Silvio Silvério da Silva
Anuj Kumar Chandel *Editors*

D-Xylitol

Fermentative Production, Application and Commercialization

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Sugar, xylitol, honey and buffalo's milk are all sweet, but incomparable is the sweetness of the Lord*

Sheikh Farid

*Added by the editors related to the context of this book

About the Editors

Professor Silvio Silvério da Silva was born on 31 December 1959. He is a professor at the Department of Biotechnology, Engineering School of Lorena, University of São Paulo, Brazil. He completed his doctorate in Biochemical and Pharmaceutical Technology from the University of São Paulo (USP) and GBF German Research Centre for Biotechnology, Germany in 1994. Prof. Silva is a reviewer of several reputed scientific journals and also offers ad hoc consulting services to various institutions. He has published more than 130 papers in peer reviewed international journals and presented more than 455 scientific papers in international conference proceedings. He has also 18 book chapters to his credit. He has recorded two patents on technological processes for xylitol production. Prof. Silva has guided 10 doctoral students, 25 masters' dissertations and 55 scientific initiation students in the area of Applied Microbiology, Biochemical Engineering and Food Science and Technology. He has successfully completed 19 research projects funded by the Brazilian Government and private funding agencies. Prof. Silvio has been actively working on fermentative production of xylitol for 24 years.

His area of research is Microbial Technology harnessing the potential of lignocellulosic feedstock for the production of xylitol and bioethanol. He has visited several international research institutions and universities of various countries for the exchange of scientific knowledge on the production of value-added products from lignocellulose feedstock.

Dr. Anuj K. Chandel completed his doctorate in 2009 from Jawaharlal Nehru Technological University, Hyderabad, India. After receiving his masters in 2000 in biotechnology from the Indian Institute of Technology Roorkee, he joined Dalas Biotech Ltd. Bhiwadi for three years for the large-scale production of penicillin acylase and antibiotic intermediates. Subsequently, he worked at the University of Delhi in a nationwide research project on development of a bioethanol production process from lignocellulosic feedstock funded by the Department of Biotechnology (DBT), Ministry of Science and Technology, Govt. of India. He worked on this

project for 3 years. Later he joined Celestial Labs Ltd., Hyderabad as a research associate and pursued his doctoral studies on ethanol production from *Saccharum spontaneum* (wild sugarcane) using thermotolerant yeasts. After this, he did post doctoral studies at the University of Stellenbosch, South Africa working on development of fermentative processes for vaccine production from recombinant yeasts. Currently he is working with Prof. S. S. Silva, Engineering School of Lorena, University of São Paulo as a post doctoral researcher for the development of a fuel ethanol production process from sugarcane bagasse in a thematic project funded by FAPESP. He is the author of 35 articles in peer-reviewed journals and 12 book chapters.

Preface

Hemicellulose, the second most abundant polysaccharide in nature, is well suited for the production of value-added products like xylitol, ethanol, protein rich food and fodder due to its enormous availability, low cost and environmentally benign process. In general, the major fraction in hemicellulose is pentosans (xylan), and the microbial conversion of xylan into xylitol is now possible on an industrial scale. D-Xylitol is found in low content as a natural constituent of many fruits and vegetables. It is a five-carbon sugar polyol and has been used as a food additive and sweetening agent to replace sucrose, especially for non-insulin dependent diabetics. It has multiple beneficial health effects such as the prevention of dental caries, osteoporosis and acute otitis media. In industries, it has been produced by chemical reduction of D-xylose, presented in xylan. Advancements in biotechnology such as screening of microorganisms, manipulation of pentose-utilizing microorganisms by molecular biology-based approach modifications, developments in fermentation processes and downstream processing could enhance the production of xylitol. Commercially, cheaper sources of carbohydrates, derived from photosynthetic biomass and modified fermentation conditions, could lead to more cost-effective production of xylitol. These methodologies would open new markets and create new applications of xylitol. This book was written keeping in mind the fundamental aspects of hemicellulose break-down into its monomeric constituents: D-xylose utilization for xylitol production by different bioconversion methods, xylitol recovery and its analysis in laboratories, economic evaluation and diverse applications of xylitol.

This book has been divided into five parts. Part I deals with the different kinds of hydrolytic methods applied to different kinds of biomass sources for xylose recovery and detoxification of xylose rich hydrolysates. In this part, three chapters are included. Chapters 1 and 2 highlight the hydrolytic methods for hemicellulosic fraction of various lignocellulosic materials. For depolymerisation of the hemicellulosic fraction of the plant cell wall, generally acid-catalysed processes are employed at high temperature and pressure. During the deconstruction of hemicelluloses, other unwanted products such as furans, phenolics and weak acids are also generated in addition to sugars. It is necessary to eliminate these inhibitors

from the hydrolysates prior to fermentation in order to get satisfactory product yields and productivities. Chapter 3 summarizes the different methods explored for detoxification of lignocellulose hydrolysates. Part II aims to explore the microorganisms, media formulations, and fermentation methods, as well as the enzymatic production of xylitol and bioenergetics analysis for xylitol production. This part constitutes the major part of the book and contains six chapters. Microbial strains, particularly yeasts used for xylitol production, metabolic pathways, physiological pathways, strain improvement methods, statistical optimization of various influential parameters, fermentation strategies and enzymatic production of xylitol have been discussed in detail in Chaps. 4–8, respectively. In addition, bioenergetic analysis of xylitol production (carbon balance and xylitol yields, and productivities from different kind of substrates adopting various fermentative strategies) employing different microorganisms has been summarized in Chap. 9. Part III describes the xylitol recovery and the analytical methods explored for xylitol quantification. Chapter 10 is concerned with xylitol recovery and crystallization from chemical synthesis and biotechnologically-based production strategies. Chapter 11 presents an appraisal on the analytical methods for xylitol quantification. Analysis on economic feasibility of biotechnological production of xylitol and market demand has been summarized in Part IV. Chapter 12 describes the key factors which influence the large-scale production of xylitol. This chapter concludes the technological barriers and methods to overcome for successful xylitol production on an industrial scale. Chapter 13 provides an overview on commercialization of xylitol, economic analysis of fermentative production of xylitol and recovery and a brief research on market demands of xylitol in future. Finally, Part IV is dedicated to the applications (medical and non-medical) of xylitol. Chapter 14 describes the application of xylitol in food/feed-based industrial sectors and summarizes the health benefits of xylitol. Chapter 15 provides an overview on the medical applications of xylitol in addition to promising future applications which may have impact on increased xylitol demands.

We sincerely believe that this book should cater to the needs of graduate and post graduate students, researchers of the biochemistry, microbiology, biotechnology, biochemical engineering, pharmacy, medicine, scientists and engineers both in academia and industry and business entrepreneurs. We would like to thank our colleagues MGA Felipe, Ines C. Roberto, Walter de Carvalho, Attilio Converti, M. Vitolo, Hou-Rui Zhang, Om V. Singh, Ricardo de Freitas Branco, R. C. L. B Rodriguez, Larissa Canilha, Solange I. Mussatto and Felipe F. A. Antunes for their constant help and encouragement. We also appreciate the timely help of our departmental staff (Nicanor, Paulo Roberto, Nadir, Walkiria, Isnaldi and Cibele) for the completion of this book. We are also thankful to doctoral and masters students of our department for their necessary help. We are grateful to the publishers for their necessary help and cooperation. Anuj would like to express sincere thanks to his wife, Meenakshi, and son, Abhay, for their patience and immense support during editing of this book. Silvio Silvério is also thankful to his wife, Deyse, and daughter, Isabela, for their cooperation while editing this book.

Editors are also thankful to EEL/USP for providing necessary facilities and basic infrastructure. We are grateful to the Government funding agencies of Brazil, particularly FAPESP, CNPq and CAPES, for the financial assistance to our lab to carry out the research work on various aspects of lignocellulose biotechnology. Last, but certainly not least, we welcome the reader's opinions and suggestions to improve future editions. Readers' benefits will be the best reward for the authors.

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Part V Applications of Xylitol

Part I
Biomass Hydrolysis for Xylose Recovery and Detoxification

Chapter 1
Deconstruction of the Hemicellulose Fraction from Lignocellulosic Materials into Simple Sugars

Francisco M. Gírio, Florbela Carvalheiro, Luís C. Duarte and Rafał Bogel-Łukasik

Abstract Hemicelluloses hold a great promise for the production of added-value compounds in the biorefinery framework. Specifically, the xylan-rich hemicelluloses from hardwoods and agro-industrial residues present themselves as effective feedstock choices for the biotechnological production of xylitol. This paper reviews the various hemicellulose structures present in such materials and critically evaluates the available processing options to produce xylose-rich fermentable hydrolysates. Currently, acid-based processes still present the best trade-off between operation easiness and xylose yield and recovery. Nevertheless, concerns regarding the impact of the fractionation processes on the overall upgradability of all biomass fractions (namely, cellulose and specially lignin) may turn the route to other strategies. Specifically, the combined/sequential use of processes targeting hemicellulose dissolution and hydrolysis might hold great promise for the economical production of pentoses.

Keywords Lignocellulose · Hemicellulose · Hydrolysis · Enzymatic hydrolysis · Xylose

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1.1 Introduction

Xylose is the second most common monosaccharide in nature. It appears in the hemicellulosic fraction of mainly hardwoods and herbaceous materials from which it is not easily removed. As such, the availability of cheap xylose-rich solutions is one of the major bottlenecks for the production of xylitol.

In recent years, many diverse hydrolytic technologies and various biological and non-biological process options have been developed, both for the removal and/or hydrolysis of hemicelluloses. Although these processes have been developed mainly as pre-treatment processes to facilitate the enzymatic hydrolysis of cellulose, recently they have become to be understood as fractionation processes by themselves, which strive (and succeed) to obtain high sugars (pentoses and mainly xylose) recovery, that enable a more complete/extensive biomass upgrade/valorization. Unfortunately, due to the chemical complexity of hemicelluloses, the hydrolysates obtained typically contain not only the hemicellulosic sugars, but also structural aliphatic or phenolic acids, as well as other compounds such as furaldehydes, and other weak acids produced from sugars degradation that can potentially act as inhibitors to the microbial xylitol production.

In this paper, a review on the main hemicellulosic features is presented, followed by a critical appraisal of hemicelluloses fractionation processes and their potential influence on xylitol production.

1.2 Structure and Composition of Xylose Containing Hemicelluloses

From the chemical point of view, components of lignocellulosic materials (LCM) can be classified as structural components and extractives. The first make up the structure of cell walls and are responsible for the shape of the cells and for most of the physical and chemical properties of LCM. These include insoluble polymeric components such as cellulose, hemicellulose and lignin. Their removal requires the use of chemical and/or mechanical treatments in order to produce depolymerization or partial dissolution.

Other polymeric components present in lesser extent and often varying quantities are pectines, starch and proteins.

Concerning the organization of structural polymers, the cell wall consists of a microfibrilar cellulose backbone enveloped by hemicellulose molecules organized as successive ribbon-like structures, with lignin occupying the empty spaces between the hemicellulose molecules (Pereira et al. 2003).

Hemicellulose is a physical barrier which surrounds the cellulose fibers and can protect the cellulose. Many fractionation methods were shown to be able to remove hemicelluloses, although most of these processes partly remove the lignin as well.

The chemical composition of LCM varies according to the origin, species and genetic and environmental factors. In general, hemicellulose content is higher in herbaceous materials such as agricultural residues. Hardwoods have the highest cellulose content whereas lignin content is usually higher in softwoods.

Hemicelluloses are a heterogeneous class of polymers representing, in general, 15–35 % of plant biomass and which may contain pentoses (*β*-D-xylose, α-L-arabinose), hexoses (*β*-D-mannose, *β*-D-glucose, α-D-galactose) and/or uronic acids (α-D-glucuronic, α-D-4-*O*-methylgalacturonic and α-D-galacturonic acids). Other sugars such as α-L-rhamnose and α-L-fucose may also be present in small amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups. Homopolymers of xylose, so-called homoxylans, only occur in seaweeds (red and green algae), and will not be discussed further. The most relevant hemicelluloses are xylans and glucomannans, with xylans being the most abundant. The structure of hemicelluloses present in hardwoods, grasses and cereals is presented in Table 1.1.

Xylans are the main hemicellulose type in these materials, although small amounts of glucomannans may occur. Upon hydrolysis, the hemicelluloses break down into their monomers (Table 1.2).

1.2.1 Glucuronoxylans

Glucuronoxylans (GX) (*O*-acetyl-4-*O*-methyglucuronoxylan) are the main hemicellulose of hardwoods, which can also contain small amounts of glucomannans (GM). In hardwoods, GX represent 15–30 % of their dry mass (Alén 2000) and consist of a linear backbone of *β*-D-xylopyranosyl units (xyl*p*) linked by *β*-(1,4) glycosidic bonds. Some xylose units are acetylated at C2 and C3, and one in ten molecules has an uronic acid group (4-*O*-methylglucuronic acid) attached by α-(1,2) linkages. The percentage of acetyl groups ranges between 8 and 17 % of total xylan, corresponding, on average, to 3.5–7 acetyl groups per 10 xylose units (Alén 2000). The 4-*O*-methylglucuronic side groups are more resistant to acids than the Xylp and acetyl groups. Besides these main structural units, GX may also contain small amounts of L-rhamnose and galacturonic acid. The latter increases the polymer resistance to alkaline agents. The average degree of polymerization (DP) of GX is in the range of 100–200 (Pereira et al. 2003).

