:340–344
148. Guerra NP, Agrasar AT, Macias CL, Bernardez PF, Castro LP (2007) Dynamic mathematical models to describe the growth and nisin production by *Lactococcuslactis* subsp. *lactis* CECT 539 in both batch and re-alkalized fed-batch cultures. J Food Eng 82:103–113
149. Parada JL, Caron CR, Medeiros ABP, Soccol CR (2007) Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. Braz Arch Biol Technol 50:521–542
150. Jung I, Oh MK, Cho YC, Kong IS (2011) The viability to a wall shear stress and propagation of *Bifidobacterium longum* in the intensive membrane bioreactor. Appl Microbiol Biotechnol 92:939–949
151. Song S-H, Kim T-B, Oh H-I, Oh D-K (2003) Optimization of *Bifidobacterium longum* growth by use of calcium carbonate-alginate beads. World J Microbiol Biotechnol 19:727–731
152. Jalili H, Razavi H, Safari M, Amrane A (2010) Kinetic analysis and effect of culture medium and coating materials during free and immobilized cell cultures of *Bifidobacterium animalis* subsp. *lactis* Bb 12. Electron J Biotechnol 13:(3)http://www.ejbiotechnology.info/content/vol13/issue3/full/4/(2010)
153. Jalili H, Balannec B, Razavi H, Amrane A (2011) Unstructured model for free and immobilized cell culture without pH control of *Bifidobacterium animalis* subsp. *lactis* Bb 12-inhibitory effect of the undissociated organic acids. Biochem Eng J 58–59:184–188
154. Kongo JM, Gomes AM, Malcata FX (2006) Manufacturing of fermented goat milk with a mixed starter culture of *Bifidobacterium animalis* and *Lactobacillus acidophilus* in a controlled bioreactor. Lett Appl Microbiol 42:595–599

155. Mathys S, Meile L, Lacroix C (2009) Co-cultivation of a bacteriocin-producing mixed culture of *Bifidobacterium thermophilum* RBL67 and *Pediococcus acidilactici* UVA1 isolated from baby faeces. J Appl Microbiol 107:36–46
156. Goderska K (2012) Different methods of probiotics stabilization. In: Rigobelo EC (ed) Probiotics. InTech, Rijeka. doi:10.5772/50313
157. Meng XC, Stanton C, Fitzgerald GF, Daly C, Ross RP (2008) Anhydrobiotics: the challenges of drying probiotic cultures. Food Chem 106:1406–1416
158. Tymczyszyn EE, Diaz R, Pataro A, Sandonato N, Gomez-Zavaglia A, Disalvo EA (2008) Critical water activity for the preservation of *Lactobacillus bulgaricus* by vacuum drying. Int J Food Microbiol 128:342–347
159. Forest P, Kulozik U, Schmitt M, Bauer S, Santivarangkna C (2012) Storage stability of vacuum-dried probiotic bacterium *Lactobacillus paracasei* F19. Food Bioprod Process 90:295–300
160. Bauer SAW, Schneider S, Behr J, Kulozik U, Foerst P (2012) Combined influence of fermentation and drying conditions on survival and metabolic activity of starter and probiotic cultures after low-temperature vacuum drying. J Biotechnol 159:351–357
161. Nag A, Das S (2013) Improving ambient temperature stability of probiotics with stress adaptation and fluidized bed drying. J Func Foods 5:170–177
162. To BCS, Etzel MR (1997) Spray drying, freeze drying, or freezing of three different lactic acid bacteria species. J Food Sci 62:576–585
163. Saarela M, Virkajärvi I, Alakomi H-L, Mattila-Sandholm T, Vaari A, Suomalainen T, Mättö J (2005) Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of *Bifodobacterium animalis* ssp. *lactis* cells produced without milk-based ingredients. J Appl Microbiol 99:1330–1339
164. Burns P, Vinderola G, Molinari F, Reinheimer J (2008) Suitability of whey and buttermilk for the growth and frozen storage of probiotic lactobacilli. Int J Dairy Technol 61:156–164
165. Celik OF, O'Sullivan JO (2013) Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria. J Dairy Sci 96:3506–3516
166. Yang C, Zhu X, Fan D, Mi Y, Luo Y, Hui J, Su R (2012) Optimizing the chemical composition of protective agents for freeze-drying *Bifidobacterium longum* BIOMA 5920. Chin J Chem Eng 20:930–936
167. Modesto M, Mattarelli P, Biavati B (2004) Resistance to freezing and freeze-drying storage processes of potential probiotic bifidobacteria. Ann Microbiol 54:43–48
168. Bruno FA, Shah NP (2003) Viability of two freeze-dried strains of *Bifidobacterium* and of commercial preparation at various temperatures during prolonged storage. J Food Sci 68:2336–2339
169. Teixeria P, Castro H, Mohacsi-Frakas C, Kirby R (1997) Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. J Appl Microbiol 83:219–226
170. Linders LJM, Kets EPW, de Bont JAM, van't Riet van K (1998) Combined influence of growth and drying conditions on the activity of dried *Lactobacillus plantarum*. Biotechnol Prog 14:537–539
171. Behdoudi-Jobbehdar S, Soukoulis C, Yonekura L, Fisk I (2013) Optimization of spray-drying process conditions for the production of maximally viable microencapsulated *L. acidophilus* NCIMB 701748. Drying Technol 31:1274–1283
172. Shokri Z, Fazeli MR, Ardjmand M, Mousavi SM, Gilani K (2015) Factors affecting viability of *Bifidobacterium bifidum* during spray drying. Daru 23:7
173. Corcoran BM, Ross RP, Fitzgerald GF, Stanton C (2004) Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. J Appl Microbiol 96:1024–1039
174. Prasad J, McJarrow P, Gopal P (2003) Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. Appl Environ Microbiol 69:917–925

175. Santivarangkna C, Kulozika U, Poerst P (2007) Alternative drying processes for the industrial preservation of lactic acid starter cultures. Biotechnol Prog 23:302–315
176. Shamekhi F, Shuhaimi M, Ariff A, Manap YA (2013) Cell viability of microencapsulated *Bifidobacterium animalis* subsp. *lactis* under freeze-drying, storage and gastrointestinal tract simulation conditions. Folia Microbiol 58:91–101
177. Dianawati D, Shah NP (2011) Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 during storage at room temperature. J Food Sci 76:M592–M599
178. Ding WK, Shah NP (2007) Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. J Food Sci 72:M446–M450
179. Weinbreck F, Bodnár I, Marco ML (2010) Can encapsulation lengthen the shelf-life of probiotic bacteria in dry products? Int J Food Microbiol 136:364–367
180. Pop OL, Brandau T, Vodnar DC, Socaciu C (2012) Study of *Bifidobacterium lactis* 300b survival during encapsulation, coating and freeze-drying process and the release in alkaline media. Bull Univ Agric Sci Vet Med 69:372–379
181. Zhang F, Li XY, Park HJ, Zhao M (2013) Effect of microencapsulation methods on the survival of freeze-dried *Bifidobacterium bifidum*. J Microencapsul 30:511–518
182. Lian WC, Hsiao HC, Chou CC (2002) Survival of bifidobacteria after spray drying. Int J Food Biotechnol 74:79–86
183. Dianawati D, Shah NP (2011) Enzyme stability of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze-drying and during storage in low water activity at room temperature. J Food Sci 76:M463–M471
184. Dianawati D, Mishra V, Shah NP (2012) Role of calcium alginate and mannitol in protecting *Bifidobacterium*. Appl Env Microbiol 78:6914–6921
185. Fávaro-Trindade CS, Grosso CR (2002) Microencapsulation of *Lactobacillus acidophilus* and *Bifobacterium lactis* and evaluation of their survival at pH values of the stomach and in bile. J Microencapsul 19:485–494
186. Saarela M, Rantala M, Hallamaa K, Nohynek L, Virkahärvi I, Mättö J (2004) Stationary phase acid and heat treatments for improvement of the viability of probiotic lactobacilli and bifidobacteria. J Appl Microbiol 96:1205–1214
187. Savijoki K, Suokko A, Palva A, Valmu L, Kalkkinen N, Varmanen P (2005) Effect of heat-shock and bile salts on protein synthesis of *Bifodobacterium longum* revealed by (35S) methionine labelling and two dimensional gel electrophoresis. FEMS Microbiol Lett 248:207–215
188. Ventura M, Canchaya C, Zhang Z, Fitzgerald GF, van Sinderen D (2007) Molecular characterization of *hsp*20, encoding a small heat shock protein of *Bifidobacterium breve* UCC2003. Appl Environ Microbiol 73:4695–4703
189. Ruiz L, Ruas-Madiedo P, Gueimonde M, de los Reyes-Gavilán CG, Margolles A, Sánchez B (2011) How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. Genes Nutr 6:307–318
190. Nguyen HT, Razafindralambo H, Blecker C, N'Yapo C, Thonart P, Delvigne F (2014) Stochastic exposure to sub-lethal high temperature enhances exopolysaccharides (EPS) excretion and improves *Bifidobacterium bifidum* cell survival to freeze-drying. Biochem Eng J 88:85–94

Adv Biochem Eng Biotechnol (2016) 156: 433–464
DOI: 10.1007/10_2016_6

Published online: 19 July 2016

Anaerobes as Sources of Bioactive Compounds and Health Promoting Tools

Gashaw Mamo

Abstract Aerobic microorganisms have been sources of medicinal agents for several decades and an impressive variety of drugs have been isolated from their cultures, studied and formulated to treat or prevent diseases. On the other hand, anaerobes, which are believed to be the oldest life forms on earth and evolved remarkably diverse physiological functions, have largely been neglected as sources of bioactive compounds. However, results obtained from the limited research done so far show that anaerobes are capable of producing a range of interesting bioactive compounds that can promote human health. In fact, some of these bioactive compounds are found to be novel in their structure and/or mode of action.

Anaerobes play health-promoting roles through their bioactive products as well as application of whole cells. The bioactive compounds produced by these microorganisms include antimicrobial agents and substances such as immunomodulators and vitamins. Bacteriocins produced by anaerobes have been in use as preservatives for about 40 years. Because these substances are effective at low concentrations, encounter relatively less resistance from bacteria and are safe to use, there is a growing interest in these antimicrobial agents. Moreover, several antibiotics have been reported from the cultures of anaerobes. Closthioamide and andrimid produced by *Clostridium cellulolyticum* and *Pantoea agglomerans*, respectively, are examples of novel antibiotics of anaerobe origin. The discovery of such novel bioactive compounds is expected to encourage further studies which can potentially lead to tapping of the antibiotic production potential of this fascinating group of microorganisms.

Anaerobes are widely used in preparation of fermented foods and beverages. During the fermentation processes, these organisms produce a number of bioactive compounds including anticancer, antihypertensive and antioxidant substances. The

G. Mamo (✉)
Biotechnology, Center for Chemistry & Chemical Engineering, Lund University, 221 00 Lund, Sweden
e-mail: gashaw.mamo@biotek.lu.se; gashaw.mamo1@gmail.com

well-known health promoting effect of fermented food is mostly due to these bioactive compounds. In addition to their products, whole cell anaerobes have very interesting applications for enhancing the quality of life. Probiotic anaerobes have been on the market for many years and are receiving growing acceptance as health promoters. Gut anaerobes have been used to treat patients suffering from severe *Clostridium difficile* infection syndromes including diarrhoea and colitis which cannot be treated by other means. Whole cell anaerobes are also studied to detect and cure cancer. In recent years, evidence is emerging that anaerobes constituting the microbiome are linked to our overall health. A dysfunctional microbiome is believed to be the cause of many diseases including cancer, allergy, infection, obesity, diabetes and several other disorders. Maintaining normal microflora is believed to alleviate some of these serious health problems. Indeed, the use of probiotics and prebiotics which favourably change the number and composition of the gut microflora is known to render a health promoting effect. Our interaction with the microbiome anaerobes is complex. In fact, not only our lives but also our identities are more closely linked to the anaerobic microbial world than we may possibly imagine. We are just at the beginning of unravelling the secret of association between the microbiome and human body, and a clear understanding of the association may bring a paradigm shift in the way we diagnose and treat diseases and disorders. This chapter highlights some of the work done on bioactive compounds and whole cell applications of the anaerobes that foster human health and improve the quality of life.

Keywords Active peptides, Anaerobe, Antibiotic, Anticancer, Bacteriocin, Bacteriotherapy, Microbiome

Contents

Abbreviations

APCs	Antigen-presenting cells
CLA	Conjugated linoleic acid

CNS	Central nervous system
COGs	Clustered Orthologous Groups
DHT	Dihydrotestosterone
EDTA	Ethylenediaminetetraacetic acid
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
HSAF	Heat stable antifungal factor
LA	Linoleic acid
MDR-TB	Multi-drug-resistant tuberculosis
MIC	Minimum inhibitory concentrations
MRSA	Methicillin-resistant *Staphylococcus aureus*
NRPS	Non-ribosomal peptide synthetase
PCR	Polymerase chain reaction
PKS	Polyketide synthase MIC
PSA	Polysaccharide A
SIRS	Systemic inflammatory response syndrome
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

1 Introduction

Man has made great strides in treating different kinds of diseases, and today diseases that were once considered lethal are no longer life threatening. However, life style changes, environmental pollution, emergence of new viral and microbial pathogens, appearance of drug resistance among known infectious organisms, lack of treatment for some old diseases, etc. are still affecting human health. For instance, a sizable proportion of the current generation has become more sedentary than their parents and grandparents, spending more time in environments with limited physical activity and often with extended sitting not only during working but also when travelling in cars or trains. Studies have revealed that sedentary life is becoming a major risk factor for obesity, cancer, diabetes, depression, cardiovascular diseases, etc. [1, 2]. Another example in this line could be the emerging drug resistance among pathogens. Microbes are wily and learn quickly how to survive in the presence of antibiotics. Mismanagement of drugs such as widespread use of antibiotics and related non-compliance, improper medication, and over-prescription has contributed to the emergence of drug resistance among pathogens [3] and today antimicrobial resistance is a growing global concern. Drug resistance is not only emerging frequently but is also expanding at an alarming rate. For instance, from 1987 to 2004 the percentage of penicillin resistance among *S. pneumoniae* infections that causes meningitis and pneumonia grew from 0.02% to 20%, a staggering 1000-fold increase [4]. In recent years, about 440,000 new cases of multidrug-resistant tuberculosis (MDR-TB) appear annually, causing at

least 150,000 deaths [5], and extensive drug-resistant tuberculosis (XDR-TB) has been reported in 64 countries [6]. The emergence and spread of drug resistance among pathogens has not only resulted in an increasing number of human casualties but also incurs an enormous economic loss. In the USA alone, drug resistant bacterial infection has brought an annual loss of 34 billion US dollars [7]. Health challenges emanating from anthropogenic activities or from different natural causes should be treated or prevented to avoid both human life and economic losses. Thus, man has been engaged in a continuous fight against disease. This fight has attracted a great deal of attention in every generation, evolving over time from simple traditional to highly complex scientific treatments.

It is a widely accepted notion that man's fight against disease should be multimodal in nature, ranging from change in life style to implementing advanced medical technologies. However, in the fight against disease, the use of drugs is very vital and it is one of the most important components that cannot be compromised. The success of drug discovery often depends on the availability of new lead and precursor bioactive compounds, substances that exhibit biological activity. In fact, the search for these substances is at the core of modern day drug research. The rate of drug discovery from traditional producers such as actinomycetes, hyphomycetes and plants which have been in focus of pharmaceutical research for decades is declining [8, 9]. The low rate of discovery has become a great concern, especially in treatment of infections where the existing drugs become inefficient [10, 11] and the pipeline for new antibiotics is running disappointingly low [8, 12, 13]. Thus, it is important to have strategies to ensure availability of new and effective drugs. One among the different possible strategies could be searching for new drug producers from previously unexplored or less explored groups of organisms [14].

Microorganisms have been among the most important sources of bioactive compounds ranging from antibiotics to anticancer drugs [15–17]. Aerobic soil microorganisms account for nearly all the available drugs of microbial origin. On the other hand, anaerobes, which are a dominant group of organisms thriving in different habitats, have received very little attention as sources of bioactive compounds. These microorganisms have remarkably diverse metabolic features, allowing them to produce a wide range of biochemicals [18, 19], an ability that has attracted researchers and industries to produce chemicals from renewable resources. However, the potential of anaerobes for the production of bioactive compounds has not received proper attention. If anaerobes were explored properly, we may probably be able to see new drugs on pharmacy shelves. Besides being potential sources of drugs, whole cells of anaerobes are also believed to play health-promoting roles. The human body harbours a highly diverse group of anaerobes and shares a bond with these organisms. Understanding this complex interaction and co-existence of the microflora and the body undoubtedly helps in promoting human health. In this respect, an exemplary achievement worth mentioning is the use of supplementary diets containing anaerobic microorganisms (i.e. probiotics) to improve human health.

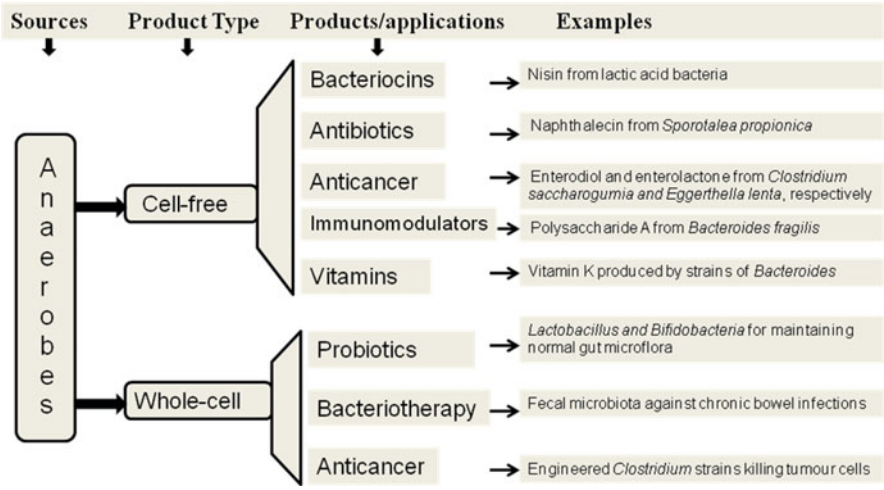


Fig. 1 Cell-free and whole-cell health promoting applications of anaerobes with some examples

Anaerobes have great potential in promoting human health and in this chapter some of the relevant research findings on production of bioactive compounds and whole cell applications (Fig. 1) that support this health-promoting role are discussed. Among whole cell applications the chapter focuses on bacteriotherapy of infection and cancer. Moreover, it deals with human microbiome and its potential role in the future medicine.

2 Bioactive Compounds of Anaerobes

Bioactive compounds, substances that exhibit biological activities by exerting an effect on or triggering a response in organisms, are highly diverse in their structure and chemical nature. These compounds could be natural or synthetic in nature. However, in this chapter, the term bioactive compound refers only to substances produced by biological entities and which have activities with a potential effect of treating or preventing disease and promoting good health.

Synthetic chemistry has been used to produce efficient drugs; however, bioactive compounds of natural origin with their remarkable diversity in chemical structure remain to be the most important sources of drugs. Bioactive compounds of biological origin can be used directly (without modification) or serve as lead compounds to synthesize more potent drugs chemically. Over 50% of the drugs being used currently are of biological origin or are synthesized using biologically active substances as lead compounds [20, 21]. More than 60% of anticancer drugs are of biological origin [22].

The global demand for drugs and health-promoting tools is already high and is even expected to increase further because of the emergence of new diseases, declining effectiveness of the existing drugs, absence of cure for many diseases,

and the upsurge of human population. Microorganisms have been the major source of bioactive compounds of pharmaceutical importance. Unfortunately, drug screening and development research have been focused only on limited groups of organisms. However, in recent years, it seems that the search for bioactive compounds is expanding to organisms that have never been in focus before.

Anaerobes are believed to be the first organisms to inhabit our planet; they are highly diverse and known to possess unique metabolic features. Although their metabolic diversity and ability to synthesize different chemicals are interesting features to prospect for bioactive compounds, only limited studies have been made so far. This could be partly because of the difficulty of cultivating anaerobes. However, the advancement of molecular biology, the development of new and efficient bioactive compound screening systems, and the accuracy and sophistication of analytical tools have minimized some of the difficulties associated with screening of anaerobes for bioactive compounds, and hopefully this can lead to further screening and concomitant discovery of novel bioactive compounds in the time ahead. A highlight of the research done on anaerobes as sources of bioactive compounds is given below.

2.1 Antimicrobial Agents

Some organisms are known for producing protein based bioactive compounds. Among these, bacteriocins and peptides are becoming increasingly important in promoting human health. Bacteriocins and peptides are made of unique sequences of amino acids and in the literature both are often referred to as peptides. However, these substances differ in some aspects as shown in Table 1 and are treated in this chapter as distinct groups. Peptides can be modified or unmodified and in this chapter it is the unmodified (except for simple cyclization) peptide that is referred to as peptide. The non-ribosomally synthesized and modified antimicrobial peptides belong to antibiotics.

Table 1 Differences between bacteriocins and active peptides

Features	Bacteriocins	Peptides
Synthesis	Ribosomal	Non-ribosomal. If ribosomal, they are released from polypeptide chains by proteolytic degradation
Gene	Encoded by specific genes	Not directly encoded by specific genes
Number of amino acids	Often 30–60	Often 3–20
Function	Antimicrobial	Antimicrobial, antihypertensive, cyto- and immune-modulation, antioxidative, etc.
Mode of action	Depolarizes cell membranes or inhibits cell wall synthesis	Chelates metal ions or binds to bacterial surfaces, interacts with appropriate receptors, exhibiting hormone-like activity
Producers	Bacteria and few fungi	Prokaryotes and Eukaryotes

Bacteriocins

The dramatic increase in the frequency of drug resistance among pathogens, the desire to reduce the use of chemical preservatives and conventional antimicrobial agents are rolling the search wheels for alternative antimicrobial substances that can be used for clinical applications as well as preservation of food and non-food products. Bacteriocins are among the promising alternative antimicrobial agents produced by all major lineages of bacteria [23]. In addition to bacteria, small cyclic bacteriocins, amatoxins (which inhibit RNA polymerase II) and phallocidins (which stabilize the F-actin) have been reported from fungi [24, 25]. A variety of bacteriocins has been reported from anaerobes isolated from ruminal, faecal, food, environmental, etc. samples [26–38]. Among the large number of bacteriocins reported, the most studied are those from lactic acid bacteria. This is mainly because of the GRAS (generally recognized as safe) status of lactic acid bacteria which makes it ideal for several applications including food preservation.

Characterization studies revealed that bacteriocins exhibit a variable spectrum, mode of action, molecular mass, amino acid sequence, amino acid modification, and biochemical properties. Some of these properties, such as their chemical structure, molecular size and mode of action, are used to group bacteriocins into classes. However, depending on the parameters (properties) considered, different classification schemes have been proposed. Some have categorized bacteriocins into two classes [39] although others categorize them in three [40] or four classes [41, 42]. Some examples of bacteriocins belonging to different classes, and properties that characterize each class are given in Table 2.

Bacteriocins exhibit interesting antimicrobial properties, which make them ideal alternatives to antibiotics for certain applications. For instance, bacteriocins exist in fermented food products we consume and can even be produced in our guts by bacteria inhabiting the gastrointestinal tract, which makes them safe to use. These molecules exhibit very low toxicity and, being produced by GRAS bacteria, bacteriocins can be readily used in foods without even prior purification [43]. The other desirable attribute from the point of view of their application is that most of them are effective at very low concentration.

Relatively few bacteriocins are found to be active against a wide spectrum of microorganisms [43]. The great majority exhibit a narrow spectrum of activity, often killing only closely related species of the producer strain. Such a narrow spectrum of activity could be of interest to target only a certain group of pathogenic bacteria. For instance, some microbes living in the human body (e.g. lactic acid bacteria) are known to have a beneficial role. Lately, it has become clear that the use of broad-spectrum antimicrobial agents that kill such beneficial microbes results in "collateral damage" which has been exemplified by the growing incidence of atopic and autoimmune diseases [44, 45]. It may be worth mentioning that most bacteriocins effectively inhibit Gram-positive pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* [46]. However, only few bacteriocins are active against Gram-negative pathogens. On the other hand, in vitro studies have shown

Table 2 Examples of bacteriocins of anaerobes belonging to different classes

Class	Properties	Examples	Producer anaerobe
Class I	Post-translationally modified peptides containing lanthionine or methyl-lanthionine	Nisin	*Lactococcus lactis*
		Lacticin 3147	*Lactococcus lactis*
		Butyrivibriocin AR10	*Butyrivibrio fibrisolvens* AR10
Class II	Small thermostable, non-modified proteins (with the exception of disulfide bridge linkages), non-lanthionine containing membrane active peptide	Pediocin PA-1	*Pediococcus acidilactici* PAC1.0
		Sakacin A	*Lactobacillus sakei*
		Lactacin F	*Lactobacillus acidophilus*
		Lactococcin G	*Lactococcus lactis* LMGT-2081
Class III	Heat labile, cell-wall-degrading peptide	Enterolysin	*Enterococcus faecium*
		Helveticin I	*Lactobacillus helveticus*
Class IV	Cyclic globular, thermostable, helical, and post-translationally modified proteins, ranging between 35 and 70 amino acids	Enterocin F4-9	*Enterococcus* sp.
		Glycocin F	*Lactobacillus plantarum*

that bacteriocins that are not naturally active against Gram-negative bacteria can effectively kill them if used together with membrane destabilizing substances such as EDTA and detergents [47]. Thus, at least potentially, it may be possible to extend the spectrum of bacteriocin activity with the help of additives.