1.2.2 Arabinoglucuronoxylans

Arabinoglucuronoxylans (AGX) (arabino-4-*O*-metylglucuronoxylans) are the major components of non-woody materials such as agricultural crops. They also consist of a linear *β*-(1,4)-D-xylopyranose backbone containing 4-*O*-metil-α-D-glucopiranosyl uronic acid and α-L-arabinofuranosyl linked by α-(1,2) and α-(1,3)

Table 1.1 Main types of xylose containing polysaccharides present in hemicelluloses of hardwoods, grasses and cereals (*Gramineae*) (adapted and modified from Gírio et al. 2010)

Polysaccharide type	Biological origin	Amount[a]	Units		
			Backbone	Side chains	Linkage
Xyloglucan (XG)	Hardwoods, grasses	2–25	β-D-Glc*p* β-D-Xyl*p*	β-D-Xyl*p* β-D-Gal*p* α-L-Ara*f* α-L-Fuc*p* Acetyl	β-(1 → 4) α-(1 → 3) β- (1 → 2) α-(1 → 2) α-(1 → 2)
Glucuronoxylan (GX)	Hardwoods	15–30	β-D-Xyl*p*	4-*O*-Me-α-D-GlcpA Acetyl	α-(1 → 2)
Arabinoglucuronoxylan (AGX)	Grasses and cereals, softwoods	5–10	β-D-Xyl*p*	4-*O*-Me-α-D-GlcpA β-L-Ara*f*	α-(1 → 2) α-(1 → 3)
Arabinoxylans (AX)	Cereals	0.15–30	β-D-Xyl*p*	α-L-Ara*f* Feruloy	α-(1 → 2) α-(1 → 3)
Glucuronoarabinoxylans (GAX)	Grasses and cereals	15–30	β-D-Xyl*p*	α-L-Ara*f* 4-*O*-Me-α-D-GlcpA Acetyl	α-(1 → 2) α-(1 → 3)

DP degree of polymerization
[a]% on dry biomass

Table 1.2 Hemicellulose composition of various lignocellulosic materials that have been already used for xylitol production[a,b,c]

Raw material	Xyl	Ara	Man	Gal	Rha	UA	AcG	References
Hardwoods								
Aspen	18–27.3	0.7–4.0	0.9–2.4	0.6–1.5	0.5	4.8–5.9	4.3	Fengel and Wegener (1983), Grohmann et al. (1985), Schell et al. (1999), Taherzadeh et al. (1997)
Birch	18.5–24.9	0.3–0.5	1.8–3.2	0.7–1.3	0.6	3.6–6.3	3.7–3.9	Fengel and Wegener (1983), Taherzadeh et al. (1997)
Eucalypt	14–19.1	0.6–1	1–2.0	1–1.9	0.3–1	2	3–3.6	Garrote et al. (1999b), Kabel et al. (2002), Miranda and Pereira (2002), Pereira (1988)
Oak	21.7	1.0	2.3	1.9	–	3	3.5	Conner (1984)
Poplar	17.7–21.2	0.9–1.4	3.3–3.5	1.1	–	2.3–3.7	0.5–3.9	Allen et al. (2001a), Fengel and Wegener (1983), Torget and Hsu (1994)
Agricultural and agro-industrial materials								
Barley bran	29.2	5.7	–	–	–	–	1.9	Cruz et al. (2001)
Brewery's spent grain	15–23.4	8–10.2	0	1	0	2	0.8–1.1	Carvalheiro et al. (2004b), Garrote et al. (1999b)
Corn cobs	28–35.3	3.2–5.0	–	1–1.2	1	3	1.9–3.8	Garrote et al. (2001b), Kabel et al. (2002), Ropars et al. (1992), Torget et al. (1991)
Corn fiber	21.6	11.4	–	4.4	–	–	–	Allen et al. (2001b)
Corn stover	14.8–25.2	2–3.6	0.3–0.4	0.8–2.2	–	–	1.7–1.9	Lee (1997), Torget et al. (1991, 2000)
Distilled grape marc	8.3	1.5	–	–	–	–	1.5	Salgado et al. (2012b)
Pistachio shells	33–50	0	–	–	–	–	5.4	Sazaki et al. (2009)
Rice straw	14.8–23	2.7–4.5	1.8	0.4	–	–	–	Lee (1997), Roberto et al. (2003)

(continued)

Table 1.2 (continued)

Raw material	Xyl	Ara	Man	Gal	Rha	UA	AcG	References
Sorghum straw	21.5	5.5	–	–	–	–	–	Tellez-Luis et al. (2002)
Sugar cane bagasse	20.5–25.6	1.3–6.3	0.5–0.6	1.6	–	–	2.6–	Aguilar et al. (2002), Canilha et al. (2011), Neureiter et al. (2002)
Vineshoot trimmings	12.8	0.9	–	–	–	–	5.3	Bustos et al. (2004), Salgado et al. (2012a)
Wheat straw	19.2–21.0	2.4–3.8	0–0.8	1.7–2.4	–	–	1.7–2.6	Carvalheiro et al. (2009), Kabel et al. (2007), Nabarlatz et al. (2007)

Xyl xylose, *Ara* arabinose, *Man* mannose, *Gal* galactose, *Rha* rhamnose, *UA* uronic acids, *AcG* acetyl groups

[a] Non-glycosidic units

[b] Expressed as g/100 g of dry material

[c] The percentages of oses were, in some cases, calculated from the corresponding "polymers"

glycosidic bonds (Timell 1965; Woodward 1984). The typical ratio arabinose:glucuronic acid:xylose is 1:2:8 (Alén 2000). Conversely to hardwoods xylan, AGX might be less acetylated, but may contain low amounts of galacturonic acid and rhamnose. The average DP of AGX ranges between 50 and 185 (Pereira et al. 2003).

1.2.3 Xyloglucans

Xyloglucans (XG) are quantitatively predominant hemicellulosic polysaccharide types in the primary cell walls of hardwoods (mainly in *dicotyledonae* and less in *monocotyledonae*) (de Vries and Visser 2001) and can also appear in small amounts in grasses. Xyloglucans consist of β-1,4-linked D-glucose backbone with 75 % of these residues substituted at *O*-6 with D-xylose. l–Arabinose and D-galactose residues can be attached to the xylose residues forming di- or triglycosyl side chains. Also L-fucose has been detected attached to galactose residues. In addition, xyloglucans can contain *O*-linked acetyl groups (Maruyama et al. 1996, Sims et al. 1996). Xyloglucans interact with cellulose microfibrils by the formation of hydrogen bonds, thus contributing to the structural integrity of the cellulose network (Carpita and Gibeaut 1993; de Vries and Visser 2001).

1.2.4 Arabinoxylans

Arabinoxylans (AX) represent the major hemicellulose structures of the cereal grain cell walls. In AX, the linear β-(1,4)-D-xylopyranose backbone is substituted by α-L-arabinofuranosyl units in the positions 2-O and/or 3-O and by the α-D-glucopyranosyl uronic unit or its 4-*O*-methyl derivative in the position 2-O (Brillouet et al. 1982; Shibuya and Iwasaki 1985). *O*-acetyl substituents may also occur (Ishii 1991; Wende and Fry 1997). Arabinofuranosyl residues of AX may also be esterified with hydroxycinnamic acid residues, e.g. ferulic and p-coumaric acids (Wende and Fry 1997). Dimerization of esterified phenolic compounds may also lead to inter- and intramolecular cross-links of xylan. The physical and/or covalent interactions with other cell-wall constituents restricts the extractability of xylan. In lignified tissues, for example, xylan is ester linked through its uronic acid side chains to lignin (Ebringerová et al. 2005).

1.2.5 Complex Heteroxylans

Complex heteroxylans (CHX) are present in cereals, seeds, gum exudates and mucilages and they are structurally more complex (Stephen 1983). In this case the

β-(1,4)-D-xylopyranose backbone is decorated with single uronic acid and arabinosyl residues and also various mono- and oligoglycosyl side chains.

1.3 Selective Fractionation of Hemicelluloses

The main process options for the selective fractionation of hemicelluloses from biomass include the use of acids, water (liquid or steam), alkaline agents and organic solvents. More recently, novel processes using inorganic salts, solid acids, supercritical fluids and ionic liquids have also been gaining increasing interest.

Alkaline agents and organic solvents are not selective towards hemicellulose as they also remove lignin, which in turn can hinder the bioconversion process since lignin-derived compounds are usually microbial growth inhibitors. And hence they will not be described in depth in this work. Also, the highly complex structure of the LCM materials, as described above, does not easily enable a direct hemicellulose enzymatic hydrolysis of the untreated biomass. As such, this later approach is rarely carried out, and it will be mainly discussed as a means for the hydrolysis of previously dissolved hemicelluloses, e.g. soluble oligosaccharides.

Therefore, acid/water/steam processes are the most commonly applied to yield a selective hydrolysis of hemicelluloses and producing hemicellulose-rich liquids totally or partially hydrolyzed into monomeric and/or oligomeric sugars (see below). In the latter case, a subsequent hydrolysis step is required to further hydrolyze the oligosaccharides into monosaccharides (Table 1.3). Depending on the operational conditions, degradation products from sugars (furan derivatives and aliphatic acids) and to a less extent, from lignin (phenolics) can also be formed. These compounds may also inhibit the fermentation processes, leading to lower xylitol yields and productivities and, therefore, might be required to carry out a prior detoxification treatment.

The following sections review the fundamental modes of action of relevant methods for the selective recovery of pentoses from biomass.

1.3.1 Hydrothermal Treatments

The simpler hemicellulose fractionating processes are the non-catalyzed ones, also called hydrothermal processes. These mainly include two process configurations: (1) liquid hot water (autohydrolysis) and (2) steam-explosion treatments. As no chemical catalysts are added, they are both economic and environmentally attractive. The operational conditions used vary widely and mainly depend on the process configuration. Typically, autohydrolysis is carried out at slightly lower temperatures, but for longer periods as compared to steam explosion that is operated at higher pressures.

Table 1.3 Hemicellulosic hydrolysates composition (g l^{-1}) obtained from diverse lignocellulosic raw materials used for xylitol production

Material	Hydrolysis process	Xyl	Glc	Ara	Gal	Man	Acetic	Formic	Furfural	HMF	Phenolics	References
Hardwoods												
Aspen	Dilute acid hydrolysis	15.3	6.3	0.9	0.8	1.2	0.6	–	–	–	–	Prior et al. (1989)
Eucalypt	Dilute acid hydrolysis	30.0	1.5	2.8	3.7	1.0	10	–	–	–	–	Ferrari et al. (1992)
Eucalypt	Dilute acid hydrolysis	18.0	3.6	0.6	–	–	5.2	–	<0.5	–	–	Parajó et al. (1997)
Eucalypt	Dilute acid hydrolysis	19.2	2.5	0.4	–	–	5.0	–	–	–	–	Canettieri et al. (2001)
Eucalypt	Dilute acid hydrolysis	17.1	1.7	2.1	–	–	5.2	–	0.50	<0.1	–	Cruz et al. (2001)
Eucalypt	Autohydrolysis + Dilute acid posthydrolysis	17.1	1.7	2.1	–	–	5.2	–	<0.50	–	–	Diz et al. (2002)
Oak	Dilute acid hydrolysis	43.5	9.0	0	3.3	2.9	10.9	–	0.30	0.9	–	Perego et al. (1990)
Agricultural and agro-industrial materials												
Barley bran	Dilute acid hydrolysis	35.6	5.9	7.1	–	–	2.4	–	0.30	<0.1	–	Cruz et al. (2001)
Brewery's spent grain	Dilute acid hydrolysis	13.2	0.32	8.21	–	–	0.60	–	0.02	0.03	–	Mussatto and Roberto (2006)
Brewery's spent grain	Dilute acid hydrolysis	26.7	4.0	12.8	–	–	1.5	0.23	0.29	0.02	0.91	Carvalheiro et al. (2004a)
Brewery's spent grain	Autohydrolysis + Dilute acid posthydrolysis	14.7	5.4	6.2	–	–	1.32	0.52	0.51	0.05	1.16	Carvalheiro et al. (2005)
Brewery's spent grain	Autohydrolysis + Enzymatic hydrolysis	8.7	7.7	4.4	–	–	1.4				1.14	Duarte et al. (2004)
Corn cobs	Dilute acid hydrolysis	35.3	3.2	4.6	–	–	3.7	–	0.30	<0.1	–	Cruz et al. (2001)
Corn cobs	Autohydrolysis + enzymatic hydrolysis	24	1.75				3.88					Vázquez et al. (2001)
Corn stover	Dilute acid Hydrolysis	12.11	1.11				1.06		2.2	<0.1		Mancilha and Karim (2003)
Corn leaves	Dilute acid hydrolysis	22.5	2.0	3.6	–	–	2.3	–	0.30	<0.1	–	Cruz et al. (2001)
Rice straw	Dilute acid hydrolysis	16.2	6.0	2.2	–	–	0.63	–	–	–	–	Roberto et al. (1994)
Rice straw	Dilute acid hydrolysis	16.4	4.4	2.4	–	–	1.4	–	0.41	<0.1	–	Roberto et al. (1994)

(continued)

Table 1.3 (continued)

Material	Hydrolysis process	Xyl	Glc	Ara	Gal	Man	Acetic	Formic	Furfural	HMF	Phenolics	References
Distilled grape marc	Dilute acid hydrolysis	9.3	2.2	1.1			1.6	–	0	0		Salgado et al. (2012b)
Pistachio shells	Dilute acid hydrolysis	41.8	0.98	0.6			8.1	1.1	2.2			Sazaki et al. (2009)
Sorghum straw	Dilute acid hydrolysis	19.1	6.7	5.3			1.1		0.8	–	–	Sepulveda-Huerta et al. (2006)
Sorghum straw	Dilute acid hydrolysis	17.89	2.1	1.81			1.87		0.04	1.56	2.12	Sene et al. (2011)
Sugarcane bagasse	Dilute acid hydrolysis	46.0	3.0	5.0	–	–	10.0	–	0.60	<0.1	–	van Zyl et al. (1988)
Sugarcane bagasse	Dilute acid hydrolysis	18.5	1.2	1.7	–	–	6.5	–	<0.1	<0.1	–	Rodrigues et al. (1999)
Sugarcane bagasse	Dilute acid hydrolysis	26.4	5.5	2.1	–	–	5.5	–	<0.5	<0.1	–	Rodrigues et al. (1998)
Sugarcane bagasse	Dilute acid hydrolysis	18.5	5.1	–	–	–	3.7	–	2.0	<0.1	–	Pessoa et al. (1996)
Grape marc	Dilute acid hydrolysis	9.3	2.2	1.1			1.6	–				Salgado et al. (2012b)
Vine trim wastes	Dilute acid hydrolysis	14.7	10.7	1.6	4.6							Garcia-Dieguez et al. (2011)
Wheat straw	Dilute acid hydrolysis	15.4	4.4	2.2	–	–	1.2		0.53	<0.1	–	Canilha et al. (2006)
Wheat straw	Autohydrolysis + dilute acid posthydrolysis	10.2	1.3	2.0	1.6	1.1	2.4	1.5	0.8	0.1	1.67	Duarte et al. (2009)