One of the remarkable features of bacteriocins is their activity against clinically important pathogens, including drug resistant strains [48, 49]. Moreover, resistance of pathogens to bacteriocins is relatively low, which may be partly because of their mode of action which is different from the common therapeutic drugs. Interestingly, because bacteriocins are proteinaceous, unlike other drugs, it is possible to engineer and fine-tune their activities or properties by manipulating the gene sequence encoding them. It has been demonstrated that it is possible to improve the efficiency, stability and specificity of bacteriocins through genetic engineering [42, 50–53]. The molecular biology techniques allow not only fine-tuning of the properties of bacteriocins but also help to produce the bacteriocin at higher titres in heterologous over-expression systems and this can possibly downsize the production cost.

Although there have been a wide variety of bacteriocins reported from aerobes and anaerobes with interesting properties for application in food, cosmetic and

drink preservation, to date only two bacteriocins of anaerobic origin – nisin and pediocin PA-1 – are available on the market. Nisin has been marketed by Danisco as Nisaplin since the early 1980s and it is the only bacteriocin approved by WHO as food preservative [46]. Kerry Bioscience markets Pediocin PA-1.

In recent years, in addition to its preservative application, bacteriocins have also been considered as alternative antibiotics to treat infections [39, 54, 55]. However, the primary focus so far has been on the treatment of animals but not humans. In this regard, the use of thiostrepton in ointments to treat dermatological problems of domestic animals and the use of nisin to prevent mastitis can be mentioned as existing commercial examples of bacteriocins being used as antibiotic alternatives [39]

Peptides

A large number of bioactive peptides have been reported from eukaryotic organisms. The majority of these peptides regulate a range of physiological functions through their hormone-like activity [56]. However, some peptides produced by eukaryotes are for defence against microbial and viral infections and insect predation [24, 57–60]. These peptides are released from larger precursor proteins and are often composed of 3–20 amino acids [61]. For instance, enzymatic hydrolysis of milk protein has been shown to release antimicrobial peptides. Peptides released from β-casein by the protease of *Lactobacillus helveticus* have shown a broad spectrum antimicrobial activity against a range of pathogens [62]. Similarly, peptides obtained from hydrolysis of yoghurt α-casein have shown potent antimicrobial activity [63, 64].

Microorganisms not only modify proteins in food and release active peptides, but are also able to synthesize antimicrobial peptides non-ribosomally. Most of these peptides contain non-proteinaceous components such as lipid or carbohydrate. The majority of these modified compounds are lipopeptides, i.e. peptides with lipid components, and belong to a group of surface active agents often referred to as biosurfactants, which in addition contains glycolipids, phospholipids and lipopolysaccharides. These peptides are known in the literature as antibiotic peptides and are discussed below.

Antibiotics

Antibiotics are relatively low molecular weight secondary metabolites synthesized by complex metabolic pathways of microorganisms. Often aerobic bacteria belonging to genus *Bacillus* and *Streptomyces* are encountered producing these compounds. Different fungal strains such as those belonging to *Penicillium* and *Cephalosporium* are also known as antibiotic producers. It is interesting that most of the antibiotics that have been used for decades are peptide driven [65]. Often these peptides contain more than one amino acid moiety synthesized by multienzyme complexes rather than through the conventional ribosome mediated

process. The well-known antibiotics such as penicillin, vancomycin, cephalosporin, etc. and their derivatives are peptide-driven antibiotics. Penicillin contains L-cysteine, D-valine and monosubstituted acetic acid; cephalosporin C comprises L-cysteine, D-α-aminoadipic acid, α,β-dehydrovaline and acetic acid, and vancomycin and teicoplanin are glycopeptides which have sugar-substituted peptide backbones.

In addition to the classical small sized antibiotics, microorganisms also produce an array of complex antibiotics. Some of these non-ribosomally synthesized antibiotics are cyclic in structure [66, 67]. One group of these cyclic peptides are known as cyclic lipopeptides, which exhibit remarkable antibiotic properties. Lipopeptides are amphiphilic in nature, containing a fatty acid tail linked to a short cyclized oligopeptide of 7–10 amino acids, which cyclizes by linking a lactone ring to a β-hydroxy fatty acid. These compounds are diverse and have been classified into various types based on their amino acid number and composition, fatty acid chain length, and structure [68, 69]. Lipopeptides such as daptomycin, micafungin, caspofungin and anidulafungin have already reached the status of commercial antibiotic [68, 70].

Although the great majority of antimicrobial peptides have been reported from aerobic microorganisms, studies have also shown that similar peptides can be produced by anaerobes. Lipopeptides have been reported from human intestinal flora strains of *Citrobacter* and *Enterobacter* [71]. A lipopeptide has been purified from the culture of a strictly anaerobic bacterium, *Anaerophaga thermohalophila*, isolated from environmental samples (oil separation tank) [72]. *Lysobacter* spp. are known to produce a number of bioactive compounds such as tripropeptin [73] and heat stable antifungal factor (HSAF) [74]. The tripropeptins (Fig. 2a) are a group of structurally related cyclic lipodepsipeptides which are active against Gram-positive bacteria including MRSA and, to a lesser extent, VRE [73, 75]. Out of the eight amino acids that compose tripropeptins, five of them are non-proteinogenic and are believed to be synthesized by non-ribosomal peptide synthetase (NRPS) [75, 76]. Strains of *Lysobacter* are also known to produce another group of antimicrobial compounds known as WAP-8294A [77]. These compounds are active against a range of Gram-positive organisms and the most potent one is WAP-8294A2 (Fig. 2b), which shows a great similarity in structure, pharmacological and toxicological properties to one of the most successful lipopeptides, daptomycin [75].

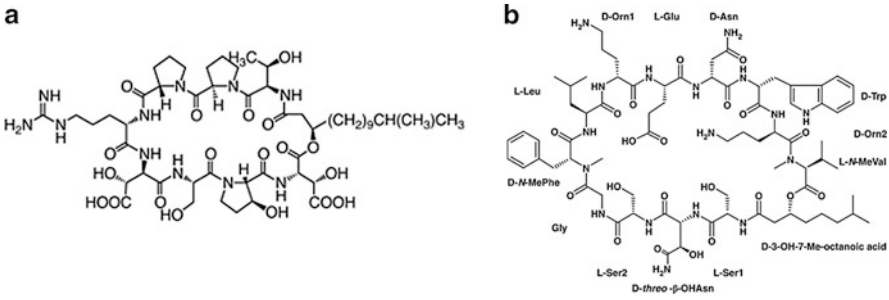


Fig. 2 (**a**) Antimicrobial agent tripropeptin C. (**b**) WAP-8294A2 (**1**)

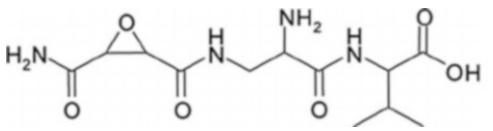


Fig. 3 Structure of herbicolin I [2-amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine]

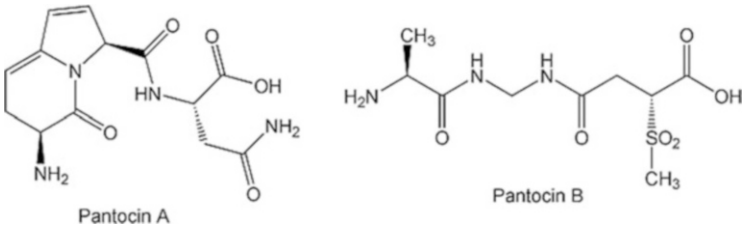


Fig. 4 Chemical structures of pantocin A and B

The closely related anaerobes *Pantoea agglomerans* and *P. vagans* are known for their interesting antibiotics. These bacteria produce herbicolin I (Fig. 3) which belongs to the dapdiamide family of peptides and has shown activity against *Erwinia amylovora*, *Pseudomonas* spp., *Serratia marcescens* and *Candida albicans* [78]. In addition to herbicolin I, *P. agglomerans* produces pantocin A and B (Fig. 4) [79, 80], relatively small molecules that are active against Gram-negative bacteria by inhibiting the histidine [81, 82] and arginine [79] biosynthesis, respectively. Strains of *P. agglomerans* are also known to produce the pseudo peptide antibiotic andrimid [83] (Fig. 5a), a new class of antibiotics with a novel mode of action, i.e., preventing the initiation of prokaryotic fatty acid biosynthesis by inhibiting the bacterial acetyl-CoA carboxylase [84]. This unique mode of action allows it to act against Gram-negative and Gram-positive pathogens including the drug resistant MRSA, VRE and *Klebsiella pneumoniae* at remarkably low minimum inhibitory concentrations (MICs) (<1 μg/mL) [85]. A glycolipid biosurfactant, rhamnolipid, with antimicrobial activity has also been reported from *Pantoea* sp. A-13 isolated from a sample collected in Antarctica [86].

The entomopathogenic bacteria *Xenorhabdus* produce different antimicrobial agents [87]. Xenocoumacin 1 exhibits potent antimicrobial activity not only against several Gram-positive and Gram-negative bacteria but also against the pathogenic fungal strain *Cryptococcus neoformans* [88, 89]. Furthermore, two antimicrobial agents, xenematide (Fig. 5b) and nematophin (Fig. 5c), have been isolated from the culture of *Xenorhabdus*. Xenematide exhibits a broad spectrum of activity against Gram-positive and Gram-negative bacteria, whereas nematophin is a narrow spectrum antibiotic which is more potent against *Staphylococcus aureus* [88, 90].

It is interesting that at least some of these anaerobes reported to produce antibiotics are themselves pathogens or live in association with another organism. For example, *P. agglomerans* is a plant pathogen and *Xenorhabdus* infects nematodes and insects. *Lysobacter capsici*, which inhibits the growth of *Fusarium*

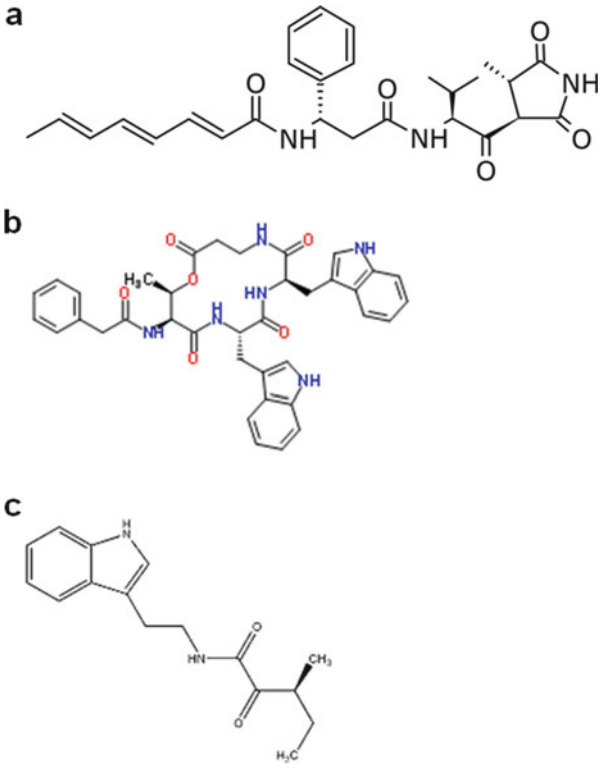


Fig. 5 Antibiotics: (**a**) andrimid; (**b**) xenematide; (**c**) nematophin

oxysporum, *Botrytis cinerea*, *Pythium ultimum*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides* and *Botryosphaeria dothidea*, is a plant-associated microbe [91]. This may indicate that anaerobes which are pathogenic or those living within other organisms such as plants or animals can be good sources of antibiotics and should not be excluded from drug prospecting screening studies. Moreover, based on what is summarized in the preceding paragraphs, it seems that members of the genus *Pantoea*, *Lysobacter* and *Xenorhabdus*, are endowed with the ability to produce very interesting antimicrobial agents and hence further studies on them may be rewarding.