Xyl xylose, *Glc* glucose, *Ara* arabinose, *Gal* galactose, *Man* mannose, *HMF* hydroxymethylfurfural

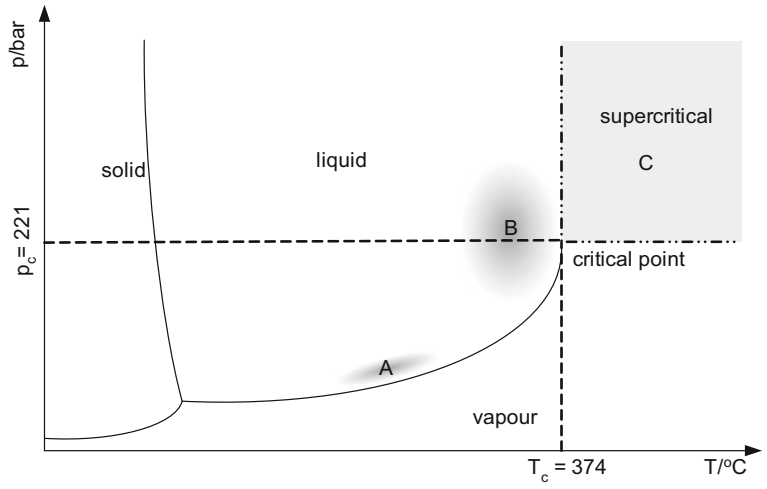

Fig. 1.1 Typical ranges for different water-based processes as a function of temperature and pressure. **a** Autohydrolysis, LHW. **b** Subcritical conditions. **c** Supercritical conditions. *Lines* represent the phase diagram for water (reproduced from Gírio et al. 2010 with permission)

1.3.1.1 Autohydrolysis

The autohydrolysis process uses compressed hot water (pressure above saturation point). This process is also called Liquid Hot Water treatment (LWH), as this is the water state for the operation temperatures usually used (between 150 and 230 °C) (Garrote et al. 1999a, b) and pressure (Fig. 1.1). Reaction time may vary from seconds up to hours, depending mainly on temperature. Solids concentration, usually referred to as the liquid-to-solid ratio (LSR), may range between 2 and 100 (w/w) (Garrote et al. 1999a; Santos et al. 2011; van Walsum et al. 1996), although the most common values are around 7–10 (Carvalheiro et al. 2004b, 2009). Higher LSR are usually associated with continuous reactors whereas lower LSR are, in general, used in the processes that employ steam. The potential LSR also depends on the density of the raw material. Autohydrolysis has a similar mechanism to dilute-acid hydrolysis. Both are hydronium ion catalyzed processes. The catalysts in autohydrolysis are hydronium ions generated in situ by water auto-ionization and acetic acid resulting from acetyl substituents of hemicelluloses, the later having a much higher contribution to the hydrolysis (Carrasco 1989; Heitz et al. 1986). It has also been suggested that uronic acids may also contribute to the formation of hydronium ions (Conner 1984) but their role in the hydrolysis is still not completely understood.

This process has been successfully applied to several LCM including hardwoods, e.g. *Eucalyptus globulus* (Garrote and Parajó 2002; Gullón et al. 2008), energetic cultures, e.g. *Arundo donax* (Caparros et al. 2007), and agricultural and agro-industrial materials, e.g. corn cobs (Garrote et al. 2008), wheat straw

(Carvalheiro et al. 2009), rice husks (Vegas et al. 2008b), and brewery's spent grain (Carvalheiro et al. 2004b).

A relatively high hemicellulose recovery in the range of 55–84 %, together with low levels of inhibitory by-products, has been obtained (Aoyama et al. 1995; Boussarsar et al. 2009; Carvalheiro et al. 2009; Garrote and Parajó 2002). Cellulose and lignin are not significantly affected, yielding a cellulose- and lignin enriched solid phase (that can be used in other processes) together with a liquid fraction with a relative low concentration of potential fermentation inhibitors. Owing to the mild pH the corrosion problems are reduced, and the steps of acid recycling and precipitates removal are no longer necessary. This reduces both the capital and operational costs with beneficial consequences on the environment, compared to other hydrolytic technologies.

The main drawback of this process is that the dissolved pentoses appear mainly in oligomeric form (Allen et al. 2001a, b; Aoyama et al. 1995; Boussarsar et al. 2009; Carvalheiro et al. 2009; Garrote and Parajó 2002), requiring a further posthydrolysis step in order to obtain monomeric pentoses to be converted to xylitol. However, it has the advantage of being highly selective towards hemicelluloses.

1.3.1.2 Steam Explosion

Steam explosion has been described as a thermomechanochemical process where the breakdown of structural components is aided by heat in the form of steam (thermo-), shear forces due to the expansion of moisture (mechano-), and hydrolysis of glycosidic bonds (chemical) once it is (self)-catalyzed (e.g. by the biomass derived acetic acid, and possibly by added catalysts) (Chornet and Overend 1988). Experimentally, the material is heated (preferably to temperatures below 240 °C) using high-pressure steam up to a few minutes. The steam condenses under the high pressure thereby "wetting" the material, which is then "exploded" when the pressure within the reactor is rapidly released. The forces resulting from decompression lead to a disaggregation of lignocellulosic matrix, breaking down inter- and intra-molecular linkages (Carrasco 1989).

The effectiveness of steam explosion treatment has been studied for many lignocellulosic materials. Moreover, some steam explosion processes were even implemented at pilot and industrial scale, mostly used as pre-treatment methods for Kraft pulps bleaching in the paper and pulp industry. The most well known is the STAKE continuous steam explosion digester (Ballesteros et al. 2000; Kokta 1991).

Most steam treatments induce high hemicellulose solubility with reported sugar recoveries between 45 and 69 % for non-catalyzed operation (Ballesteros et al. 2002; Heitz et al. 1991; Martín et al. 2008; Ruiz et al. 2008) and only slight lignin dissolution. Unfortunately, the main drawback is that the hemicellulosic sugars are mainly in the form of oligosaccharides A way to overcome this is to impregnate the biomass with acid catalysts, namely, H_2SO_4 or SO_2, prior to the operation.

In this case, a higher pentose yield is expected. For example, when 1% H_2SO_4 is added, maximum pentose yield can increase about 30 % for olive tree pruning (Cara et al. 2008). This brings the process close to the acid hydrolysis conditions.

1.3.2 Acid Hydrolysis

1.3.2.1 Direct Acid Hydrolysis of Biomass

Acid catalyzed processes can be, in general, divided in two approaches, based on concentrate-acid/low temperature and dilute-acid/high temperature hydrolysis. Sulfuric acid is the most common acid employed although other mineral acids such as hydrochloric, nitric and trifluoracetic acids have also been assayed (Ramos 2003). In dilute-acid processes phosphoric acid and weak organic acids such as acetic, maleic, succinic and citric acids, have also been reported (Carvalho et al. 2004; Gamez et al. 2006; Mosier et al. 2001).

Concentrate-acid processes enable the hydrolysis of both hemicelluloses and cellulose. The dissolution of polysaccharides is reached using different acid concentrations, like 72 % H_2SO_4, 41 % HCl or 100 % TFA (Fengel and Wegener 1983). HCl and TFA have the advantage of being more easily recovered. Concentrate-acid based processes have the advantage of allowing operating at low/medium temperatures, leading to reduction in the operational costs. Under appropriate operation conditions the formation of degradation products can be low, although its formation rate can be severely affected by slight changes in temperature (Camacho et al. 1996). One of the key steps of these processes is the acid recovery, which is mandatory for their economic viability (Goldstein 1983). Moreover, the equipment corrosion is an additional disadvantage. Nevertheless, in recent years a renewable interest in these processes has been observed (Zhang et al. 2007).

Dilute-acid processes are more specific towards hemicellulose, rendering the cellulose un-hydrolyzed (but not unaffected), in the solid fraction, amenable for a further enzymatic or acid hydrolysis. Typically, hemicellulose hydrolysis is carried out using sulfuric acid in the range 0.5–1.5 % (w/w) and temperatures above 121–160 °C. Dilute-acid processes usually yield sugar recoveries from hemicelluloses above 70 % up to >95 % (Allen et al. 2001a; Carvalheiro et al. 2004a; Marzialetti et al. 2008; Monavari et al. 2009). However, both for dilute- and concentrate-acid hydrolysis approaches, the acid has to be removed/neutralized before fermentation, yielding large amounts of waste (gypsum and others) leading also to a loss of hydrolysates as well.

Compared to the concentrate-acid hydrolysis, one of the advantages of dilute-acid hydrolysis is the relatively low acid consumptions, limited problems associated with equipment corrosion and less energy demanding for acid recovery. Under controlled conditions, the levels of the degradation compounds generated can also be low.

Table 1.4 Typical conditions for acid posthydrolysis of autohydrolysis liquors in order to obtain monomeric sugars from oligosaccharides

Raw material	Initial treatment	Catalyst concentration (%)	Reaction time (min)	Temperature (°C)	References
Sugarcane	Autohydrolysis	6.5	120	100	Saska and Ozer (1995)
Sugarcane bagasse	Autohydrolysis	4	60	120	Allen et al. (1996)
Douglas fir (*Pseudotsuga menziesii*) wood chips	SO_2-catalyzed steam explosion	0–3	60	120	Shevchenko et al. (2000)
Eucalyptus globulus wood	Autohydrolysis	0.5–2	0–602	105.5–135	Garrote et al. (2001a)
Corn cobs	Autohydrolysis	0.5–2		101.5–135	Garrote et al. (2001c)[a]
Corn cobs	Autohydrolysis	0.5	165	125	Rivas et al. (2002)[a]
White fir (70 %) and ponderosa pine (30 %)	SO_2-catalyzed steam explosion	3	60	121	Boussaid et al. (2001)
Brewery's spent grain	Autohydrolysis	1–4	15–60	121	Duarte et al. (2004)

[a] No optimization was performed

As an alternative to the conventional dilute-acid processes, the addition of CO_2 to aqueous solutions, taking advantage of the carbonic acid formation, has been described (van Walsum and Shi 2004), but the results obtained were not very encouraging yet.

1.3.2.2 Acid Hydrolysis of Soluble Oligosaccharides

Another application of the acid hydrolysis is related to the posthydrolysis step after mild processes. Actually, the most effective treatments for the selective fractionation of hemicelluloses render the soluble hemicellulose, either totally or in a very significant amount, in oligomeric form (see autohydrolysis and alkaline processes). Since the most efficient xylitol producing microorganisms are not able to assimilate XOS, their hydrolysis is a compulsory requirement.

The posthydrolysis options for the XOS hydrolysis can be reduced to acid (Allen et al. 1996; Boussaid et al. 2001; Duarte et al. 2004, 2009; Garrote et al. 2001a, c; Saska and Ozer 1995; Shevchenko et al. 2000), or enzymatic catalyzed processes (see below).

The main factors affecting monosaccharide recovery in dilute-acid posthydrolysis are catalyst concentration, reaction time, and temperature (Table 1.4).

This process has been applied to hydrolysates obtained from many types of lignocellulosic materials including hardwoods (Garrote et al. 2001a) and herbaceous materials (Allen et al. 1996; Duarte et al. 2004, 2009; Garrote et al. 2001c; Saska and Ozer 1995). The main catalyst reported is sulfuric acid (Duarte et al. 2004, 2009; Garrote et al. 2001a, c; Saska and Ozer 1995; Shevchenko et al. 2000). Under fully optimized posthydrolysis conditions, sugar recovery can reach values as high as 100 % (Duarte et al. 2004, 2009; Garrote et al. 2001a, c; Saska and Ozer 1995; Shevchenko et al. 2000).

1.3.2.3 Solid (Super) Acids

Super acids may be defined as acids stronger than 100 % sulfuric acid (also known as Brønsted superacids), or as acids that are stronger than anhydrous aluminum trichloride (also known as Lewis superacids). Solid superacids are composed of solid media that are treated with either Brønsted or Lewis acids. Although the use of solid superacids for hydrolyzing oligosaccharides and polysaccharides was already proposed in the 1980s (Hahn-Hägerdal et al. 1984; Kim and Lee 1986), only recently has there been a considerable renewed interest in their use, and the main focus has been on the study of the hydrolysis of pure cellulose.

Solid acids are environmental benign catalysts with respect to corrosiveness, safety and waste (Okuhara 2002), that are better proton donors than pure sulfuric acid for hydrolysis of polysaccharides and exhibit higher selectivity towards the hydrolytic reaction. Other advantages of these catalysts are the use of lower temperatures and pressures, together with a decrease in water usage, which improves process water economy. Furthermore, particulate catalysts can be readily separated from the sugar solution for reuse without loss of activity and with lower energy consumption. The main process variable to be controlled is the water availability (Yamaguchi et al. 2009), as it influences the effective acid concentration and biomass/catalyst contact.

Examples of solid acids studied for biomass fractionation include niobic acid ($Nb_2O_5.nH_2O$), H–mordenite (zeolite), Nafion NR50 (perfluorosulfonated ionomer), Amberlyst-15 (polystyrene-based cation-exchangeable resin with SO3H), sulfonated activated-carbon, and amorphous carbon bearing SO_3H, COOH and OH (Chung et al. 1992; Onda et al. 2009; Suganuma et al. 2008), as well as other materials such as bentonite, kaolin and acid-treated alumina (Blair et al. 2009). The latter materials and amorphous carbon bearing SO_3H, COOH and OH have been reported to effectively treat the solid biomass at relatively mild temperatures ($\leq$100 °C).

In the case of the amorphous carbon bearing SO_3H, COOH and OH, this better catalytic performance is attributed to the ability of this material to adsorb β-1,4 glucan, which does not adsorb to other solid acids (Kitano et al. 2009). The process seems to be not very selective. As to the polysaccharides, both cellulose and hemicellulose are dissolved (to mono and oligosaccharides) with close to 100 % recoveries. Lignin may mainly remain insoluble (Suganuma et al. 2008; Yamaguchi et al. 2009), or also be dissolved (Blair et al. 2009), in what is probably a function of the catalyst nature.