Among the anaerobes, it is the facultative anaerobes that have been studied as sources of antimicrobial agents, which might be because of the relative ease of cultivating them in contrast to the obligate ones. Although there are not many reports on antibiotics produced by strictly anaerobic bacteria, few interesting compounds have been reported. A good example could be closthioamide (Fig. 6a), produced by the strictly anaerobic microbe *Clostridium cellulolyticum*. Closthioamide is symmetric and very rich in thioamide moieties, which are rare in nature. This metabolite exhibits strong activity against Gram-positive pathogens, including the drug resistant strains MRSA and VRE at nanomolar concentration [92, 93]. Given that closthioamide represents an unprecedented antibiotic with

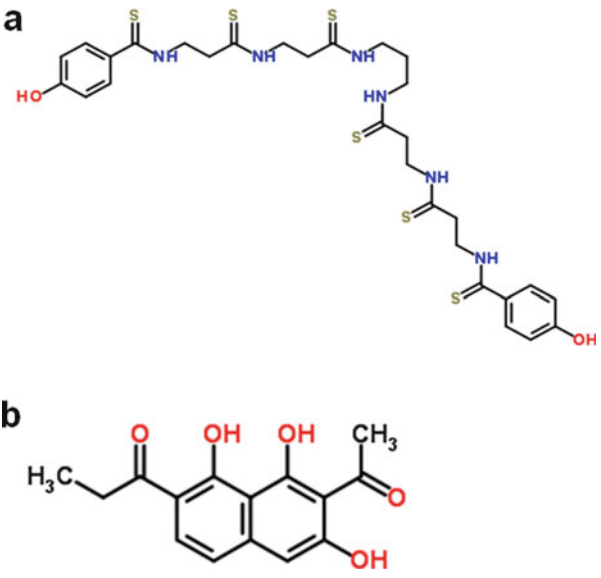


Fig. 6 Chemical structures of antibiotics from obligate anaerobes: (**a**) closthioamide; (**b**) naphthalecin

regard to its structure, composition and source of isolation, one expects a novel mode of action, which has yet to be proven. A novel, low-molecular-weight antibiotic dubbed naphthalecin (Fig. 6b) is also reported from a strictly anaerobic termite gut bacterium *Sporotalea propionica* [94]. Naphthalecin is a narrow spectrum antibiotic active against Gram-positive bacteria. Previously, a low molecular weight antibiotic reutericyclin was reported from a lactic acid bacteria (facultative anaerobe) *Lactobacillus reuteri* [95]. A closely related strain, *L. rhamnosus*, has also been reported to produce a bioactive compound that inhibits the growth of clinically important strains of *Staphylococcus aureus*, *Klebsiella pneumonia*, *Burkholderia cepacia* and *Escherichia coli* [96].

Almost all of the aforementioned antimicrobial agents from anaerobes are obtained from functional screening studies. In addition, molecular techniques have also started to contribute towards expanding the exploration of anaerobes. For instance, genomic studies have revealed the metabolic potential of anaerobes in making different bioactive compounds [97–99]. Such genome analysis of *Clostridium* strains confirmed the presence of many polyketide synthase (PKS) and/or non-ribosomal peptide synthetase (NRPS) gene clusters which mediate the synthesis of polyketides and non-ribosomal peptides, respectively [98, 100]. Non-ribosomal peptides are among large families of natural products with diverse structures and functions including antimicrobial activity [101]. The finding of PKS and NRPS gene clusters in different groups of microorganisms [98] reveals the unnoticed antibiotics production potential of anaerobes and possibly heralds the coming of a new era of anaerobes in drug discovery. However, the mere presence of genes encoding PKS and NRPS does not lead to easy and direct antibiotic production.

Production of secondary metabolites by anaerobic microorganisms is challenging and, at the level of screening, it is not easy to optimize production conditions. Thus, it is easier to screen first with PCR for genes that encode PKS and NRPS or search in genome sequence for these genes. Once the presence of genes encoding PKS and/or NRPS is confirmed, a thorough optimization should be carried out to produce and characterize the active metabolite. This strategy may lead to the discovery of novel antimicrobial agents from anaerobes. In fact, the discovery of closthioamide may somehow reflect the merit of this approach. The secondary metabolite biosynthetic gene clusters of *Clostridium* strains remain silent under standard cultivation conditions [100], which suggest that certain specific conditions are required to be activated [102]. Thus, the presence of PKS and NRPS in members of genus *Clostridium* cannot easily lead to production of the active substance but gives a green light for optimization. For example, *C. cellulolyticum* has been subjected to over 30 stress conditions such as antibiotic, heat shock and heavy metal stress, but only in one condition, upon addition of an aqueous soil extract to the culture was the production of the novel compound closthioamide initiated [93].

Several anaerobes are known to produce surface-active agents, although the compounds are not well characterized and it is difficult to know to which antimicrobial agent category the compounds belong. An example of this could be the surface-active agent produced by *Clostridium pasteurianum* [103]. Isolation and further characterization of such kinds of unidentified bioactive compounds of anaerobic origin may contribute to discovery of novel bioactive compounds. Considering that most of the interesting antibiotic discoveries from anaerobes happened in the last decade, it seems that the prospecting of antibiotics from anaerobes is just beginning. Because of their impressive phylogenetic and physiological diversity, and novelty of some of the discovered compounds, it would not be surprising if several new antibiotics are revealed and at least some of them find their way onto the market in the near future.

2.2 Immunomodulators

In addition to what has been described above, a number of studies have elucidated the contribution of anaerobes to our wellbeing through production of other bioactive compounds. Among these compounds are immunomodulators, substances that modulate or regulate the immune system. Anaerobes inhabiting the human gut are known for their impressive role in enhancing human health. In addition to protecting our bodies against pathogens, these anaerobes are also believed to play an immune boosting role. Studies have revealed that the gut microflora regulate the density and population of intestinal immune cells by influencing the development of the gut lymphoid tissues that mediate a variety of immune functions [104]. In line with this, immunity improvement upon consumption of probiotic bacteria has been reported [105, 106]. As most of the probiotic bacteria are members of the gut bacterial commensals, it strengthens the notion that the gut microbiota influences the immune system. Indeed, this has been supported experimentally by analysing

the fecal flora of patients suffering from severe systemic inflammatory response syndrome (SIRS), which were found to have extremely low counts of *Bifidobacterium* and *Lactobacillus* [107].

One of the important bacteria which plays an important role in human immune system is *Bacteroides fragilis*. This human commensal anaerobe produces a capsular exopolysaccharide known as polysaccharide A (PSA) which is capable of activating T cell-dependent immune responses and hence influences the host immune system development and homeostasis [108, 109]. Ochoa-Reparaz and co-workers [110, 111] have reported that *Bacteroides fragilis* cells or its PSA protected mice models (of autoimmune encephalomyelitis) against disease of the central nervous system (CNS) in which the neuron myelin sheath is damaged. Moreover, recent studies show that PSA is capable of activating intestinal sensory neurons [112]. This may indicate the strong correlation between the bacterial effects on the nervous system and on the immune system that seems to be in bidirectional communication.

The gut bacteria produce ligands such as peptidoglycans, lipopolysaccharides, lipopeptides and lipotechnoic acids, which bind to receptor proteins and activate the inflammatory signalling pathways [113]. The signal initiates the antigen-presenting cells (APCs) known as dendritic cells to induce the primary immune response to defend the body. Some of these anaerobes produce other bioactive compounds with recognized health effects [114, 115]. Conjugated linoleic acid (CLA) is among these substances that are important to the immune system. CLA refers to isomers of linoleic acid found predominantly in meat and milk of ruminants and often related to several health benefits [116]. CLA has demonstrated efficacy as an anti-inflammatory and an immune modulator substance in mouse and pig models of colitis [117]. Strains of the anaerobic bacteria *Clostridium*, *Propionibacterium*, *Butyrivibrio*, *Bifidobacterium* and *Lactobacillus* are able to convert linoleic acid (LA) into CLA isomers [118, 119] and hence these anaerobes positively contribute to the host immune system, especially when CLA rich food is not in the diet. *Lactobacillus* strains are most commonly used for production of dietary CLA [120].

2.3 *Compounds Active Against Cancer, Hypertensive and Cardiovascular Diseases*

Gut microflora has been known to release bioactive compounds from the food we consume. There are a number of peptides that can be of great interest in the treatment of different diseases. Enzymatic hydrolysis of proteins found in foods releases bioactive peptides containing two to nine amino acids [121], which have positive effect on the digestive, cardiovascular, immune and nervous systems of humans. Anaerobic microorganisms have been used in the production of different kinds of fermented foods, and during the fermentation process they modify the food protein with the help of their enzymes and release active peptides which have a favourable impact on human physiology and health. It has been known that consumption of

fermented foods containing active peptides has beneficial health benefits, including antihypertensive, antioxidative and antithrombotic effects [122–124].

Most starter cultures used in the dairy industry such as *Lactobacillus plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. helveticus*, *L. delbrueckii* ssp. *bulgaricus*, *L. lactis* and *Streptococcus thermophilus* are proteolytic, and hence the use of these organisms in dairy products generates bioactive peptides. These anaerobes produce cell wall-bound proteinase and different intracellular peptidases – endopeptidases, aminopeptidases, tripeptidases and dipeptidases which release antihypertensive, immunomodulatory and antioxidative peptides from milk proteins [125–127]. The two well-known antihypertensive peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) are released from milk protein during *L. helveticus* fermentation [128, 129]. Not only the microorganisms but also their isolated proteases have been successfully used to release bioactive peptides from the milk proteins [130]. In a similar way, a vast array of active peptides can be potentially produced from a variety of proteins existing in other foods, which may also render health-promoting effects.

Besides releasing bioactive compounds from food, intestinal bacteria can also synthesize bioactive compounds. For instance, the gut anaerobes produce equol (Fig. 7a) from isoflavones. Equol is believed to have many beneficial effects including improving bone health [131, 132], anti-prostate cancer [133], and male pattern baldness and acne because of its dihydrotestosterone (DHT) blocking effect [134]. Similarly, these bacteria synthesize enterodiol (Fig. 7b) and enterolactone (Fig. 7c) from lignans which might minimize the risk of breast cancer, cardiovascular disease, endometrial and ovarian cancer, osteoporosis and prostate cancer. However, further studies are needed to confirm these claims. Another example of bioactive compound production by gut bacteria is the synthesis of urolithins (Fig. 7d) from ellagic acid. Preliminary evidence from recent studies indicates that urolithins have anticarcinogenic antioxidant, antiinflammatory and antimicrobial effects [114, 135].

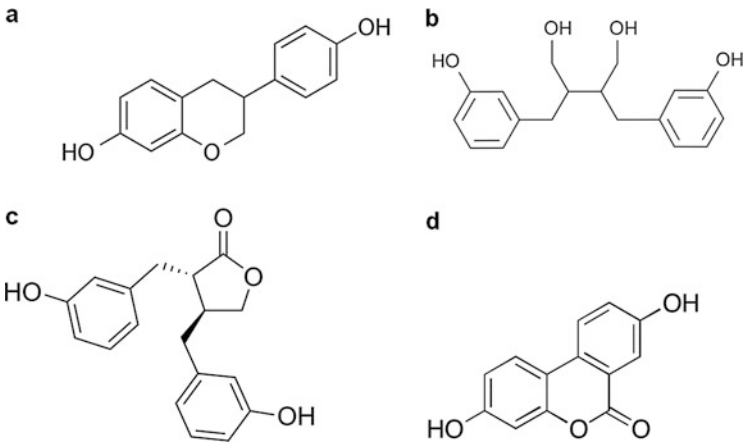


Fig. 7 Bioactive compounds produced by anaerobes from precursor molecules: (**a**) equol; (**b**) enterodiol; (**c**) enterolactone; (**d**) urolithins

2.4 Vitamins

Vitamins are among the important organic substances that the body needs for healthy life. They serve as vital coenzymes that regulate different cellular reactions. Unfortunately, humans lack the biochemical machinery necessary for the synthesis of most vitamins and are dependent on exogenous sources. We acquire vitamins from our natural food and, lately, as vitamin supplement tablets and fortified food and drinks. Anaerobes, which are part of the human microbiota [136, 137] or those isolated from non-human samples [138–140], are known to produce vitamins which can promote human health. Those anaerobes thriving in our gut such as members of the genus *Bacteroides* and *Eubacterium* produce vitamin K and the group B vitamins including thiamine, nicotinic acid, biotin, riboflavin, folates, pyridoxine, panthotenic acid and cobalamin [136, 137], and directly nourish our body. Metagenome analysis of the human distal gut microflora revealed the presence of Clustered Orthologous Groups (COGs), which are involved in the production of several essential vitamins [141]. This shows the possible contribution of these anaerobes in fulfilling the need for vitamins to some extent. Most of the production and absorption of microbial vitamins occurs in the colon [142], and the absorption of vitamins produced by the gut microflora contributes to systemic vitamin levels [143] and possibly minimizes the drastic effects of vitamin deficiency.

Anaerobes can be sources of dietary vitamins in isolated or crude form. Vitamin-producing anaerobes can be cultivated in bioreactors and the vitamins isolated for use. However, in some cases isolation of the vitamins is not necessary; for instance, when vitamin-producing GRAS anaerobes are used to ferment food, the vitamins produced during fermentation can be directly consumed with the food [144, 145].

3 Application of Whole Cell Anaerobes in Health Promotion

Besides providing bacteriocins or antibiotics, live anaerobes have also been used in many applications that promote human health, among which their use as probiotics, cancer treatment agents and bacteriotherapy agents has attracted considerable attention.

The application of anaerobes in food production and preservation is one of the oldest biotechnological methods. The ingestion of some probiotic anaerobes such as strains of *Lactobacillus*, *Bifidobacteria* and *Propionibacteria* is claimed to provide a tremendous health benefit. Among other advantages, these probiotics not only prevent but also treat pathogen-induced diarrhoea, maintain normal gut microflora, treat chronic inflammatory diseases and manage atopic and autoimmune diseases. However, because several reviews [104, 146–148] and books [149] have been written on anaerobic probiotics, and a chapter [150] is dedicated in this book, this particular application of anaerobes is not assessed in the present chapter.

3.1 Anaerobes in Cancer Treatment

Despite all the efforts and progress made, treatment of cancer still remains one of the biggest medical challenges. One of the greatest problems of cancer treatment is the selectivity of the therapy. It is relatively easy to kill cancer cells in vitro; however, to kill selectively the tumour cells in vivo is more complicated. Different methods have been used in cancer therapy to deliver therapeutic agents, but effective and selective delivery to target sites remains a complex task. Poor blood supply and high interstitial fluid pressure have contributed to the challenge of effective and selective delivery of therapeutic agent.

One of the strategies used to deliver therapeutic agents selectively to tumours is based on the nature of the microenvironments at tumour sites. The low oxygen tension or hypoxia has been a characteristic property of the tumour microenvironment [151, 152]. Tumour cells are fast growing and their consumption of oxygen is higher than that of cells in normal tissue. Moreover, when tumour cells are growing aggressively, the blood supply falls because of the disorganization of the newly formed blood vessels [153], which leads to nutrient depletion, acidity and poor oxygen availability. Studies have revealed that the oxygen level in normal tissue is about 3–9%, whereas the oxygen concentration in tumours is only about 0.3% [154]. This hypoxic microenvironment and poor blood supply reduce the efficiency of the conventional cancer treatments [151]. For instance, radiation therapy requires good oxygen levels for effective killing of the cells; however, the significantly lower oxygen level makes tumour cells three times more resistant to radiation than well aerated cells [154].

Targeting the hypoxic microenvironment of human tumours is expected to contribute to advancement of cancer therapy. This is where the application of anaerobic bacteria has emerged as an effective treatment strategy. Anaerobic bacteria are not only able to grow selectively in the hypoxic environment but they can also penetrate into the tumour necrotic part. Bacteria belonging to the genera *Clostridium* [155–158], *Salmonella* [159, 160], *Bifidobacterium* [161, 162] and *Escherichia* [163] have been shown to localize and proliferate selectively in tumours. The bacteria target different intratumoural regions through specific chemotaxis, preferential growth and hypoxic germination [164]. For example, the facultative anaerobes *Salmonella* and *Escherichia* strains use specific chemotaxis and preferential growth to colonize tumours, whereas the obligate anaerobes *Clostridium* and *Bifidobacterium* primarily rely on the mechanism of hypoxic germination/growth.

The wild-type bacteria used in cancer treatment can colonize the tumour, slow down its growth and destroy it, at least potentially. However, clinical trials have revealed that the colonization could be insufficient [165, 166] or the invasion of the tumour may be accompanied by severe toxicity [167]. Thus, in recent years there have been efforts to address these problems using molecular biology techniques to engineer the anaerobes. Some of the engineered bacteria have been able to colonize target tumour regions efficiently and deliver therapeutic payloads effectively

[168]. For instance, when the ribose/galactose chemoreceptor is deleted, the bacterial cells effectively colonize the tumour [169]. Genetically modified bacteria with engineered therapeutic delivery including controlled release of cytotoxin, secretion of enzymes (e.g. cytosine deaminase) that activate pro-drugs, and production of bioactive compounds that eventually kill the tumour cells without affecting the normal tissue [164, 170, 171] have been studied. Moreover, engineered bacteria expressing a cell signalling protein tumour-necrosis-factor-α which induces apoptotic cell death, antibodies that inhibit hypoxia-inducible-factor-1-α which plays a role in the adaptation of tumour cells to the hypoxic microenvironment, α-hemolysin which kills tumour cells or interleukin-2 which is widely used in cancer immunotherapy have been tried to inhibit tumour development [164, 168, 170–173]. The results of these studies show that anaerobes can potentially be used to fight cancer effectively. Indeed, there have been showcases where bacterial treatments have resulted in very impressive results.

Anaerobes are not only used in treatment but also for early detection of cancer. Recently, the probiotic strain *Escherichia coli* Nissle 1917 was engineered and orally administered to indicate liver metastasis through generation of easily measurable signals in urine [174]. This is very encouraging as early detection of cancer makes the treatment relatively easy. It seems that the combination of the diagnostic and therapeutic use of anaerobes in the treatment of cancer can open a new window of hope for cancer therapy.

3.2 Gut Anaerobes in Bacteriotherapy

A wide variety of microorganisms live inside our bodies and others gain entry with the air we breathe, the food we eat and the liquid we drink. Some are beneficial, others do not produce any trouble or benefit although the infectious strains can cause diseases. Bacteria inhabiting our gastrointestinal tract (GIT) are believed to play an important role in maintaining a healthy environment and prevent some gastrointestinal diseases or suppress the growth of infectious microorganisms. For these reasons, some of the human gut microflora have been used to treat disease and foster human health. For instance, strains of *Lactobacillus* and *Bifidobacterium* have been used to treat gastrointestinal disorders [175]. This approach of using live bacteria to treat disease is known as bacteriotherapy.

Many probiotic commercial products are relatively simple and contain known culture(s) of live anaerobic bacteria. However, such simple probiotic formulations cannot solve some health problems which can potentially be treated by probiotics. In such a scenario, when one does not have a clear picture which bacteria or bacterium can alleviate the problem, it may be ideal to use preparations containing diverse groups of bacteria. In bacteriotherapy, the most efficient and elaborate mix of human gut bacteria is the entire fecal flora containing a vast array of microorganisms. The use of human fecal flora as a therapeutic agent is specifically known as fecal bacteriotherapy [176], which has been used sporadically for over five

decades. Fecal bacteriotherapy is used to treat chronic bowel infections, often as a last resort treatment for patients suffering from severe *Clostridium difficile* syndromes such as diarrhoea, colitis and pseudomembranous colitis [175, 177]. There have also been encouraging results when the fecal flora is used to treat inflammatory bowel disease, chronic constipation and irritable bowel syndrome [178, 179]. The *C. difficile* infection epidemics have triggered an increased use of human fecal bacteria to treat the infection and the associated syndromes. Because of its high efficacy, more patients become willing to take human faecal bacteria and lately many clinics have started offering the service. In recent years, applications of bacteriotherapy against other than *C. difficile* infection have emerged. This is mainly because of the human microbiome research results emerging with exciting insights.

4 Microbiome: The Invisible Organ with Visible Health Impact

The human body harbours a diverse group of microorganisms that colonize almost every body part, ranging from the skin to the deepest recesses of our guts. The trillions of microorganisms that live in our bodies are collectively referred to as human microbiome. It is believed that there are at least 10 times more bacterial cells than human body cells. However, because of their minute size, the microbiome accounts for only about 1–3% of our body weight. The great majority of these microorganisms are anaerobes which inhabit the gastrointestinal tract (GIT). Along the tract, the diversity and cell count of the gut microflora varies. The bacterial count is less than 10^3/mL in the stomach, but further down in the GIT (the colon) it reaches to 10^{11}–10^{12}/mL and here the anaerobes outnumber the aerobes by up to 1000-fold [180–183].

To start with, the gut microflora is primarily composed of bifidobacteria which are believed to be selected by the milk feed during the first weeks of infant life [184, 185]. As we grow up, Firmicutes (genera of lactic acid bacteria and Clostridia) and Bacteroidetes become dominant, whereas species belonging to Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia are in a minority [186]. It is estimated that there could be more than 1,000 species in an adult gut microbiota which comprises the permanent (autochthonous) inhabitants and the acquired (allochthonous or transient) flora obtained from the environment or diet.

The relation between the microbiome and the human body seems mutualistic rather than pathogenic. The association is the result of a long co-evolution where human body and microbiome become interdependent [187]. In this association, the bacteria benefited from the nutrient-rich and stable provided by the host and the human body benefited from the roles played by the bacteria in developing and maintaining a healthy system [188, 189]. Lately, the diversity and role of human microbiome has been the focus of a growing number of research activities, and

fascinating insights have started to emerge. It has become known that the microbiome plays a major role in humans: nutrition, protection from infection, metabolism, development of epithelial and systemic immunity, immune modulation and fat storage regulation [115, 190–199]. Thus, the microbiome can be considered as an invisible organ, and examples of human systems that are influenced by anaerobes are shown in Fig. 8. Keeping a proper balance between the microbiome and our body is vital for allowing the microbes to play their role and maintain our health. Indeed, compositional variation of the gut microbiome has been related to several diseases such as obesity, inflammatory immune disorders and cancer [114, 190, 200]. Several health problems such as the metabolic syndrome which is a bunch of abnormalities such as high blood pressure, accumulation of excess fat especially around the waist, increased blood sugar level, and abnormal cholesterol that raise the risk for diabetes and cardiovascular diseases as well as neurodevelopmental disorders, autoimmune and allergic diseases are believed to be linked to the microbiota [177]. In recent years, research on human microbiota is gearing towards the use of bacteria not only to treat but also prevent some diseases. For instance, it has been reported that the probability of developing asthma in children having lower microbiota diversity is significantly higher than children with higher diversity [201]. In fact, further microbiota analysis has shown specifically that children who have low abundance of members of the anaerobic bacteria *Faecalibacterium*, *Rothia*, *Veillonella* and *Lachnospira* during the first 3 months of their lives are at risk of developing asthma [202]. Delivery of these bacteria to germ-free mice has prevented airway inflammation and hence supports the possible role of these anaerobes in avoiding asthma development. The potential is clear and in the future these bacteria may be used as probiotics or vaccines to prevent asthma, an age-old disease affecting several hundred million people. Similarly, swabbing the gut microflora of fat and thin mice has shown that the thin mice increase weight whereas the fat ones shed weight [203], and this possibly shows that such swabbing

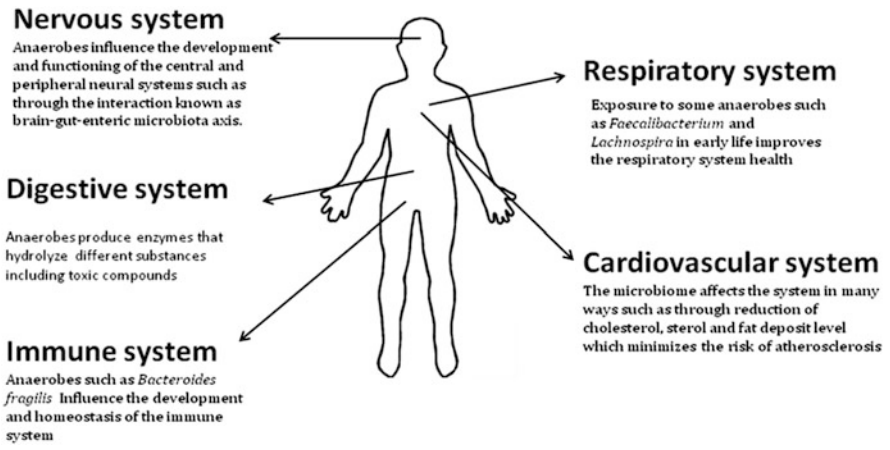


Fig. 8 The human systems influenced by the microbiome

is one key component in regulation of body weight and hence preventing diseases associated with it.

The consumption of probiotics, prebiotics and polyphenols are known to influence the numbers and types of microbes inhabiting the gut. Diet composition [204] and administration of broad-spectrum antibiotics [205] are also among factors that influence the microbiome diversity. Thus, proper diet, consumption of dietary modifiers, and whenever possible avoiding use of broad-spectrum antibiotics may help to maintain a healthy microbiota. When the balance is disrupted and the microbiome becomes dysfunctional, probiotic intervention could be important as it may positively influence the gut ecosystem and homeostasis, and hence maintain a proper host metabolism and ultimately promote the host health.

Although the use of bacteria for microbiota-related disorders is currently in its infancy, as more results trickle in we expect to see in the time ahead new and unexpected applications in the treatment of diseases and that could herald a paradigm shift in diagnosing and treating a number of important diseases.