Recent studies are focused on the use of solid acids together with ionic liquids, exploring possible synergies (Rinaldi et al. 2008; Zhang and Zhao 2009).

1.3.3 Inorganic Salts

In recent years the utilization of inorganic salts for biomass (pre)-treatment has also been gaining interest. Several studies reported that inorganic salts could increase hydrolysis rate of hemicellulose and cellulose in biomass during dilute acid processing (Marcotullio and de Jong 2010; Marcotullio et al. 2011; Nguyen and Tucker 2002) despite that inorganic salts alone (KCl, NaCl, $CaCl_2$, $MgCl_2$ or $FeCl_3$) and especially the latter, increase the degradation rate of pentoses (Liu and Wyman 2006). They can also be very effective both on hemicellulose removal from LCM as well as on the increase of cellulose digestibility (Liu et al. 2009; Sun et al. 2011; Zhao et al. 2011).

The main salts used for biomass fractionation are $FeCl_3$, $FeSO_4$, $Fe(NO_3)_3$, $Al_2(SO_4)_3$, $AlCl_3$, and $MgSO_4$ and they have been tested in several LCM such as corn stover (Zhao et al. 2011), corn stover silages (Sun et al. 2011), wheat straw (Marcotullio et al. 2011) and barley straw (Kim et al. 2010). However, the most effective salt seems to be $FeCl_3$ that could be an interesting option for acid replacement. Salt solutions of $FeCl_3$, in particular, showed a strong effect on the hemicellulose removal, reaching as high as 100 %, and the yield of monomeric and oligomeric xylose in the liquid fraction can reach values higher than 90 % (Liu and Wyman 2006; Marcotullio et al. 2011; Nguyen and Tucker 2002; Sun et al. 2011). In general, the most important fraction is represented by monomeric sugars. Besides the high hemicellulose removal and recovery, cellulose is almost not affected by these treatments and its enzymatic digestibility is significantly improved as compared to other processing methods (Liu and Wyman 2006; Marcotullio and de Jong 2010; Zhao et al. 2011). This novel method has some particular advantages, such as high reaction rate, it is less corrosive than acids, inorganic salts are easy to recycle and due to the mild pH further neutralization of the hydrolysates can even be avoided.

1.3.4 Ionic Liquids

Nowadays ionic liquids (ILs) have become the real laboratory scale alternative to the conventional media in biomass fractionation processes. ILs are organic salts with melting points below 100 °C. Their tailored properties such as high thermal stability (Domanska and Bogel-Łukasik 2005), great solvent power (Bogel-Łukasik et al. 2010; Conceição et al. 2012; Forte et al. 2011, 2012) negligible vapor pressure (Paulechka et al. 2003) and others allow use of them in the manner that makes the processes more "green and sustainable." A claimed advantage of using ILs is the possibility of a complete and very selective dissolution of wood in its native form, which has opened new possibilities to fractionate, derivatise and process LCM.

The series of ILs are particularly useful in dissolution of cellulose (Zakrzewska et al. 2010). The first work in this research area was presented in 2002 by Rogers and co-workers (Swatloski et al. 2002) who demonstrated that some imidazolium ILs dissolve up to 25 wt% of cellulose, forming the highly viscous solutions. They also suggested that dissolution occurs via breaking the extensive hydrogen bonding network of the polysaccharide by the anion of the IL. The most comprehensive review on this subject has been published recently (Zakrzewska et al. 2010). Among several important parameters influencing the solvent power of ILs in the relation to LCM is water content. It was found that water considerably decreased the solubility of saccharides by promoting the re-aggregation of the polymer's chains through the competitive hydrogen bonds (Conceição et al. 2012; Swatloski et al. 2002). The influence of water is particularly important in case of further modification because the aggregation decreases accessibility, and thus the reactivity of the polymer. On the other hand, the same feature allows for the easy regeneration of the already dissolved carbohydrates forming the solution, by the simple addition of water, alcohol or acetone (Swatloski et al. 2002).

The major efforts in the dissolution of lignocellulose-based materials were focused on the employment of chloride ILs, as the chloride anion acts as a strong proton acceptor in the interaction between the IL and the hydroxyl groups of the carbohydrate. Nevertheless, the high melting point and viscosity of most of the chloride salts causes the processing of carbohydrates to be expensive and inefficient. This is the reason why the newly designed ILs exhibiting a low melting temperature, a low viscosity and a sufficient polarity are preferably being investigated. ILs built up from carboxylate anions show relatively low viscosities and stronger (than chloride ions) hydrogen bonding basicity (Fukaya et al. 2006; Ohno and Fukaya 2009). Additionally, they dissolve a considerably higher amount of cellulose more efficiently at lower temperatures than chloride ILs. Nevertheless, the low thermal stability, due to the decarboxylation, is considered to be an important limitation in the employment of this class of ILs. A different example showed that 1-ethyl-3-methylimidazolium dimethylphosphate ([emim][$(MeO)_2PO_2$]) revealed a moderate ability to destroy the crystalline structure of the carbohydrate as well. It was also shown that ILs containing dialkylimidazolium cation and dicyanamide anion, are also good solvents for mono- and more bulky carbohydrates (Liu et al. 2005). One of the most recent works shows that at temperatures which can be easily achieved at the industrial side, the solubility of saccharides in thiocyanate and hydrogen sulfate imidazolium based ILs is considerably high and can reach even 50 wt% at 110 °C (Conceicao et al. 2012).

ILs have been shown as very effective in cellulose dissolution (Ohno and Fukaya 2009), although the solubility study of hemicellulose and lignin in ILs was reported rarely and it is required to be investigated in detail. Nevertheless, presented examples show that there are two possible approaches for wood fractionation. One of which leads to the complete dissolution followed by selective precipitation of the different components (Edye and Doherty 2008; Fort et al. 2007; Sun et al. 2009). The second assumes the selective dissolution of one or more of the components as it is reported on the examples of selective dissolution of lignin and (hemi)cellulose (Lee et al. 2009; Sievers et al. 2009).

1.3.5 Supercritical Fluids

Supercritical fluid (SCF) is a compound above its critical temperature, *Tc*, and critical pressure, *pc*, but below the pressure required to condense it into a solid (Jessop and Leitner 1999). As the temperature increases the liquid becomes less dense due to thermal expansion and as the pressure increases the gas becomes denser. Once the densities become equal, the phase distinction between liquid and gas disappears and the critical point has been reached. The most popular supercritical fluids are carbon dioxide ($Tc = 31.0$ °C, $pc = 73.8$ bar), water ($Tc = 374.0$ °C, pc = 221.0 bar) and propane ($Tc = 96.7$ °C, $pc = 42.5$ bar) (Jessop and Leitner 1999). Above the critical point, there is only one gas phase. The properties of gases above the critical conditions take values that are somewhat in between the values usually taken by liquids and gases. Therefore supercritical fluids demonstrate important advantages for use in chemical processes. Additionally, supercritical fluids show tunable properties such as partition coefficients and solubility. Small changes in temperature or pressure close to the critical point can result in up to 100-fold changes in solubility, which simplifies separation.

Up to now there have been only a few literature reports about the pre-treatment of lignocellulosic material by SCF, mostly by supercritical water (Miyafuji et al. 2005; Zetzl et al. 2011), or by supercritical CO_2 (Kim and Hong 2001). Water under sub or supercritical conditions behaves very differently from water under normal pressure and temperature. The typical regions for different water-base processes in temperature–pressure scale are shown in Fig. 1.1. Supercritical water or Liquid Hot Water (LHW) treatments can be expected to be superior in economic efficiency because the feasibility of hydrolysis with water is facilitated by the fact that water develops acidic characteristics at high temperatures (Schacht et al. 2008). Particularly in the subcritical range of temperature and pressure ($P < 210$ bar, $T < 380$ °C), the ion product and the hydrolysis capacity of H_2O increases with increasing temperature, and at 250 °C the ion product for water, *K*w, reaches a maximum of 6.34×10^{-12}, resulting in a pH of 5.5 for water at a temperature of 220 °C. Thus hemicellulose could be completely separated from the lignocellulose, and enzymatic digestibility of cellulose can be significantly increased by treating the lignocellulosic material under such conditions (Kim and Lee 2006; Sasaki et al. 2003). Furthermore, it was found that the amount of dissolved hemicellulose rises with increasing temperature and time of the treatment. However, the hydrolyzed hemicellulose monomers undergo further degradation to furfural or HMF and other toxic by-products; therefore it should also be taken into account.

The use of pure supercritical CO_2 did not cause any significant change in microscopic morphology of wood (Ritter and Campbell 1991), but it can significantly increase cellulose hydrolysis (Kim and Hong 2001). Addition of organic acids to CO_2 allows enhancing the yield of the process. What is more, the mineral acids can be avoided and corrosiveness of the acid catalyzed process is significantly reduced. Furthermore, the employment of CO_2 usually reduces the temperature of the process that diminishes the xylose degradation and increases the yield of the reaction (Zheng et al. 1998). Additionally, the employment of CO_2 is also relevant in separation of the products (Persson et al. 2002; Schacht et al. 2008).

Summarizing, the employment of highly dense (either sub or supercritical) fluids is beneficial for the hemicellulose recovery but there is still a wide range of improvements to be achieved before these fluids will be implemented in a larger scale.

1.3.6 Alkaline Treatments

Conversely to acid or hydrothermal processes, alkaline-based methods target both lignin and hemicellulose fractions. The alkaline treatments can be divided into two major groups, depending on the catalyst used, namely, treatments that use sodium, potassium, or calcium hydroxides and those that use ammonia.

Hydrolytic methods such as organosolv, alkaline, ionic liquid, etc., primarily remove lignin. However, during such operations, a significant amount of hemicellulose is also degraded. However, it is difficult to separate hemicellulose sugars from the lignin rich solution aiding several purification and extraction steps ameliorating the cost of the overall process.

In general, sodium and calcium hydroxide (lime) treatments are not used as methods to sole produced hemicellulosic liquors. The main effect of the alkali treatment is the increase of cellulose digestibility. These treatments are applied either as a primary treatment directly to the biomass (Holtzapple et al. 1999; Mussatto et al. 2007, 2006; Parajó et al. 1996a; Silverstein et al. 2007; Wyman et al. 2005) or as a subsequent treatment to the acid hydrolyzed biomass (Moldes et al. 2002; Vázquez et al. 1992a) to increase cellulose digestibility, but the use of alkali treatment before an acid hydrolysis has also been reported (Parajó et al. 1996b).

The major representatives of ammonia-based processes are the AFEX (ammonia fiber explosion/expansion) treatment, and the ammonia recycling percolation (ARP) processes. Although both of these can dissolve hemicelluloses, AFEX seems to be efficient for herbaceous and agricultural residues (Balan et al. 2008; Belkacemi et al. 1998; Chundawat et al. 2007; Holtzapple et al. 1991, 1992; Moniruzzaman et al. 1996; Teymouri et al. 2005) and works moderately well on hardwoods (Holtzapple et al. 1992; Mosier et al. 2005; Ogier et al. 1999; Wheals et al. 1999). The mechanism is a combination of chemical and physical effects that induces the cleavage of the lignin-carbohydrate complex (Chundawat et al. 2007), hemicellulose hydrolysis, and cellulose decrystallization, and both polysaccharides are recovered in monomeric and oligomeric form. But, even after enzymatic hydrolysis, xylo-oligosaccharides (XOS) typically account for more than 35 % of the soluble hemicellulose (Lau and Dale 2009). As the hydrolysates obtained may have a high glucose content, this process has not been used yet to obtain hydrolysates for xylitol production. Nonetheless, this process seems to be more appropriate for other fermentation purposes than for producing hydrolysates for xylitol production, as not only a significant fraction of xylose is not available, but a potentially present glucose may induce a fermentative diauxic behavior.

Conversely, ARP is more selective towards hemicellulose dissolution (reaching values between 40 and 60 %) whereas the cellulosic fraction is almost not

significantly dissolved (<10 %). Nevertheless, hemicellulose is recovered mainly in the oligomeric form. Another further disadvantage of this process is its low selectivity, as delignification can also be high (Kim et al. 2003, 2008; Kim and Lee 2005).

The main constraints of the ammonia-based processes are the cost associated with ammonia, its recovery, and the safety issues regarding its use (Ogier et al. 1999). On the other hand, the economy is positively influenced by the high total sugar yields achieved (although many of which in oligomeric form).

1.3.7 Wet Oxidation

Wet oxidation was first described as a biomass pre-treatment involving oxygen or air and water at elevated temperatures and pressure, promoting the oxidation of lignin and decomposing it to CO_2, H_2O and carboxylic acids (Bjerre et al. 1996; Klinke et al. 2002). Combining alkaline agents (in particular Na_2CO_3) it is possible to get higher rates of soluble hemicellulose, up to 82 % xylan for sugarcane bagasse (Bjerre et al. 1996). The hemicellulosic sugars remain mainly in the oligomeric form, and although there is a low formation of furan-aldehydes, a significant formation of carboxylic acids still exist (Bjerre et al. 1996; Klinke et al. 2002; Martín et al. 2008).

1.3.8 Organosolv

In the organosolv process the direct action of water dissolved organic solvents (such as ethanol, methanol, or acetone), usually in combination with an acid, acts together to dissolve the lignin and hydrolyze the hemicellulose fraction (Kin 1990; Pan et al. 2005; Vázquez et al. 1992b; Zhao et al. 2009). The process temperatures may vary from room temperature up to 205 °C, depending mainly on the organic solvent use. Overall economy is strongly dependent on the solvent recycling. Among the most effective processes are those based on water:ethanol blends catalyzed by sulfuric acid (Pan et al. 2005, 2006). The operational temperature usually ranges between 180 and 200 °C. Ethanol has the advantage that it can be easily recycled by distillation. Under these conditions hemicellulosic sugars can be recovered from the water-soluble stream, where about half are in oligomeric form and require posthydrolysis. As a further drawback, there are the significant amounts of furfural that can also be produced, which implies a lower recovery of pentoses.

1.3.9 Enzymatic Hydrolysis

The use of enzymes directly in the untreated biomass is not efficient, and hemicellulases are not an exception. Therefore, a pre-treatment as the ones described above are always necessary (Polizeli et al. 2005).

Table 1.5 Relevant enzymatic activities for hydrolysis of pentose-rich hemicelluloses (adapted from Carvalheiro et al. 2008 and the NC-IUBMB website)

EC	Enzyme	Hydrolysed linkage	Substrate	Main product	Comments
3.1.1.72	Acetyl xylan esterase	Ester bond	Side groups	Acetic acid	
3.1.1.73	Feruloyl esterase	Ester bond	Side groups	Ferulic acid	
3.2.1.8	Endoxylanase (endo-1,4-β-xylanase)	Internal β-1,4	Main chain	Oligomers	
3.2.1.32	Endo-1,3-β-xylanase	Internal β-1,3	β(1,3)-xylan	Xylobiose, Xylotriose and Xylotetraose	This enzyme is found mostly in marine bacteria, which break down the β(1,3)-xylan found in the cell wall of some green and red algae
3.2.1.37	β-Xylosidase	Terminal β-1,4 (non-reducing end)	Oligomers	Xylose	
3.2.1.55	α-*N*-arabinofuranosidase	Terminal α-1,2; α-1,3	Side groups	Arabinose	
3.2.1.72	Xylan 1,3-β-xylosidase	Terminal β-1,3 (non-reducing end)	β(1,3)-xylan	Xylose	Hydrolysis of successive xylose residues from the non-reducing termini of (1 → 3)-β-D-xylans
3.2.1.120	Oligoxyloglucan β-glycosidase	Terminal β-1,4 (non-reducing end)	Oligoxyloglucans	Isoprimeverose[a]	
3.2.1.99	Arabinan endo-1,5-α-L-arabinanase	Internal α-1,5	Linear or branched arabinans	Oligomers	Acts best on linear 1,5-α-L-arabinan. Also acts on branched arabinan
3.2.1.131	Xylan α-1,2-glucuronosidase	(1 → 2)-α-D-(4-O-methyl)glucuronosyl links	Main chain of hardwood xylans		
3.2.1.136	Glucuronoarabinoxylan endo-1,4-β-xylanase				High activity towards feruloylated arabinoxylans from cereal plant cell walls
3.2.1.139	α -Glucuronidase	Terminal α-1,2	Side groups	Methylglucuronic acids	

(continued)

Table 1.5 (continued)

EC	Enzyme	Hydrolysed linkage	Substrate	Main product	Comments
3.2.1.150	Oligoxyloglucan reducing-end-specific cellobiohydrolase	Terminal β-1,4 (non-reducing end)	Xyloglucans consisting of a β-1,4-linked glucan carrying α-D-xylosyl groups on O-6 of the glucose residues	Cellobiose	To be a substrate, the first residue must be unsubstituted, the second residue may bear a xylosyl group, whether further glycosylated or not, and the third residue, which becomes the new terminus by the action of the enzyme, is preferably xylosylated, but this xylose residue must not be further substituted
3.2.1.151	Xyloglucan-specific endo-β-1,4-glucanase	Internal β-1,4	Xyloglucan	Xyloglucan,	oligosaccharides
3.2.1.155	Xyloglucan-specific exo-β-1,4-glucanase	Terminal β-1,4	Xyloglucans	Oligosaccharides	It is not known whether the cleavage takes place at the reducing or non-reducing end of the polymer
3.2.1.156	Oligosaccharide reducing-end xylanase	Terminal β-1,4 (reducing end)	Main chain	Xylose,	
3.2.1.177	α-D-xyloside xylohydrolase (α-xylosidase)	Terminal, α-D-xylose residues (reducing ends)		α-D-xylose	

[a] α-xylo-(1 $\rightarrow$ 6)-β-D-glucosyl

Hemicellulases encounter their major application on the hydrolysis of soluble hemicelluloses, either in polymeric or oligomeric form, e.g. as the ones derived from hydrothermal or alkaline treatments. Both these forms retain much of the inherent hemicellulose complexity and therefore the action of several enzyme activities is usually required for complete hydrolysis (Table 1.5). In fact, the hydrolysis extent is largely governed by the structure of the substrate, e.g. the degree and pattern of substitution as well as the degree of polymerization (Tenkanen 2004). Combining hemicellulases in simultaneous or step-wise fashion increases the possibility of achieving the required results.

These enzymes act synergistically, since endoxylanase activity is highly dependent on the presence of debranching enzymes and vice versa (Puls and Poutanen 1989; Saha 2003; Vázquez et al. 2001; Walch et al. 1992), as well as chain length and degree of substitution (Li et al. 2000). Furthermore, several compounds typically present in hydrolysates can significantly reduce enzyme activity (Cantarella et al. 2004; Dekker 1988; Hörmeyer et al. 1987; Walch et al. 1992) thus turning this process even less competitive. Phenolic compounds are particularly toxic, once they easily form cross-linkage with proteins, inactivating them (Zahedifar 1996), and as such non-selective processes removing hemicellulose and lignin may impose further constraints.

As such, enzymatic hydrolysis of hemicellulose has been mainly studied as a process for oligosaccharide hydrolysis (Akpinar et al. 2010; Belkacemi and Hamoudi 2003; Belkacemi et al. 2002; Duarte et al. 2004; Puls et al. 1985; Vázquez et al. 2001, 2002; Vegas et al. 2008a; Walch et al. 1992), and less for the hydrolysis of alkali extracted xylan (Akpinar et al. 2007).

The main advantage of enzymatic posthydrolysis over acid processes are the milder operation conditions (temperature, and pH), which lead to reaction media free of further sugar degradation compounds that can limit microbial performance, together with potential economic advantages related to energy savings and equipment cost; nevertheless, this imposes the necessity for sterile operation. Finally, enzyme costs as compared to acid costs have also favored the chemical process.

1.4 The Fractionation Selectivity Index

Each fractionation technology has advantages and disadvantages, and the “ideal”, here understood as the process that allows for the total selective recovery of hemicellulose without contamination from cellulose and lignin derived compounds, will not exist. But, although the extent of the recoveries of the three distinct biomass fractions is a function of the raw material, the calculation of the selectivity of a given process can be useful to further characterize the processes and to help choose between them. As such, we introduce here the hemicellulose Fractionation Selectivity Index, Hemicellulose FSI:

Table 1.6 Quantification of the hemicellulose selectivity index for various materials and processes

Material	Process	HFSI	References
Brewery's spent grain	Autohydrolysis + Posthydrolysis	0.68	Duarte et al. (2004)
Brewery's spent grain	Dilute acid hydrolysis	0.83	Carvalheiro et al. (2004a)
Brewery's spent grain	Autohydrolysis	0.55	Carvalheiro et al. (2004b)
Distilled grape marc	Dilute acid hydrolysis	0.80	Salgado et al. (2012b)
Pistachio shells	Dilute acid hydrolysis	0.91	Sazaki et al. (2009)
Vineshoot trimmings	Dilute acid hydrolysis	0.75	Salgado et al. (2012a)
Wheat straw	Autohydrolysis	0.37	Carvalheiro et al. (2009)

HFSI hemicellulose fractionation selectivity index

$$\text{Hemicellulose FSI} = \frac{R_{\text{Hemicellulose}}}{R_{\text{Hemicellulose}} + R_{\text{Cellulose}} + R_{\text{Lignin}}} \tag{1.1}$$

Where, R_i are the recoveries of the given fraction in the relevant stream, typically the liquid stream. The individual recoveries are calculated as the percentage ratio of effectively recovered fraction to the initial composition. This index varies between 1 for a totally selective process and zero for a process that does not enable a hemicellulose recovery. A value close to 0.3 would be typical for a non-selective process. To simplify the calculations, and since xylose is typically the most significant hemicellulosic sugar in hardwoods and straws, as well as the most difficult component to be hydrolyzed, together with the consideration that hemicellulose-derived glucose content is much lower than cellulose-derived glucose, Eq. 1.1 can be simplified to Eq. 1.2:

$$\text{Simplified FSI} = \frac{R_{\text{Xyl}}}{R_{\text{Xyl}} + R_{\text{Glc}} + R_{\text{Lignin}}} \tag{1.2}$$

Equation 1.2 has the further advantage of taking into account directly the monomeric xylose (the substrate for xylitol production) and glucose, and not any other sugar forms, namely, the oligomeric that require further hydrolysis, thus also reflecting efficiency. As lignin recovery in the liquid fraction can be somewhat more difficult to quantify experimentally, an approximate estimation may be used, if required, e.g. 100-RLignin_Solid fraction.

The application of this index to the fractionation of different raw materials by the described methods is presented in Table 1.6 based on literature data. As it can be seen, dilute acid hydrolysis, independently of raw material seems to be the most selective process. Conversely, autohydrolysis suffers from the fact that xylose is mainly recovered in oligomeric form, and although the use of a posthydrolysis process increases overall selectivity, it does not attain the high performance exhibited by dilute acid hydrolysis.

Table 1.7 Advantages and disadvantages of the most relevant fractionation methods for xylose recovery from hemicelluloses (adapted and modified from Gírio et al. 2010)

Desirable feature	Concentrate acid	Dilute acid	Solid (super)acids	Steam explosion	Autohydrolysis	Supercritical fluids	Ionic liquids	Inorganic salts
High hemicellulosic monosaccharides production	++	++	+	0	−	−	0/+	+
Low hemicellulosic oligosaccharides production	+	+	+	0	−	−	0/+	0
High chemicals recycling	−	−	+	0	n.r.	n.r./+	+	−
Low inhibitors formation	−	−	0	0	0	0	−	0
Low corrosion problems	−	−	0	0	0	−/0	−/0	0
Low need for chemicals	−	−	0/+	0	++	++	+	−
Low neutralization requirements	−	−	−/0	0	n.r.	n.r.	0	+
Low investment costs	+	+	0	−	+	−	+	0
Low operational costs	−	0	0	++	+	−	−	+
Low energy use	0	−	+	0	0	+	++	+

+ advantage, − disadvantage, 0 neutral, n.r. not relevant

1.5 Conclusions and Future Recommendations

As can be drawn from the discussion above, there is no single method that can fulfill all the requirements for effective hemicellulose recovery (Table 1.7). This becomes an even more demanding challenge when the upgradeability of the other fractions are take into consideration, as it should be, especially in the biorefinery framework.

Despite its drawbacks, currently the dilute acid hydrolysis process is still the dominating technology for the production of fermentable xylose-rich hydrolysates. But, clearly, other strategies are needed. One option is the design of strategies using combined/sequential processes targeting separately dissolution and hydrolysis. In this scenario, the use of processes such as liquid hot water, steam or supercritical fluids when associated with a posthydrolysis step that converts oligomers into monomeric xylose may be advantageous. These processes are potentially cheaper, milder, and can be turned more selective for the recovery of hemicellulose, inducing less corrosion and chemicals spending. The development of novel (post) hydrolysis processes using, e.g. solid (super) acids, ionic liquids, inorganic salts, or enzymes turn now to be especially appealing, enabling more environmentally friendly processes. Actually, future developments are further foreseen on these methodologies that will mold them into better competitors to the dilute acid hydrolysis.

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Chapter 2
Dilute Acid Hydrolysis of Agro-Residues for the Depolymerization of Hemicellulose: State-of-the-Art

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Abstract Geo-political, long-term economic and sustainable concerns are promoting researchers and entrepreneurs to harness the potential of lignocellulosic feedstock (LCF) into industrially significant products. Agro-residues (sugarcane bagasse, wheat straw, rice straw, corn stover, etc.) constitute the principal fraction of LCF and are available in large amounts globally. The judicious exploration of agro-residues into important products such as D-xylitol, an artificial sweetener, may provide a strong platform for its sustainable supply to the medical and non-medical applications-based sectors. Pretreatment of agro-residues by dilute acid hydrolysis is an inevitable process for the depolymerisation of hemicellulosic fraction into xylose and other sugars. Dilute acid catalyses hemicellulose fractionation at high temperature within short reaction times. Significant developments have been made in the past towards the chemical hydrolysis of agro-residues, particularly for the hemicellulose breakdown. Critical parameters such as acid load, temperature, residence time and solid-to-liquid ratio play pivotal roles in the kinetics of dilute acid hydrolysis of agro-residues. Furthermore, reactor configurations such as counter-current, plug-flow, percolation and shrinking-bed have been designed in order to maximize the sugars recovery with minimum inhibitors generation. This chapter reviews the process parameters, kinetics, methods and reactor engineering for the dilute acid catalysed processes employed for agro-residues.

Keywords Agro-residues · Dilute acid hydrolysis · Hemicellulose depolymerization · Pretreatment · Reaction kinetics · Reactor design

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2.1 Introduction

The low cost and renewable nature of agro-residues such as sugarcane bagasse, wheat straw, rice straw, corn stover, etc. represent the ideal feedstock for their conversion into value-added products by biotechnological routes (Mantanis et al. 2000; Mussatto and Teixeira 2010). These agro-residues, like other lignocellulosic materials, are constituted primarily of lignin, hemicellulose(s) and cellulose. The carbohydrate fraction (hemicellulose and cellulose) can be depolymerised into sugars which act as a primary carbon source for the microbial biocatalysts for the production of xylitol, ethanol, organic acids, industrial enzymes, etc. (Carvalheiro et al. 2008; Mussatto and Teixeira 2010; Chandel et al. 2011a). Figure 2.1 demonstrates the process configuration for dilute acid hydrolysis of various agro-residues into monomeric constituents and subsequently their conversion into value-added products by microbial fermentation. Due to the large variations of the chemical compositions of agro-residues, especially polysaccharides and lignin, it is important to choose an appropriate method of biomass fractionation for their effective biotechnological utilization (Howard et al. 2003). In this context, the complex cellular structure of the agro-residues must be fractionated into C6 or C5 sugars with minimum side (or by)-products (Mosier et al. 2005). Thus the procedure applied will be a hydrolysis and/or a pretreatment, depending on the purpose of the released sugars.

During the acid hydrolysis of the hemicellulose fraction of plant cell wall, sugars are liberated in addition to some undesired compounds. The recovered solid residue, so-called cellulignin, is readily available for the subsequent cellulases-mediated enzymatic hydrolysis into glucose. Thus the pretreatment step involving dilute acid facilitates the enzymes accessibility to the cellulose for maximizing sugars recovery (Chandel et al. 2011b). Dilute acid hydrolysis is a simple and fast method to obtain hemicellulosic hydrolysates. These hydrolysates mainly contain xylose (80 % of the sugar content in hemicellulosic fraction), arabinose, glucose, galactose and mannose in conjunction with cell wall-derived inhibitors such as furans, phenolics, weak acids and others (Canilha et al. 2006, 2008; Chandel et al. 2007a).