5 Conclusion

Anaerobes are relatively less explored. However, the limited research done so far has clearly shown the enormous potential of these microorganisms in promoting and maintaining human health. Anaerobes improve the quality of our lives not only through production of bioactive compounds, but those living in our bodies (microbiome) are functioning as one of our organs, playing a complex role to ensure our very existence. Anaerobes have been successfully tested to treat a wide range of diseases and new research on the microbiome is generating even more impressive data and insight, which may bring a major breakthrough in the diagnosis and treatment of several diseases. So far, the use of prebiotics, probiotics and synbiotics has shown health promoting effect. This could be just the tip of the iceberg. Elucidation of the complex interdependent association of the human microbiome and human body is just starting, yet with impressive indications that can potentially shape the future medicine.

Acknowledgement Part of this work was supported by the Swedish Research Council via the Research Link Grant.

References

1. Hill JO, Wyatt HR, Reed GW, Peters JC (2003) Obesity and the environment: where do we go from here? Science 299:853–855
2. Owen N, Sparling PB, Healy GN, Dunstan DW, Matthews CE (2010) Sedentary behavior: emerging evidence for a new health risk. Mayo Clin Proc 85:1138–1141

3. Penesyan A, Gillings M, Paulsen IT (2015) Antibiotic discovery: combatting bacterial resistance in cells and in biofilm communities. Molecules 20:5286–5298
4. Laxminarayan R, Malani A (2007) Extending the cure: policy responses to the growing threat of antibiotic resistance. Earthscan
5. WHO (2010) Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response
6. WHO (2011) Antimicrobial resistance. http://www.who.int/mediacentre/factsheets/fs194/en/
7. Gerberding J (2003) The resistance phenomenon in microbes and infectious disease vectors: implications for human health and strategies for containment. National Academic Press, Washington, pp 210–215
8. Davies J (2006) Where have all the antibiotics gone? Can J Infect Dis Med Microbiol 17: 287–290
9. Scannell JW, Blanckley A, Boldon H, Warrington B (2012) Diagnosing the decline in pharmaceutical R&D efficiency. Nat Rev Drug Discov 11:191–200
10. Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, Bartlett JG, Edwards J (2008) The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. Clin Infect Dis 46:155–164
11. WHO (2014) Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva
12. Payne DJ, Gwynn MN, Holmes DJ, Pompliano D (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov 6:29–40
13. So AD, Gupta N, Brahmachari SK, Chopra I, Munos B, Nathan C, Outterson K, Paccaud JP, Payne DJ, Peeling RW, Spigelman M, Weigelt J (2011) Towards new business models for R&D for novel antibiotics. Drug Resist Updat 14:88–94
14. Pidot SJ, Coyne S, Kloss F, Hertweck C (2014) Antibiotics from neglected bacterial sources. Int J Med Microbiol 304:14–22
15. Bérdy J (2005) Bioactive microbial metabolites. J Antibiot 58:1–26
16. Cragg GM, Newman DJ (2013) Natural products: a continuing source of novel drug leads. Biochim Biophys Acta 1830:3670–3695
17. Newman DJ, Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 75:311–335
18. Morris JG (1994) Obligately anaerobic bacteria in biotechnology. Appl Biochem Biotechnol 48:75–106
19. Weusthuis RA, Lamot I, van der Oost J, Sanders JPM (2011) Microbial production of bulk chemicals: development of anaerobic processes. Trends Biotechnol 29:153–158
20. Bernstein AS, Ludwig DS (2008) The importance of biodiversity to medicine. JAMA 300: 2297–2299
21. Brown DG, Lister T, May-Dracka TL (2014) New natural products as new leads for antibacterial drug discovery. Bioorg Med Chem Lett 24:413–418
22. Hussain S, Fareed S, Ansari S, Khan S (2012) Marine natural products: a lead for anti cancer. Indian J Geo Mar Sci 41:27–39
23. Riley MA, Wertz JE (2002) Bacteriocins: evolution, ecology, and application. Annu Rev Microbiol 56:117–137
24. Cascales L, Craik DJ (2010) Naturally occurring circular proteins: distribution, biosynthesis and evolution. Org Biomol Chem 8:5035–5047
25. Walton JD, Hallen-Adams HE, Luo H (2010) Ribosomal biosynthesis of the cyclic peptide toxins of Amanita mushrooms. Biopolymers 94:659–664
26. Arihara K, Cassens RG, Luchansky JB (1993) Characterisation of bacteriocins from *Enterococcus faecium* with activity against *Listeria monocytogenes*. Int J Food Microbiol 19:123–134
27. Balciunas EM, Al Arni S, Converti A, Leblanc JG, Oliveira RPDS (2015) Production of bacteriocin-like inhibitory substances (BLIS) by *Bifidobacterium lactis* using whey as a substrate. Int J Dairy Technol 68:1–6

28. Barefoot SF, Nettles CG (1993) Antibiosis revisited: bacteriocins produced by dairy starter cultures. J Dairy Sci 76:2366–2379
29. Chakchouk-Mtibaa A, Elleuch L, Smaoui S, Najah S, Sellem I, Abdelkafi S, Mellouli L (2014) An antilisterial bacteriocin BacFL31 produced by *Enterococcus faecium* FL31 with a novel structure containing hydroxyproline residues. Anaerobe 27:1–6
30. Cole ST, Garnier T (1993) Molecular genetic studies of UV-inducible bacteriocin production in *Clostridium perfringens*. In: Sebald M (ed) Genetics and molecular biology of anaerobic bacteria. Springer, New York, pp 248–254
31. Iverson WG, Mills NF (1976) Bacteriocins of *Streptococcus bovis*. Can J Microbiol 22: 1040–1047
32. Lauková A, Mareková M, Javorsky P (1993) Detection and antimicrobial spectrum of a bacteriocin-like substance produced by *Enterococcus faecium* CCM4231. Lett Appl Microbiol 16:257–260
33. Maqueda M, Galvez A, Bueno M, Sanchez-Barrena MJ, Gonzalez C, Albert A, Rico M, Valdivia E (2004) Peptide AS-48: prototype of a new class of cyclic bacteriocins. Curr Protein Pept Sci 5:399–416
34. Meghrous J, Euloge P, Junelles AM, Ballongue J, Petitdemange H (1990) Screening of *Bifidobacterium* strains for bacteriocin production. Biotechnol Lett 12:575–580
35. Miranda CMS, Farias LM, Carvalho MAR, Damasceno CAV, Totola AH, Tavares CAP, Cisalpino EO, Vieira EC (1993) Purification and partial characterization of a bacteriocin isolated from *Bacteroides ovatus* H47. Can J Microbiol 39:169–174
36. Southern JA, Katz W, Woods DR (1984) Purification and properties of a cell-bound bacteriocin from a *Bacteroides fragilis* strain. Antimicrob Agents Chemother 25:253–257
37. Toba T, Yoshioka E, Itoh T (1991) Potential of *Lactobacillus gasseri* isolated from infant faeces to produce bacteriocin. Lett Appl Microbiol 12:228–231
38. Wannun P, Piwat S, Teanpaisan R (2014) Purification and characterization of bacteriocin produced by oral *Lactobacillus paracasei* SD1. Anaerobe 27:17–21
39. Cotter PD, Ross RP, Hill C (2013) Bacteriocins – a viable alternative to antibiotics? Nat Rev Microbiol 11:95–105
40. Zacharof MP, Lovitt RW (2012) Bacteriocins produced by lactic acid bacteria: a review article. APCBEE Procedia 2:50–56
41. Heng NCK, Tagg JR (2006) What's in a name? Class distinction for bacteriocins. Nat Rev Microbiol 2006:4. doi:10.1038/nrmicro1273-c1
42. Sanchez-Hidalgo M, Montalban-Lopez M, Cebrian R, Valdivia E, Martinez-Bueno M, Maqueda M (2011) AS-48 bacteriocin: close to perfection. Cell Mol Life Sci 68:2845–2857
43. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3:777–788
44. Blaser M (2011) Antibiotic overuse: stop the killing of beneficial bacteria. Nature 476: 393–394
45. Willing BP, Russell SL, Finlay BB (2011) Shifting the balance: antibiotic effects on host–microbiota mutualism. Nat Rev Microbiol 9:233–243
46. Sobrino-Lopez A, Martin-Belloso O (2008) Use of nisin and other bacteriocins for preservation of dairy products. Int Dairy J 18:329–343
47. Prudêncio CV, dos Santos MT, Vanetti MCD (2015) Strategies for the use of bacteriocins in Gram-negative bacteria: relevance in food microbiology. J Food Sci Technol 52:5408–5417
48. Ghodhbane H, Elaidi S, Sabatier JM, Achour S, Benhmida J, Regaya I (2015) Bacteriocins active against multi-resistant gram negative bacteria implicated in nosocomial infections. Infect Disord Drug Targets 15:2–12
49. Piper C, Cotter PD, Ross RP, Hill C (2009) Discovery of medically significant lantibiotics. Curr Drug Discov Technol 6:1–18
50. Arnusch CJ, Bonvin AM, Verel AM, Jansen WT, Liskamp RM, de Kruijff B, Pieters RJ, Breukink E (2008) The vancomycin-nisin(1–12) hybrid restores activity against vancomycin resistant enterococci. Biochemistry 47:12661–12663

51. Field D, Hill C, Cotter PD, Ross RP (2010) The dawning of a 'Golden era' in lantibiotic bioengineering. Mol Microbiol 78:1077–1087
52. Levengood MR, Knerr PJ, Oman TJ, van der Donk WA (2009) *In vitro* mutasynthesis of lantibiotic analogues containing nonproteinogenic amino acids. J Am Chem Soc 131: 12024–12025
53. Ross AC, McKinnie SM, Vederas JC (2012) The synthesis of active and stable diaminopimelate analogues of the lantibiotic peptide lactocin S. J Am Chem Soc 134:2008–2011
54. Goldstein BP, Wei J, Greenberg K, Novick R (1998) Activity of nisin against *Streptococcus pneumoniae*, in vitro, and in a mouse infection model. J Antimicrob Chemother 42:277–278
55. van Staden AD, Brand AM, Dicks LM (2012) Nisin F-loaded brushite bone cement prevented the growth of *Staphylococcus aureus* in vivo. J Appl Microbiol 112:831–840
56. Dziuba M, Darewicz M (2007) Food proteins as precursors of bioactive peptides – division into families. Food Sci Technol Int 13:393–404
57. Garcia AE, Camarero JA (2010) Biological activities of natural and engineered cyclotides, a novel molecular scaffold for peptide-based therapeutics. Curr Mol Pharmacol 3:153–163
58. Pelegrini PB, Franco OL (2005) Plant γ-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins. Int J Biochem Cell Biol 37:2239–2253
59. Selitrennikoff CP (2001) Antifungal proteins. Appl Environ Microbiol 67:2883–2894
60. Terras FR, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Rees SB, Torrekens S, Van Leuven F, Vanderleyden J (1995) Small cysteine rich antifungal proteins from radish: their role in host defense. Plant Cell 7:573–588
61. Korhonen H, Pihlanto-Leppälä A (2001) Milk protein-derived bioactive peptides – novel opportunities for health promotion. IDF Bull 363:17–26
62. Minervini F, Algaron F, Rizzello CG, Fox PF, Monnet V, Gobbetti M (2003) Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed caseins of milk from six species. Appl Environ Microbiol 69: 5297–5305
63. Hayes M, Ross RP, Fitzgerald GF, Hill C, Stanton C (2006) Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. Appl Environ Microbiol 72: 2260–2264
64. Hayes M, Ross RP, Fitzgerald GF, Hill C, Stanton C (2007) Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part I: overview. Biotechnol J 2:426–434
65. Hancock REW, Chapple DS (1999) Peptide antibiotics. Antimicrob Agents Chemother 43: 1317–1323
66. Mukherjee S, Das P, Sen R (2006) Towards commercial production of microbial surfactants. Trends Biotechnol 24:509–515
67. Rodrigues L, Banat IM, Teixeira J, Oliveira R (2006) Biosurfactants: potential applications in medicine. J Antimicrob Chemother 57:609–618
68. Mandal SM, Barbosa AEAD, Franco OL (2013) Lipopeptides in microbial infection control: scope and reality for industry. Biotechnol Adv 31:338–345
69. Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125
70. Schneider T, Müller A, Miess H, Gross H (2014) Cyclic lipopeptides as antibacterial agents – potent antibiotic activity mediated by intriguing mode of actions. Int J Med Microbiol 304: 37–43
71. Mandal SM, Sharma S, Pinnaka AK, Kumari A, Korpole S (2013) Isolation and characterization of diverse antimicrobial lipopeptides produced by *Citrobacter* and *Enterobacter*. BMC Microbiol 13:152
72. Denger K, Warthmann R, Ludwig W, Schink B (2002) *Anaerophaga thermohalophila* gen. nov., sp. nov., a moderately thermohalophilic, strictly anaerobic fermentative bacterium. Int J Syst Evol Microbiol 52:173–178