During dilute acid hydrolysis of agro-residues, parameters such as temperature, time, acid concentration and solid-to-liquid ratio play critical roles in obtaining optimum sugar recovery and minimum generation of inhibitors (Taherzadeh and Karimi 2007). The establishment of these parameters is of fundamental importance to define optimal conditions of hydrolysis to ensure the success of the process, in view of concerns taken into account since the hydrolyzate will be used as fermentation medium (Gírio et al. 2010). Thus, the knowledge about the cell wall chemical composition of agro-residues is very important in order to identify the raw materials with higher content of hemicellulose and bioprocesses using pentose sugars as substrates for the production of several chemical commodities (Saha 2003). The choice of the optimal hydrolysis conditions is very important for maximum yield of sugars and minimal formation of toxic compounds for the

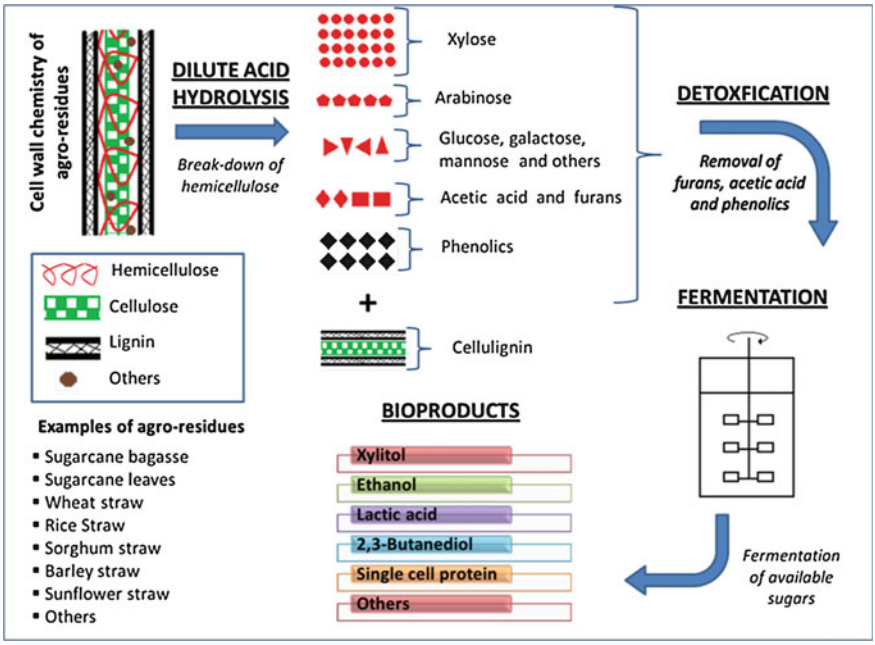


Fig. 2.1 Process configuration summarizing the dilute acid hydrolysis of various agro-residues into monomeric constituents and their conversion into value-added products

utmost production of compounds after fermentation reaction. This should be a low-cost process to ensure the sustainability of this technology and successful agro-residual feedstock management (Mantanis et al. 2000; Mussatto and Teixeira 2010; Chandel et al. 2010a). In this context, designing of reactors or changes in their configuration is an important consideration for the maximum de-polimerization of hemicellulose during dilute acid hydrolysis (Mosier et al. 2005; Thaerzadeh and Karimi 2007; Lenihan et al. 2011). The emergence of plug-flow reactors (PFR), counter-current reactors, percolation reactors and shrinking bed counter current reactors have shown promising results for dilute acid mediated hydrolysis of agro-residues (Lee et al. 1999; Taherzadeh and Karimi 2007; Lenihan et al. 2011). Among all these process configurations, counter-current reactors have shown better results for the maximum hemicellulosics breakdown with fast reaction rates consequently producing low concentration of cell-wall derived inhibitors (Lee et al. 1999). However, a considerable amount of work is still required to establish a robust and reproducible technology for the maximum hemicellulose de-polimerization at industrial scale.

This chapter discusses the chemical nature and influential parameters of dilute acid hydrolysis of agro-residues, hydrolysis kinetics with sugar recovery, and the improvements in reactor configuration for maximum hemicelluloses de-polimerization into their monomeric constituents.

2.2 Cell Wall Chemistry of Various Agro-Residues

The cell wall consists of three main components: cellulose microfibrils (with characteristic distributions and organization), hemicellulose and lignin. Also, smaller amounts of pectin, protein, extractives and ash are found. The structure of these materials is very complex, and native biomass is generally resistant to chemical/enzymatic hydrolysis (Saha 2003).

The lignocellulosic biomass chemical composition differs with the source of plant species (Table 2.1). Cellulose is the main constitute(s) of bagasses and straws. The highest content of this fraction was found for sugarcane bagasse, an average of 42.8–45.0 %. Hemicellulose is the second major compound of these biomass sources, usually between 22 and 35 % of dry mass. Celluloses and most of the hemicelluloses are structural carbohydrates as they form the bulk of the plant cell's supporting structure (Ek et al. 2009). Coffee husk showed the highest hemicellulosic content (36.7 %) of the materials available, while sunflower stalks showed the lowest (20.2 %). In general, straws showed higher lignin content than others materials. For instance, sugarcane leaves straw showed high lignin content (26–32 %). According to Ek et al. (2009), the lignin contents in non-woody plants are, however, much lower (1–20 %) than in woody plant tissues. There is also a variation in cell wall composition between individual biomass sources of the same plant species, depending on the age of plant, genetic factors and growth conditions, e.g. climatologic and geographic factors (Ek et al. 2009). Cellulose is the most common polysaccharides in nature and consists of repeating units of cellobiose. It is a glucan polymer of D-glucopyranose units, which are linked together by β-(1 → 4)-glucosidic bonds (Rowell et al. 2005). The number of glucose units in one wood cellulose molecule (i.e. the degree of polymerization) is at least 9,000–10,000 and possibly as high as 15,000 (Rowell et al. 2005). In this case, cellulose molecules aggregate with each other due to hydrogen bonding and form microfibrils, which are the building blocks of fibrils, and in turn build the cellulose fiber (Sjöström 1993).

In general, the hemicellulose fraction is a polymer that is composed of several different sugars like D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, L-rhamnose and is sometimes referred to by the sugars they contain, for example, galactoglucomannan, arabinoglucoronoxylan, arabinogalactan, glucuronoxylan, glucomannan, etc. The hemicellulose also contains acetyl and methyl substitute groups (Rowell et al. 2005). The degree of polymerization of hemicellulose is lower than cellulose, achieving an average of about 100–200 and the molecules can be highly branched (Rowell et al. 2005). Due to the combination of several sugars and for presenting majority part of amorphous structure, the hemicellulose is more soluble in water and easily degraded than cellulose. In the lignocellulosic materials the cellulose and lignin are intimately linked, because hemicellulose acts as glue between those fractions (Fengel and Wegener 1984).

Next to cellulose, lignin is the most abundant and important polymeric organic substance in the plant world. Lignin increases the mechanical strength properties

Table 2.1 Cell wall composition of various agro-residues (% of dry material)

Lignocellulosic source		Components					References
		Cellulose	Hemi-cellulose	Lignin	Extractives	Ash	
Bagasse	Sorghum	40.4	35.5	3.9	na	0.2	Dogaris et al.(2009)
	Sugarcane	42.8	25.9	22.1	6.1	1.4	Silva et al. (2010a, b, c)
		43.1	25.2	22.9	4.3	2.8	Rocha et al. (2012)
		43.8	27.0	22.6	3.9	2.0	Martín et al. (2011)
		45.0	26.0	20.0	na	2.1	Boussarsar et al. (2009)
Grains	Barley spent	27.6	32.5	13.4	12.9	3.4	Dehnavi, (2009)
	Brewery′s spent	16.8	28.4	27.8	5.8	4.6	Mussatto et al. (2007)
		21.2	30.4	22.2	na	1.1	Duarte et al. (2008)
	Coffee spent	8.6	36.7	na	na	1.6	Mussatto et al. (2011)
Husk	Oat	29.3	28.4	22.2	na	4.5	Tamanini et al. (2004)
	Rice	21.5	23.1	14.6	na	na	Megawati et al. (2011)
Straw	Corn	34.4	29.0	17.2	na	na	Bura et al. (2009)
	Rice	43.4	22.9	17.2	na	11.4	Roberto et al. (2003)
	Sorghum	35.1	24.0	25.4	na	na	Téllez-Luiz et al. (2002)
	Sugarcane	40.8	30.8	25.8	na	2.6	Mouta et al. (2011)
		33.6	28.9	31.8	na	5.7	Silva et al. (2010a, b, c)
	Wheat	33.0	33.0	20.0	na	na	Canilha et al. (2006)
Others	Corn cobs	42.7	34.3	18.4	3.0	na	Cheng et al. (2009)
	Sunflower stalks	33.8	20.2	17.3	6.9	9.6	Ruiz et al. (2008)

na not available

to such an extent that huge plants such as trees with heights to even more than 100 m can remain upright (Fengel and Wegener 1984). Lignins are complex polymers consisting of phenyl propane units linked together by ether or carbon–carbon bonds (Brunow et al. 1999). Lignins can be classified in several ways, but they are usually divided according to their structural elements (Sjöström 1993). All wood lignins consist mainly of three basic building blocks of guaiacyl, syringyl, and p-hydroxyphenyl moieties, although other aromatic units also exist in many different types of woods. There is a wide variation of structures within different wood species. The lignin content of hardwoods is usually in the range of 18–25 %, whereas the lignin content of softwoods varies between 25 and 35 % (Rowell et al. 2005).

2.3 Bioconversion of Xylose and Other Hemicellulosic Sugars into Bioactive Products

Agro-industrial wastes/byproducts represent a large portion of biomass which does not compete with food production (Doherty et al. 2011). Considering the large amount of agro-industrial byproducts discarded, the development of a technological platform for the production of any value-added product has yet to be established on a large scale (Felipe et al. 1997).

The conversion of the hemicellulosic fraction to fermentable sugars is essential for its use in various biochemical processes (Chandel et al. 2010b; Gírio et al. 2010). Due to the recalcitrant characteristic of agro-residues, a pretreatment step can depolymerise the hemicellulosic fraction into fermentable sugars such as xylose, glucose and arabinose (Mosier et al. 2005). These hemicellulosic-derived sugar solutions are used for the production of commercially significant products. A wide variety of pretreatment methods aiming at hemicellulose de-polimerization such as dilute acid hydrolysis, steam explosion, and liquid hot water pretreatment have been developed (Mosier et al. 2005). Among these methods, dilute acid mediated hydrolysis has been found more effective towards complete hemicellulose hydrolysis in short reaction times (Taherzadeh and Karimi 2007). Acid hydrolysis with dilute sulfuric acid under mild conditions has proven to be a method that is reliable, easy to operate and low cost (Shatalov and Pereira 2012).

Table 2.2 summarizes the examples of various bioproducts of commercial interest produced by microbial fermentation using the sugars derived from agro-residues.

The application of xylose in xylitol production has being widely studied. Xylitol is a polyalcohol with great importance in food, pharmaceutical and dental care industries because of its interesting properties, as its sweetness is metabolically independent of insulin and anticariogenic properties (Milessi et al. 2011). Branco et al. (2008) obtained a conversion of xylose to xylitol (0.87 g/g) using the yeast *Candida guilliermondii* in a bench reactor. Rodrigues et al. (2011) recently evaluated xylitol production (0.61 g/g) from *Pichia stipitis* YS-30 using corn stover acid hydrolysate. Hemicellulose hydrolysate from various agro-residues has shown great potential in other added value products such as ethanol (Chandel et al. 2007a; Mouta et al. 2011), citric acid (Kirimura et al. 1999), lactic acid (Ou et al. 2011), single cell protein (SCP) (Anupama and Ravindra 2000), and 2,3-butanediol (Zhao et al. 2011). Recently, Rabelo et al. (2011) evaluated the potential of hemicellulose-derived sugar solution for biogas production.

Looking at the potential of pentose sugars as fermentable feedstock for the production of commercially valuable products, the importance of pentose sugars recovery and subsequently their utilization in fermentation processes is highly remarkable.