73. Hashizume H, Igarashi M, Hattori S, Hori M, Hamada M, Takeuchi T (2001) Tripropeptins, novel antimicrobial agents produced by *Lysobacter* sp. I. Taxonomy, isolation and biological activities. J Antibiot 54:1054–1059
74. Yu F, Zaleta-Rivera K, Zhu X, Huffman J, Millet JC, Harris SD, Yuen G, Li XC, Du L (2007) Structure and biosynthesis of heat-stable antifungal factor (HSAF), a broad-spectrum antimycotic with a novel mode of action. Antimicrob Agents Chemother 51:64–72
75. Xie Y, Wright S, Shen Y, Du L (2012) Bioactive natural products from *Lysobacter*. Nat Prod Rep 29:1277–1287
76. Hashizume H, Hirosawa S, Sawa R, Muraoka Y, Ikeda D, Naganawa H, Igarashi M (2004) Tripropeptins novel antimicrobial agents produced by *Lysobacter* sp. II. Structure elucidation. J Antibiot 57:52–58
77. Kato A, Nakaya S, Kokubo N, Aiba Y, Ohashi Y, Hirata H, Fujii K, Harada K (1998) A new anti-MRSA antibiotic complex, WAP-8294A. I. Taxonomy, isolation and biological activities. J Antibiot 51:929–935
78. Sammer UF, Volksch B, Mollmann U, Schmidtke M, Spiteller P, Spiteller M, Spiteller D (2009) 2-Amino-3-(oxirane-2,3-dicarboxamido)-propanoyl-valine, an effective peptide antibiotic from the epiphyte *Pantoea agglomerans* 48b/90. Appl Environ Microbiol 75: 7710–7717
79. Brady SF, Wright SA, Lee JC, Sutton AE, Zumoff CH, Wodzinsky RS, Beer SV, Clardy J (1999) Pantocin B, an antibiotic from *Erwinia herbicola* discovered by heterologous expression of cloned genes. J Am Chem Soc 121:11912–11913
80. Ishimaru CA, Klos EJ, Brubaker RR (1988) Multiple antibiotic production by *Erwinia herbicola*. Phytopathology 78:746–750
81. Jin M, Liu L, Wright SA, Beer SV, Clardy J (2003) Structural and functional analysis of pantocin A: an antibiotic from *Pantoea agglomerans* discovered by heterologous expression of cloned genes. Angew Chem Int Ed 42:2898–2901
82. Jin M, Wright SA, Beer SV, Clardy J (2003) The biosynthetic gene cluster of pantocin A provides insights into biosynthesis and a tool for screening. Angew Chem Int Ed 42: 2902–2905
83. Fredenhagen A, Tamura SY, Kenny PTM, Komura H, Naya Y, Nakanishi K (1987) Andrimid, a new peptide antibiotic produced by an intracellular bacterial symbiont isolated from a brown planthopper. J Am Chem Soc 109:4409–4411
84. Freiberg C, Brunner NA, Schiffer G, Lampe T, Pohlmann J, Brands M, Raabe M, Habich D, Ziegelbauer K (2004) Identification and characterization of the first class of potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity. J Biol Chem 279:26066–26073
85. Needham J, Kelly MT, Ishige M, Andemen RJ (1994) Andrimid and moiramides A-C, metabolites produced in culture by a marine isolate of the bacterium *Pseudomonas fluorescens*: structure elucidation and biosynthesis. J Org Chem 59:2058–2063
86. Vasileva-Tonkova E, Gesheva V (2007) Biosurfactant production by Antarctic facultative anaerobe *Pantoea* sp. during growth on hydrocarbons. Curr Microbiol 54:136–141
87. Bode HB (2009) Entomopathogenic bacteria as a source of secondary metabolites. Curr Opin Chem Biol 13:224–230
88. Lang G, Kalvelage T, Peters A, Wiese J, Imhoff JF (2008) Linear and cyclic peptides from the entomopathogenic bacterium *Xenorhabdus nematophilus*. J Nat Prod 71:1074–1077
89. McInerney BV, Taylor WC, Lacey MJ, Akhurst RJ, Gregson RP (1991) Biologically active metabolites from *Xenorhabdus* spp. Part 2. Benzopyran-1-one derivatives with gastroprotective activity. J Nat Prod 54:785–795
90. Li J, Chen G, Webster JM (1997) Nematophin a novel antimicrobial substance produced by *Xenorhabdus nematophilus* (Enterobactereaceae). Can J Microbiol 43:770–773
91. Park JH, Kim R, Aslam Z, Jeon CO, Chung YR (2008) *Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. Int J Syst Evol Microbiol 58:387–392

92. Kloss F, Lincke T, Hertweck C (2011) Highly efficient total synthesis of the *Clostridium*-derived anti-MRSA antibiotic closthioamide. Eur J Org Chem 2011:1429–1431
93. Lincke T, Behnken S, Ishida K, Roth M, Hertweck C (2010) Closthioamide: an unprecedented polythioamide antibiotic from the strictly anaerobic bacterium *Clostridium cellulolyticum*. Angew Chem Int Ed 122:2055–2057
94. Ezaki M, Muramatsu H, Takase S, Hashimoto M, Nagai K (2008) Naphthalecin, a novel antibiotic produced by the anaerobic bacterium, *Sporotalea colonica* sp. nov. J Antibiot 61: 207–212
95. Ganzle MM, Holtzel A, Walter J, Gunther J, Hammes WP (2000) Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl Environ Microbiol 66: 4325–4333
96. Salman JAS, Alimer DA (2014) Antibacterial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus rhamnosus* against some bacteria causing UTI in Iraqi women. Int J Curr Res 6:5368–5374
97. Behnken S, Hertweck C (2012) Anaerobic bacteria as producers of antibiotics. Appl Microbiol Biotechnol 96:61–67
98. Letzel AC, Pidot SJ, Hertweck C (2013) A genomic approach to the cryptic secondary metabolome of the anaerobic world. Nat Prod Rep 30:392–428
99. Seedorf H, Fricke WF, Veith B, Bruggemann H, Liesegang H, Strittmatter A, Miethke M, Buckel W, Hinderberger J, Li F, Hagemeier C, Thauer RK, Gottschalk G (2008) The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. Proc Natl Acad Sci U S A 105:2128–2133
100. Behnken S, Hertweck C (2012) Cryptic polyketide synthase genes in non-pathogenic *Clostridium* spp. PLoS One 7(1), e29609
101. Zhang J, Liangcheng D, Liu F, Xu F, Hu B, Venturi V, Qian G (2014) Involvement of both PKS and NRPS in antibacterial activity in *Lysobacter enzymogenes* OH11. FEMS Microbiol Lett 355:170–176
102. Scherlach K, Hertweck C (2009) Triggering cryptic natural product biosynthesis in microorganisms. Org Biomol Chem 7:1753–1760
103. Cooper DG, Zajic JE, Gerson DF, Manninen KI (1980) Isolation and identification of biosurfactants produced during anaerobic growth of *Clostridium pasteurianum*. J Ferment Technol 58:83–86
104. Isolauri E, Sütas Y, Kankaanpää P, Arvilommi H, Salminen S (2001) Probiotics: effects on immunity. Am J Clin Nutr 73:444S–450S
105. Akatsu H, Iwabuchi N, Xiao JZ, Matsuyama Z, Kurihara R, Okuda K, Yamamoto T, Maruyama M (2013) Clinical effects of probiotic *Bifidobacterium longum* BB536 on immune function and intestinal microbiota in elderly patients receiving enteral tube feeding. J Parenter Enteral Nutr 37:631–640
106. Gill HS, Rutherfurd KJ, Cross ML, Gopal PK (2001) Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. Am J Clin Nutr 74:833–839
107. Shimizu K, Ogura H, Goto M, Asahara T, Nomoto K, Morotomi M, Yoshiya K, Matsushima A, Sumi Y, Kuwagata Y, Tanaka H, Shimazu T, Sugimoto H (2006) Altered gut flora and environment in patients with severe SIRS. J Trauma 60:126–133
108. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122:107–118
109. Troy EB, Kasper DL (2010) Beneficial effects of *Bacteroides fragilis* polysaccharides on the immune system. Front Biosci 15:25–34
110. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, Kasper D, Kasper L (2010) Central nervous system demyelinating disease protection by the human commensal *Bacteroides fragilis* depends on polysaccharide A expression. J Immunol 185:4101–4108

111. Ochoa-Reparaz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper D, Kasper L (2010) A polysaccharide from the human commensal *Bacteroides fragilis* protects against CNS demyelinating disease. Mucosal Immunol 3:487–495
112. Mao YK, Kasper DL, Wang B, Forsythe P, Bienenstock J, Kunze WA (2013) *Bacteroides fragilis* polysaccharide A is necessary and sufficient for acute activation of intestinal sensory neurons. Nat Commun 4:1465. doi:10.1038/ncomms2478
113. O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7:688–693
114. Davis CD, Milner JA (2009) Gastrointestinal microflora, food components and colon cancer prevention. J Nutr Biochem 20:743–752
115. O'Shea EF, Cotter PD, Stanton C, Ross RP, Hill C (2012) Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid. Int J Food Microbiol 152:189–205
116. Leah D, Whigham LD, Mark E, Cook ME, Richard L, Atkinson RL (2000) Conjugated linoleic acid: implications for human health. Pharmacol Res 42:503–510
117. Bassaganya-Riera J, Hontecillas R, Horne WT, Sandridge M, Herfarth HH, Bloomfeld R, Isaacs KL (2012) Conjugated linoleic acid modulates immune responses in patients with mild to moderately active Crohn's disease. Clin Nutr 31:721–727
118. Chung SH, Kim IH, Park HG, Kang HS, Yoon CS, Jeong HY, Choi NJ, Kwon EG, Kim YJ (2008) Synthesis of conjugated linoleic acid by human-derived *Bifidobacterium breve* LMC 017: utilization as a functional starter culture for milk fermentation. J Agric Food Chem 56: 3311–3316
119. Jiang J, Bjorck L, Fonden R (1998) Production of conjugated linoleic acid by dairy starter cultures. J Appl Microbiol 85:95–102
120. Ogawa J, Kishino S, Ando A, Sugimoto S, Mihara K, Shimizu S (2005) Production of conjugated fatty acids by lactic acid bacteria. J Biosci Bioeng 100:355–364
121. Möller NP, Scholz-Arhens KE, Roos N, Sherezenmeir J (2008) Bioactive peptides and proteins from foods: indication for health effects. Eur J Nutr 47(4):171–182
122. Korhonen H, Pihlanto A (2003) Food-derived bioactive peptides – opportunities for designing future foods. Curr Pharm Des 9:1297–1308
123. Meisel H, FitzGerald RJ (2003) Biofunctional peptides from milk proteins: mineral binding and cytomodulatory effects. Curr Pharm Des 9:1289–1295
124. Raikos V, Dassios T (2014) Health-promoting properties of bioactive peptides derived from milk proteins in infant food: a review. Dairy Sci Technol 94:91–101
125. Gobbetti M, Minervini F, Rizzello CG (2004) Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. Int J Dairy Technol 57:173–188
126. Korhonen H, Pihlanto A (2001) Food-derived bioactive peptides – opportunities for designing future foods. Curr Pharm Des 9:1297–1308
127. Matar C, LeBlanc JG, Martin L, Perdigón G (2003) Biologically active peptides released in fermented milk: role and functions. In: Farnworth ER (ed) Handbook of fermented functional foods. Functional foods and nutraceuticals series. CRC Press, Florida, pp 177–201
128. Nakamura T, Hirota T, Mizushima K, Ohki K, Naito Y, Yamamoto N, Yoshikawa T (2013) Milk-derived peptides, Val-Pro-Pro and Ile-Pro-Pro, attenuate atherosclerosis development in apolipoprotein E-deficient mice: a preliminary study. J Med Food 16:396–403
129. Ueno K, Mizuno S, Yamamoto N (2004) Purification and characterization of an endopeptidase that has an important role in the carboxyl terminal processing of antihypertensive peptides in *Lactobacillus helveticus* CM4. Lett Appl Microbiol 39:313–318
130. Urista CM, Fernandez RA, Rodriguez FR, Cuenca AA, Jurado AT (2011) Review: production and functionality of active peptides from milk. Food Sci Technol Int 17:293–317
131. Tousen Y, Ezaki J, Fujii Y, Ueno T, Nishimuta M, Ishimi Y (2011) Natural S-equol decreases bone resorption in postmenopausal, non-equol-producing Japanese women: a pilot randomized, placebo-controlled trial. Menopause 18:563–574
132. Wu J, Oka J, Ezaki J, Ohtomo T, Ueno T, Uchiyama S, Toda T, Uehara M, Ishimi Y (2007) Possible role of equol status in the effects of isoflavone on bone and fat mass in