Table 2.2 Dilute acid hydrolysis of various agro-residues for the production of hemicellulosic-derived products

Agro-residue	Conditions for acid hydrolysis	Sugars (g/L)	Inhibitors (g/L)	Reference
Rice straw	1.5 % H_2SO_4, 130 °C, 30 min, S: L = 1:10	Xylose, 17.2; Glucose, 4.3; Arabinose, 3.3	Acetate, 1.43; HMF, 0.15; Furfural, 0.25	Baek and Kwon (2007)
Wheat straw	1.85 % (w/v) H_2SO_4, 90 °C, 18 h, S: L = 1:20	Xylose, 12.80 ± 0.25; D-glucose, 1.70 ± 0.30; L -arabinose, 2.60 ± 0.21	Furfural, 0.15 ± 0.02; acetic acid, 2.70 ± 0.33	Nigam (2001)
Corn stover	2.13 % H_2SO_4, 180 min, 121 °C, S: L = 1:10	Xylose, 9.09; Glucose, 2.13; Arabinose, 1.01	Acetic acid, 1.48; Furans, 0.56; Phenolics, 0.08	Cao et al. (2009)
Sugarcane bagasse	2.5 % (v/v) HCl, 140 °C, 30 min, S: L = 1:10	Total reducing sugars (TRS), 30.29	Furans, 1.89; Phenolics, 2.75; Acetic acid, 5.45	Chandel et al. (2007a)
Brewary's spent grain	Combined severity, 1.94 (3 % (w/w) H_2SO_4, 130 °C, 15 min, S:L = 8 % w/w)	Xylose, 26.7; Arabinose, 12.8; Glucose, 4.0	Acetic acid, 1.5; Formic acid, 0.23; Furfural, 0.29; HMF, 0.02; Total phenolics 0.91	Carvalheiro et al. (2004)
Sugarcane leaves straw	130 °C, 2.9 % H_2SO_4 w/v, S:L = 1:4, 30 min	Total reducing sugars (TRS), 56.5	Acetic acid, 3.19; Furfural, 0.56; HMF, 0.15	Mouta et al. (2011)
Sorghum straw	2 % H_2SO_4, 122 °C, 71 min	Xylose, 54.2; Glucose, 13.5; Arabinose, 12	Furfural, 0.2; acetic acid, 0.00	Sepúlveda-Huerta et al. (2006)
De-oiled rice bran	3.5 % v/v H_2SO_4, 120 °C, S:L = 1:10, 60 min	TRS, 38.50 ± 0.45	n.d.	Chandel et al. (2009)
Barley bran	3 % w/v H_2SO_4, 120 °C, S:L = 1:10, 60 min		n.d.	Cruz et al. (2000)

S:L solid:liquid ratio, *n.d.* not detected

2.4 Factors Influencing the Dilute Acid Hydrolysis of Agro-Residues

The achievement of high levels of sugar after pretreatment is a crucial factor for the commercial competitiveness of the use of lignocellulosic materials. Pretreatment in the form of dilute acid hydrolysis is one of the most important cost contributing factors in overall bioconversion processing of agro-residues (Yang and Wyman 2008; Chandel et al. 2010a). Furthermore, dilute acid hydrolysis facilitates the enzymatic hydrolysis of the remaining cellulosic fraction into glucose (Chandel et al. 2011b). Pretreatment of agro-residues with diluted acid has been extensively studied and found to be an efficient method for recovery of fermentable sugars (Laopainboon et al. 2010; Akpinar et al. 2009). Dilute acid hydrolysis has the advantage over concentrated acid hydrolysis due to equipment corrosion (Gírio et al. 2010). However, this process has the disadvantage of formation of cell wall-derived inhibitors such as furfural and 5-hydroxymethylfurfural (HMF), weak acids, phenolics and others evidencing the importance of the optimization of the conditions used in the dilute acid-based process (Yang and Wyman 2008; Chandel et al. 2011c).

The concentration of released sugars during pretreatment is directly dependent upon the type of lignocellulosic material, composition of substrates, temperature, time, acid concentration, solid-to-liquid ratio and the reactors employed in the process (Lee et al. 1999; Lenihan et al. 2011; Mosier et al. 2005; Taherzadeh and Karimi 2007; Xiang et al. 2003; Akpinar et al. 2009).

Table 2.3 shows the effect of influencing factors on sugars recovery after hemicellulosics hydrolysis from a variety of agro-residues. The cell wall composition of the agro-residues and the operational conditions directly influence the recovery of sugars (Shatalov and Pereira 2012). Akpinar et al. (2009) found that two different lignocellulosic materials when subjected to similar hydrolytic conditions, showed different levels of xylose recovery. Biomass with high lignin content released a smaller amount of fermentable sugars due to its structural compactness. Studies carried out at our laboratory for dilute sulfuric and oxalic acid hydrolysis of sugarcane bagasse showed efficient hydrolysis of hemicellulose (more than 80 % sugar recovery). Figure 2.2 shows the scanning electron microscopic (SEM) analysis of sulfuric acid and oxalic acid pretreated sugarcane bagasse.

A clear-cut distinction is visible in the cell wall compactness of sugarcane bagasse after pretreatment compared with native sugarcane bagasse. Dilute acids act mechanistically on hemicellulose making the overall structure quite disorganized due to the disruption of hemicellulose. Sulfuric acid is the most commonly used acid for the pretreatment of agro-residues. Laopaiboon et al. (2010) tested different acids for sugarcane bagasse hydrolysis and found the maximum sugar recovery with HCl. Li et al. (2008) used double acid hydrolysis (HCl and H_2SO_4) for the sugars recovery from lignocellulosic waste. Rocha et al. (2011) used a mixture of many acids for the hydrolysis of sugarcane bagasse and reported more than 90 % hydrolysis of the hemicellulosic fraction.

Table 2.3 Dilute acid hydrolysates of various for the production of various industrially significant products by microbial fermentation

Agro-residue hydrolysate	Sugars released (g/L)	Fermenting microorganisms	Production profile of compounds (g/g or g/L)	References
Corn stover	Glucose, 2.39; Xylose, 21.50; Arabinose, 5.12	*Pichia stipitis* YS-30	Xylitol (0.61 g/g)	Rodrigues et al. (2011)
Wheat straw	Xylose, 37	*Candida guilliermondii* FTI 20037	Xylitol (0.59 g/g)	Canilha et al. (2008)
Sugarcane bagasse	Xylose, 30	*C. guilliermondii*	Xylitol (63 % conversion)	Silva et al. (2007)
Sugarcane bagasse	Xylose, 30	*C. guilliermondii* FTI 20037	Xylitol (0.58 g/g)	Carvalho et al. (2007)
Sugarcane bagasse	Xylose, 20	*Candida shehatae* NCIM 3501	Ethanol (0.48 g/g)	Chandel et al. (2007a)
Groundnut shell	Total sugars, 57.5	*P. stipitis* NCIM 3498	Ethanol (23.5 g/L)	Gajula et al. (2010)
Wheat straw	D-xylose, 45.00 ± 0.33; D-glucose, 6.40 ± 0.21; L-arabinose 9.00 ± 0.12	*P. stipitis* NRRL Y-7124	Ethanol (0.41 g/g)	Nigam (2001)
Corn cob	D-glucose, 39.0 ± 1.5 g/k of corn cob; D-Xylose 233 ± 6.7 g/kg of corn cob	*Rhizopus oryzae* GY18	Lactic acid (355 g/kg of corn cob)	Guo et al. (2010)
Dried distillers grain (DDG)	na	*Aspergillus niger* ATCC 9142	Citric acid (5.25 g/kg of DDG)	Xie and West, (2009)
Sugarcane bagasse	D-Xylose, 47.2 ± 0.82; D-Glucose, 4.5 ± 0.17; L-Arabinose, 6.2 ± 0.29	*Candida langeronii* RLJ Y-019	Single cell protein (0.4 g/g)	Nigam (2000)

na not available

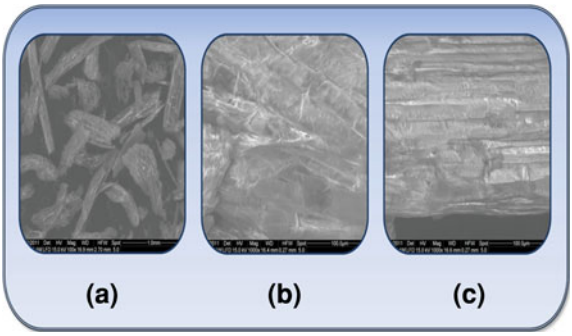


Fig. 2.2 Scanning electron microscopic (SEM) analysis of sugarcane bagasse: native (**a**), dilute sulfuric acid pretreated (**b**) and dilute oxalic acid pretreated (**c**)

Among the factors that influence the efficiency of acid hydrolysis, temperature, reaction time and acid concentration are the most widely investigated factors. However, some authors investigated the influence of particle size of biomass and solid/liquid ratio (Kapdan et al. 2011). The acid concentration is considered one of the most important factors regarding the release of sugars. High concentrations of acid may decompose the hemicellulosic structure, producing inhibitors and also causing damage to the equipment used. Therefore, an appropriate acid concentration is essential for acid hydrolysis of lignocellulose at industrial scale (Taherzadeh and Karimi 2007).

Temperature is also a crucial factor that affects directly the degradation of sugars into inhibitors, which eventually affect microbial metabolism (Akpinar et al. 2009; Chandel et al. 2007a). Temperature is directly connected to the energy waste of the process (Kim et al. 2011). In general, it is observed that mild temperature led to a significant recovery of sugars while higher temperatures caused more sugar degradation, aiding the formation of inhibitors (Yang and Wyman 2008).

According to Gírio et al. (2010), sulphuric acid/hydrochloric acid concentrations for hemicellulose hydrolysis are in the range of 0.5–1.5 % and temperatures between 121 and 160 °C. However, there is no direct recipe or formula which can be considered the same for all agro-residues. The pretreatment conditions will depend on the type and species of the used vegetal biomass. Canilha et al. (2011) found that temperature is the most important factor followed by acid concentration and time. Neureiter et al. (2002) found that the acid concentration is the most significant factor for hemicellulose hydrolysis. During acid pretreatment, more severe processes cause fast sugar degradation and thus yield poor hydrolytic efficiency (Bösch et al. 2010).

2.5 Dilute Acid Hydrolysis of Various Agro-Residues

The use of diluted acids to break down the biomass structure and recovery of the hemicelluloses derived sugars have been studied extensively with varieties of agricultural residues (Table 2.3). Rodrigues et al. (2010) obtained approximately

74 % xylose yields from sugarcane bagasse at 130 °C for 10 min using 100 mg of sulfuric acid per gram of bagasse. Sugarcane leaves straw was submitted to dilute acid hydrolysis under the optimized conditions (130 °C, 2.9 % w/v sulfuric acid, solid:liquid ratio 1:4, and 30 min of residence time) and produced 56.5 g/L total reducing sugars (Mouta et al. 2011). Akpinar et al. (2009) found the optimized set of conditions of 120 °C, 30 min and 4 % acid concentration and 133 °C, 27 min and 4.9 % of acid for the hydrolysis of sunflower stalk and tobacco stalk, respectively. Kim et al. (2011) reported the best conditions (150 °C, 16.9 min and 1.16 % of acid concentration) for the maximum hemicellulose hydrolysis of barley straw. Roberto et al. (2003) studied the parameters for diluted acid hydrolysis of rice straw and reported the best conditions (1 % H_2SO_4, 27 min at 121 °C) for maximum hydrolysis.

Rahman et al. (2006) evaluated the production of xylose from oil palm empty fruit bunch fiber using sulfuric acid. Optimum conditions (6 % H_2SO_4 concentration, 120 °C, 15 min) produced 29.4 g/L xylose concentration. However, Zhang et al. (2012) investigated conditions of combined dilute acid-catalyzed hydrolysis of oil palm empty fruit bunch for the optimum production of xylose. Maximum xylose yield (91.3 %) was obtained after hydrolysis catalyzed by 0.5 % (w/v) of H_2SO_4 and 0.2 % (w/v) of H_3PO_4 at 160 °C at a liquid to solid ratio of 20 mL/g for 10 min. In this study, the authors concluded that combined use of H_2SO_4 and H_3PO_4 showed a synergistic effect on improved hemicelluloses hydrolysis as compared to H_2SO_4 alone. Herrera et al. (2003) evaluated the production of xylose (16.2 g/L) from sorghum straw using HCl under the optimized set of conditions 6 % HCl, 122 °C for 70 min. Mussatto and Roberto (2005) carried out statistical optimization studies for dilute acid hydrolysis of brewer's spent grain and found the best conditions (liquid/solid ratio of 8 g/g, 100 mg H_2SO_4/g of dry matter, 17 min) which yielded 92.7 % extraction of hemicellulosic sugars.

2.6 Some Aspects of Kinetics of Dilute Acid Hydrolysis

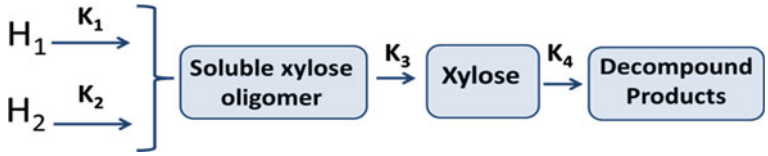
The kinetics of dilute acid hydrolysis of agro-residues mainly depends upon the temperature of reaction, acid concentration, time, substrate concentration and substrate composition (Mosier et al. 2005; Taherzadeh and Karimi 2007). There are two different phases involved in dilute acid hydrolysis—solid phase (agro-residues) and liquid phase (dilute acid-catalyst). Saeman (1945) initially proposed the kinetic model for the hydrolysis of Douglas-fir wood. This model was found as an irreversible pseudo-homogeneous first order reaction. In fact, this model was based on the cellulose conversion into glucose followed by its conversion into decomposition products:

$$\text{Glucan (s)} \xrightarrow{k_1} \text{Glucose (p)} \xrightarrow{k_2} (\text{HMF}) \tag{2.1}$$

K_1 Rate of glucan conversion into glucose
K_2 Rate of glucose decomposition into hydroxyl methyl furfural (HMF)

Both reactions can be considered as first order and irreversible. This model can also be considered for hemicelluloses hydrolysis. McMillan (1992) reported that hemicellulose hydrolysis at high temperature (more than 160 °C) is not homogeneous and has two types of kinetic patterns, i.e. fast hydrolysis and slow hydrolysis.

The kinetics of hemicellulose hydrolysis can be presented as follows (Lee et al. 1999):



where
H_1 Kinetics of hemicelluloses hydrolysis (easy to hydrolyse)
H_2 Kinetics of hemicelluloses hydrolysis (difficult to hydrolyse)

Grohman et al. (1986) proposed that the dilute acid hydrolysis of hemicellulose is a pseudo-homogenous first order reaction consisting of a fast hydrolyzing reaction and a slow hydrolyzing reaction. Lenihan et al. (2011) described the hemicellulose hydrolysis as follows:

$$\text{Hemicellulose (s)} \xrightarrow{k_1} \text{soluble sugars (p)} \xrightarrow{k_2} \text{(decomposition products)}$$

Monomers (xylose or other sugars) concentration (M) is a function of time (t) which can be represented as:

$$M = [k_1 \cdot P_0/k_2 - k_1](e^{-k_1 t} - e^{-k_2 t}) + M_0 e^{-k_2 t} \tag{2.2}$$

where
M Monomer concentration (g/L)
P Polymer concentration (g/L)
M_O Initial monomer concentration (g/L)

Consider that initial monomer concentration (M_O) is equal to zero, then Eq. (2.2) can be represented as (Lenihan et al. 2011):

$$M = [k_1.P_0/k_2 - k_1](e^{-k_1 t} - e^{-k_2 t}) \tag{2.3}$$

As described before, there are two fractions in hemicellulose polymer, i.e. fast hydrolyzing and slow hydrolyzing. The ratio between both the fractions is α. This equation can be further simplified as (Lenihan et al. 2011):

$$M = \alpha[k_1.P_0/k_2 - k_1](e^{-k}{}_1{}^t - e^{-k}{}_2{}^t) \quad (2.4)$$

If hemicelluloses hydrolysis is 100 % fast hydrolyzing then $\alpha = 1\,\mathrm{g/g}$.