postmenopausal Japanese women: a double-blind, randomized, controlled trial. Menopause 14: 866–874
133. Akaza H, Miyanaga N, Takashima N et al (2004) Comparisons of percent equol producers between prostate cancer patients and controls: case-controlled studies of isoflavones in Japanese, Korean and American residents. Jpn J Clin Oncol 34:86–89
134. Lund TD, Munson DJ, Haldy ME, Setchell KD, Lephart ED, Handa RJ (2004) Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback. Biol Reprod 70: 1188–1195
135. Espín JC, Larrosa M, García-Conesa MT, Tomás-Barberán F (2013) Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. Evid Based Complement Alternat Med, http://dx.doi.org/10.1155/2013/270418
136. Hill MJ (1997) Intestinal flora and endogenous vitamin synthesis. Eur J Cancer Prev 6: S43–S45
137. Saulnier DM, Kolida S, Gibson GR (2009) Microbiology of the human intestinal tract and approaches for its dietary modulation. Curr Pharm Des 15:1403–1414
138. Jayashree S, Jayaraman K, Kalaichelvan G (2010) Isolation, screening and characterization of riboflavin producing lactic acid bacteria from Katpadi, Vellore district. Recent Res Sci Technol 2:83–88
139. Papastoyiannidis G, Polychroniadou A, Michaelidou AM, Alichanidis E (2006) Fermented milks fortified with B-group vitamins: vitamin stability and effect on resulting products. Food Sci Technol Int 12:521–529
140. Santos F, Wegkamp A, de Vos WM, Smid EJ, Hugenholtz J (2008) High-level folate production in fermented foods by the B12 producer *Lactobacillus reuteri* JCM1112. Appl Environ Microbiol 74:3291–3294
141. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE (2006) Metagenomic analysis of the human distal gut microbiome. Science 312:1355–1359
142. Said HM, Mohammed ZM (2006) Intestinal absorption of water-soluble vitamins: an update. Curr Opin Gastroenterol 22:140–146
143. Ichihashi T, Takagishi Y, Uchida K, Yamada H (1992) Colonic absorption of menaquinone-4 and menaquinone-9 in rats. J Nutr 122:506–512
144. Gu Q, Zhang C, Song D, Li P, Zhu X (2015) Enhancing vitamin B 12 content in soy-yogurt by *Lactobacillus reuteri*. Int J food Microbiol 206:56–59
145. Rossi M, Amaretti A, Raimondi S (2011) Folate production by probiotic bacteria. Nutrients 3: 118–134
146. Chukwu EE, Nwaokorie FO, Coker AO (2014) Role of anaerobes as probiotic organisms. Int J Food Nutr Saf 5:74–97
147. Drisko JA, Giles CK, Bischoff BJ (2003) Probiotics in health maintenance and disease prevention. Altern Med Rev 8:143–155
148. Patel A, Shah N, Prajapati JB (2014) Clinical application of probiotics in the treatment of *Helicobacter pylori* infection – a brief review. J Microbiol Immunol Infect 47:429–437
149. Fuller R (ed) (2012) Probiotics: the scientific basis. Springer Science, Netherlands
150. El-Henshasy H, Malik K, AbdMalek R, Othman NZ, Elsayed EA, Wadaan M (2016) Anaerobic probiotics: the key microbes for human health. Adv Biochem Eng Biotechnol. doi:10.1007/10_2016_14
151. Brown JM, Giaccia AJ (1998) The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58:1408–1416
152. Raghunand N, Gatenby RA, Gillies RJ (2014) Microenvironmental and cellular consequences of altered blood flow in tumours. Br J Radiol 76:S11–S22
153. Folkman J, Watson K, Ingber D, Hanahan D (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 339:58–61
154. Lee CH (2012) Engineering bacteria toward tumor targeting for cancer treatment: current state and perspectives. Appl Microbiol Biotechnol 93:517–523

155. Fox ME, Lemmon MJ, Mauchline ML, Davis TO, Giaccia AJ, Minton NP, Brown JM (1996) Anaerobic bacteria as a delivery system for cancer gene therapy: in vitro activation of 5-fluorocytosine by genetically engineered clostridia. Gene Ther 3:173–178
156. Lambin P, Theys J, Landuyt W, Rijken R, van der Kogel A, van der Schueren E, Hodgkiss R, Fowler J, Nuyts S, de Bruijn E, Van Mellaert L, Anné J (1998) Colonisation of *Clostridium* in the body is restricted to hypoxic and necrotic areas of tumours. Anaerobe 4:183–188
157. Nuyts S, Van Mellaert L, Theys J, Landuyt W, Lambin P, Anne J (2002) *Clostridium* spores for tumor-specific drug delivery. Anticancer Drugs 13:115–125
158. Theys J, Landuyt AW, Nuyts S, Van Mellaert L, Lambin P, Anne J (2001) *Clostridium* as a tumor-specific delivery system of therapeutic proteins. Cancer Detect Prev 25:548–557
159. Low KB, Ittensohn M, Le T, Platt J, Sodi S, Amoss M, Ash O, Carmichael E, Chakraborty A, Fischer J et al (1999) Lipid A mutant *Salmonella* with suppressed virulence and TNFalpha induction retain tumor-targeting in vivo. Nat Biotechnol 17:37–41
160. Pawelek JM, Low KB, Bermudes D (1997) Tumor-targeted *Salmonella* as a novel anticancer vector. Cancer Res 57:4537–4544
161. Fujimori M, Amano J, Taniguchi S (2002) The genus *Bifidobacterium* for cancer gene therapy. Curr Opin Drug Discov Devel 5:200–203
162. Yazawa K, Fujimori M, Nakamura T, Sasaki T, Amano J, Kano Y, Taniguchi S (2001) *Bifidobacterium longum* as a delivery system for gene therapy of chemically induced rat mammary tumors. Breast Cancer Res Treat 66:165–170
163. Yu YA, Shabahang S, Timiryasova TM, Zhang Q, Beltz R, Gentschev I, Goebel R, Szalay AA (2004) Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. Nat Biotechnol 22:313–320
164. Jean ATS, Zhang M, Forbes NS (2008) Bacterial therapies: completing the cancer treatment toolbox. Curr Opin Biotechnol 19:511–517
165. Nemunaitis J, Cunningham C, Senzer N, Kuhn J, Cramm J, Litz C, Cavagnolo R, Cahill A, Clairmont C, Sznol M (2003) Pilot trial of genetically modified, attenuated *Salmonella* expressing the *E. coli* cytosine deaminase gene in refractory cancer patients. Cancer Gene Ther 10:737–744
166. Toso JF, Gill VJ, Hwu P, Marincola FM, Restifo NP, Schwartzentruber DJ, Sherry RM, Topalian SL, Yang JC, Stock F et al (2002) Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma. J Clin Oncol 20: 142–152
167. Minton NP (2003) Clostridia in cancer therapy. Nat Rev Microbiol 1:237–242
168. Fujimori M (2006) Genetically engineered *Bifidobacterium* as a drug delivery system for systemic therapy of metastatic breast cancer patients. Breast Cancer 13:27–31
169. Kasinskas RW, Forbes NS (2007) *Salmonella typhimurium* lacking ribose chemoreceptors localize in tumor quiescence and induce apoptosis. Cancer Res 67:3201–3209
170. Liu SC, Minton NP, Giaccia AJ, Brown JM (2002) Anticancer efficacy of systemically delivered anaerobic bacteria as gene therapy vectors targeting tumor hypoxia/necrosis. Gene Ther 9:291–296
171. Theys J, Lambin P (2015) *Clostridium* to treat cancer: dream or reality? Ann Transl Med 3: S21
172. Jean ATS, Swofford CA, Panteli JT, Brentzel ZJ, Forbes NS (2014) Bacterial delivery of *Staphylococcus aureus* α-hemolysin causes regression and necrosis in murine tumors. Mol Ther 22:1266–1274
173. Wong S, Slavcev RA (2015) Treating cancer with infection: a review on bacterial cancer therapy. Lett Appl Microbiol 61:107–112
174. Danino T, Prindle A, Kwong GA, Skalak M, Li H, Allen K, Hasty J, Bhatia SN (2015) Programmable probiotics for detection of cancer in urine. Sci Transl Med 7:289ra84
175. Ringel Y, Quigley EMM, Lin HC (2012) Using probiotics in gastrointestinal disorders. Am J Gastroenterol Suppl 1:34–40

176. Gough E, Shaikh H, Manges AR (2011) Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. Clin Infect Dis 53:994–1002
177. Borody TJ, Khoruts A (2012) Fecal microbiota transplantation and emerging applications. Nat Rev Gastroenterol Hepatol 9:88–96
178. Aroniadis OC, Brandt LJ (2013) Fecal microbiota transplantation: past, present and future. Curr Opin Gastroenterol 29:79–84
179. Colman RJ, Rubin DT (2014) Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. J Crohn's Colitis 8:1569–1581
180. Campieri M, Gionchetti P (1999) Probiotics in inflammatory bowel disease: new insights to pathogenesis or a possible therapeutic alternative. Gastroenterology 116:1246–1249
181. Linskens RK, Huijsdens XW, Savelkoul PH, Vandenbroucke-Grauls CM, Meuwissen SG (2001) The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. Scand J Gastroenterol Suppl 234:29–40
182. Simon GL, Gorbach SL (1984) Intestinal flora in health and disease. Gastroenterology 86:174–193
183. Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A 95:6578–6583
184. Fanaro S, Chierici R, Guerrini P, Vigi V (2003) Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl 91:48–55
185. Martin R, Jimenez E, Heilig H, Fernandez L, Marin ML, Zoetendal EG, Rodriguez JM (2009) Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. Appl Environ Microbiol 75:965–969
186. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638
187. Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848
188. Hooper LV, Falk PG, Gordon JI (2000) Analyzing the molecular foundations of commensalism in the mouse intestine. Curr Opin Microbiol 3:79–85
189. Hooper LV, Midtvedt T, Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 22:283–307
190. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI (2004) The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 101:15718–15723
191. Claus SP, Tsang TM, Wang Y, Cloarec O, Skordi E, Martin FP, Rezzi S, Ross A, Kochhar S, Holmes E, Nicholson JK (2008) Systemic multicompartmental effects of the gut microbiome on mouse metabolic phenotypes. Mol Syst Biol 4:219
192. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG (2007) Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc Natl Acad Sci U S A 104:7617–7621
193. Lee YK, Puong KY, Ouwehand AC, Salminen S (2003) Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J Med Microbiol 52:925–930
194. Macpherson AJ, Harris NL (2004) Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 4:478–485
195. Martin FP, Sprenger N, Montoliu I, Rezzi S, Kochhar S, Nicholson JK (2010) Dietary modulation of gut functional ecology studied by fecal metabonomics. J Proteome Res 9:5284–5295
196. Martin FP, Wang Y, Sprenger N, Yap IK, Rezzi S, Ramadan Z, Pere-Trepat E, Rochat F, Cherbut C, van Bladeren P, Fay LB, Kochhar S, Lindon JC, Holmes E, Nicholson JK (2008) Top-down systems biology integration of conditional prebiotic modulated transgenomic interactions in a humanized microbiome mouse model. Mol Syst Biol 4:205

197. Noverr MC, Huffnagle GB (2004) Does the microbiota regulate immune responses outside the gut? Trends Microbiol 12:562–568
198. Pamer EG (2007) Immune responses to commensal and environmental microbes. Nat Immunol 8:1173–1178
199. Wells JM, Rossi O, Meijerink M, van Baarlen P (2010) Microbes and health sackler colloquium: epithelial crosstalk at the microbiota-mucosal interface. Proc Natl Acad Sci U S A 108(Suppl 1):4607–4614
200. Scanlan PD, Shanahan F, O'Mahony C, Marchesi JR (2006) Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease. J Clin Microbiol 44:3980–3988
201. Abrahamsson TR, Jakobsson HE, Andersson AF, Björksten B, Engstrand L, Jenmalm MC (2013) Low gut microbiota diversity in early infancy precedes asthma at school age. Clin Exp Allergy 44:842–850
202. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, Kuzeljevic B, Gold MJ, Britton HM, Lefebvre DL, Subbarao P, Mandhane P, Becker A, McNagny KM, Sears MR, Kollmann T, Mohn WW, Turvey SE, Finlay BB (2015) Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med 7:307ra152. doi:10.1126/scitranslmed.aab2271
203. Liou AP, Paziuk M, Luevano JM Jr, Machineni S, Turnbaugh PJ, Kaplan LM (2013) Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Sci Transl Med 5(178):178ra41. doi:10.1126/scitranslmed.3005687
204. Graf D, Di Cagno R, Fåk F, Flint HJ, Nyman M, Saarela M, Watzl B (2015) Contribution of diet to the composition of the human gut microbiota. Microb Ecol Health Dis 26:26164, http://dx.doi.org/10.3402/mehd.v26.26164
205. Modi SR, Collins JJ, Relman DA (2015) Antibiotics and the gut microbiota. J Clin Invest 124:4212–4218

Index