2.7 Process Configurations Considering the Reactors used for Dilute Acid Hydrolysis

Dilute acid hydrolysis is probably one of the most common and largely explored methods to hydrolyse the hemicellulosic fraction of agro-residues or other lignocellulosic materials. Batch hydrolysis is the simplified process where substrate and dilute acid are mixed first followed by the reaction at high temperature for a certain period of time and recovery of the liquid fraction consisting of hemicellulosic-derived sugars in addition to cell wall-derived inhibitors. The acid-pretreated agro-residues are enzymatically digested to depolymerise cellulose into glucose.

Among all the available technologies, dilute acid hydrolysis followed by enzymatic hydrolysis by less expensive and more efficient cellulases has been found more promising towards the potential economics and environmental impact (Taherzadeh and Karimi 2007; Chandel et al. 2007b). Dilute acid hydrolysis was first demonstrated by Faith (1945) treating wood chips with dilute sulfuric acid (0.5 %) for 45 min at 11–12 bar pressure. Since then, a lot of developments have been seen aiming towards the development of a dilute acid hydrolysis process with less acid load, less reaction time and high temperature.

Batch processes of dilute acid hydrolysis generally show more than 80 % recovery of hemicellulose (xylose-rich hydrolysate containing arabinose, mannose, glucose and galactose) in addition to cell wall-derived inhibitors (furfurals, 5-hydroxy methyl furfural, weak organic acids, lignin derived phenolics and others). Batch hydrolysis can be carried out in a variety of vessels (Erlenmeyer flasks to stainless cylindrical steel vessels, Parr reactor, stirred tank reactor, rotating drum reactor, etc.). Lignocellulosic material and dilute acid are added in Erlenmeyer flasks or cylindrical vessels made of stainless steel which are kept in autoclave, Parr reactor or oil bath reactor for reaction at higher temperatures. The required temperature in a Parr reactor, stirred tank reactor and drum reactors is approached by providing heat or cooling water from an outside source through the jacket. Figure 2.3 shows the examples of vessels/reactors used for dilute acid hydrolysis at the Engineering School of Lorena, University of Sao Paulo (USP), Lorena, São Paulo, Brazil. Dilute acid hydrolysis is carried out in two stages.

2.7.1 First-Stage Dilute Acid Hydrolysis

The lignocellulosic material is first placed in contact with dilute sulfuric acid (0.75 %) and heated to approximately 50 °C, then transferred to the first-stage acid

Fig. 2.3 Systems explored for dilute acid hydrolysis of various agro-residues at Engineering School of Lorena (EEL), University of Sao Paulo, Lorena. **a** Large reactor of capacity 1,000 L. **b** Medium size rotator reactor. **c** Autoclave equipped for acid hydrolysis of biomass. **d** Parr reactor. **e** Oil bath reactor with stainless steel made hydrolytic vessels. **f** Medium size static reactor

impregnator where the temperature is raised to 190 °C. Approximately, 80 % of the hemicellulose and 29 % of cellulose are hydrolyzed in the first reactor. The hydrolysate is further incubated at a lower temperature for a residence time of 2 h to hydrolyse most of the oligosaccharides into monosaccharides followed by the separation of solid and liquid fractions (Chandel et al. 2007b). The solid material is then again washed with plenty of water to maximize sugar recovery. The separated solid material is sent to the second-stage acid hydrolysis reactor. Figure 2.4 shows the configuration outline of batch processes for acid hydrolysis of lignocellulosic materials.

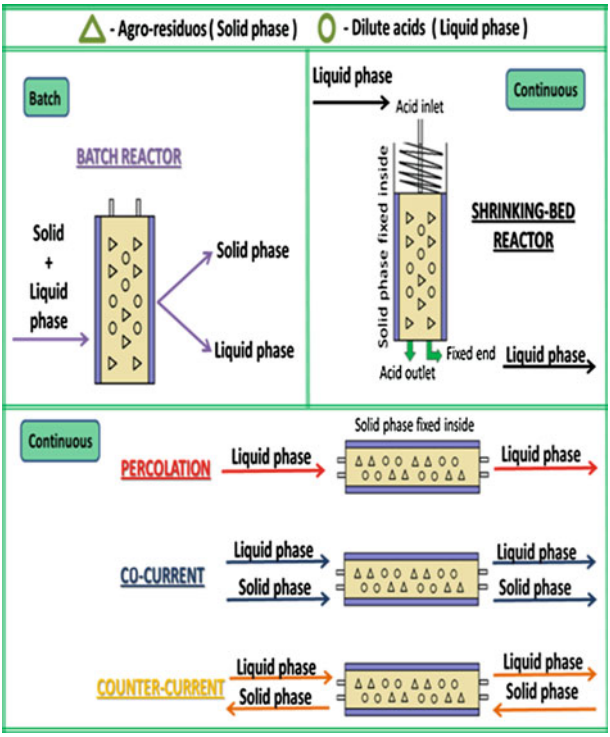


Fig. 2.4 Modified reactors configured for batch and continuous hydrolysis of agro-residues (Batch reactor, continuous reactors: counter-current, percolation reactor, plug-flow and shrinking-bed reactor)

2.7.2 Two-Stage Dilute Acid Hydrolysis

In the two-stage dilute acid hydrolysis process, first, biomass is treated with dilute acid at relatively mild conditions during which the hemicelluose fraction is hydrolyzed, and the second stage is normally carried out at a higher temperature for depolymerisation of cellulose into glucose (Nguyen et al. 2000). The liquid phase, containing the monomeric sugars is removed between the treatments, thereby avoiding degradation of monosaccharides formed. Sanchez et al. (2004) carried out the two-stage dilute acid hydrolysis using Bolivian straw material, *Paja brava*. In the first stage, *P. brava* was pretreated with steam followed by dilute sulfuric acid (0.5 or 1.0 % by wt.) hydrolysis at temperatures between 170 and 230 °C for a residence time between 3 and 10 min. The highest yield of hemicellulose-derived sugars was found at a temperature of 190 °C, and a reaction time of 5–10 min, whereas in second stage hydrolysis considerably higher temperature (230 °C) was found for hydrolysis of the remaining fraction of cellulose.

The two-stage dilute acid hydrolysis process is generally preferred over the first-stage dilute acid hydrolysis process due to less energy requirements,

minimization of sugars degradation and less derivation of inhibitors (Nguyen et al. 2000). Batch hydrolysis processes are most commonly employed for the hydrolysis of lignocellulosic materials. However, continuous processes for dilute acid hydrolysis have also been employed.

2.7.3 Continuous (Percolation Reactor, Plug-Flow Reactor, Counter-Current Reactor, Shrinking-Bed Reactor)

Figure 2.4 describes the configuration outline of the continuous dilute acid hydrolysis process employed for the acid hydrolysis of agro-residues.

2.7.3.1 Plug-Flow Reactor

Figure 2.4 shows the conceptual diagram of Plug flow reactor (PFR). In a PFR, it is quite difficult to control short residence time of biomass pretreatment (range of few seconds). Low concentration of acids is required in PFR in conjunction with high temperature (200–240 °C). Heat transfer limitations are also apparent due to uneven reaction within biomass that eventually reduces the overall sugar yield. In general, 50–70 % yield of sugars are obtained in PFRs (Lee et al. 1999; Taherzadeh and Karimi 2007).

2.7.3.2 Percolation Reactor (Packed-Bed Flow Through Reactor)

Percolation reactors are easy to use for dilute acid hydrolysis of lignocellulosic biomass due to immovable biomass and liquid fraction. In the percolation reactor, sugars can be obtained as they are formed and thus minimize the chances of sugar degradation consequently releasing a high amount of sugars (Lee et al. 1999). Figure 2.4 shows the conceptual diagram of a percolation reactor. There are many benefits to using a percolation reactor. High lignocellulosic biomass-to-liquid ratio can be used in a percolation reactor. Hydrolysate is easily removed from the solid fraction. An optimized feeding of solid and liquid is an important parameter to get the high sugars yield in the reactor. Slow feeding of lignocellulosic material will cause high sugar degradation while very fast feeding will leave the substrate untreated. In both circumstances, low sugar yield is obtained. On the basis of kinetics of hydrolysis reaction, a percolation reactor can be divided into two types: (1) a step change of temperature and optimum temperature difference, and (2) a two-stage reverse-flow percolation reactor. The former percolation strategy constituted two different basic characteristics (fast-hydrolysing and slow hydrolyzing reactions). Temperature change during the process, particularly a step change from uniform low to uniform high, showed the improved results (Lee et al.

1999). In a two-stage reverse flow percolation reactor, the biomass is first pretreated at low temperature followed by high temperature reaction. The striking difference between a step-change and two-stage reverse flow percolation reactor is stream from high temperature is again exposed to fresh biomass at low temperature. The treated solid residue in this reactor is then treated with fresh acid at high temperature. This process is regularly repeated (Lee et al. 1999; Taherzadeh and Karimi 2007).

2.7.3.3 Counter-Current Reactor

These reactors are specialized moving bed reactors in which the direction of solid and liquid are reversed. Figure 2.4 shows the conceptual diagram of a counter-current reactor. A high concentration of sugars is obtained in counter-current reactors. The major part of sugars is formed near the liquid outlet point. The stream moves relatively short distances before it is washed out of the reactor thus reducing the time period for the sugar degradation, eventually increasing the high sugars yield (Lee et al. 1999; Taherzadeh and Karimi 2007). These reactors offer great advantages over plug flow or percolation reactors.

2.7.3.4 Shrinking Bed Counter-Current Reactor

Continuous counter current reactors have the property of bed shrinking causing improvements in sugars recovery with minimized sugar degradation. The shrinking bed causes the change of solid velocity within the reactor which improves the hydrolysis efficiency (Lee et al. 1999). Figure 2.4 shows the conceptual diagram of a shrinking bed counter-current reactor.

2.8 Economics and Environmental Aspects

The major parameters which influence the cost of dilute acid hydrolysis of agro-residues are the cost of raw material, amount of catalytic agent (acid) and utility expenses (steam, cooling water, electricity and maintenance of reactors) (Yang and Wyman 2008). The cost impact of these parameters directly influences the sugar recovery, processing time and generation of inhibitors. An ideal dilute acid hydrolysed process is one which has minimum operational and functional cost while largely influencing sugars recovery with the least amount of inhibitors generation (Yang and Wyman 2008). Removal of inhibitors from acid hydrolysates prior to fermentation adds the extra cost in biomass conversion. A generalized procedural break-up of expenses in ethanol production from lignocellulosic feedstock(s) shows that the cost of raw material (almost 34 %) is the main important factor to be considered, followed by total pretreatment cost (17.5 %), boiler energy (8.5 %) and utilities and storage (5 %) (Viikari 2004). Among the

LCF, agro-residues such as sunflower stalk, *Brassica compestris* stalks, cotton stalks, sugarcane leaves, groundnut shell, etc., which have minimum food/feed value and commercial applications would be of paramount importance.

Dilute acid hydrolysis of agro-residues in batch processes usually done using low concentration of acids, high temperature, and different time variations eventually release moderate amounts of sugars recovery (80–85 %) with considerable amount of cell wall derived inhibitors (Canilha et al. 2011). Dilute acid hydrolysis using modified continuous reactors (plug flow, percolation, counter-current and shrinking-bed) generally occur at high temperature (200–240 °C) for a short time (less than 1 min) using low acid concentration (Lee et al. 1999). Counter-current operations could be more economic in terms of yielding high amounts of sugars in short times with less operational cost (Lee et al. 1999). National Renewable Energy Laboratory (NREL)-based process engineering studies have developed a fully-integrated and mature material balance and financial modules (ASPEN PLUS) process simulation which can provide the net economic analysis of dilute acid hydrolysis of various agro-residues (Mosier et al. 2005).

With regards to net impact of dilute acid hydrolysis-based protocols on the environment, it can be stated that these processes are not very environmentally friendly (Taherzadeh and Karimi 2008). However, a much diluted concentration of acids is used in dilute acid hydrolysis of agro-residues. In addition to acid load, a high temperature range also poses a negative effect and an overall impact on the environment. Hemicellulase enzyme-based catalysed processes certainly have advantages over acid catalysed processes (Gírio et al. 2010). However, looking at the high sugars recovery in short time periods, dilute acid hydrolysis is still considered as a favorite compared to enzyme-based processes for hemicellulose break-down. Considering less acid load at high temperature is a preferred method since it causes less environmental pollution compared with strong acid hydrolysis. Another significant advantage using dilute acid hydrolysis is that generation of a low amount of inhibitors eventually requires milder detoxification methods to eliminate the inhibitors.

2.9 Conclusion and Future Recommendations

Agro-residues constitute the important part of total biomass available on earth for their readily available application in bioconversion systems. However, pretreatment of agro-residues with dilute acid hydrolysis is an important step for their fullest utilization. Dilute acids catalyze the hemicellulose degradation into simpler constituents and thus increase the amenability of cellulolytic enzymes towards the hydrolysis of the cellulosic fraction into fermentable feedstock. During hemicellulose hydrolysis by dilute acid catalyzed processes, a number of inhibitors are also generated which hamper the fermentation reaction for the production of important metabolites by microorganisms. In the last 30 years, significant work has been carried out in order to establish the successful and reproducible

technological protocol for hemicellulose depolymerisation with fewer number of inhibitors. Parameters involved in dilute acid hydrolysis of agro-residues viz. acid load, substrate to liquid ratio, residence time and temperature play the decisive role in obtaining the maximum hydrolysis efficiency. Consideration of these parameters also depends upon the selection of raw material. Batch reactors have been used more often for agro-residues hydrolysis. However, various modifications in reactors have also been adopted in order to maximize hemicellulose hydrolysis within short time periods. Reactor modifications such as percolation, plug-flow, counter-current and shrinking bed have been used for acid hydrolysis of agro-residues. Counter-current reactors have shown improved results in some studies carried out at the National Renewable Energy Laboratory (NREL), Colorado, USA. Better understanding of kinetic models for their implication in these reactor modifications may further improve the hemicellulose hydrolysis. Economics and environmental impact are two important considerations for the selection of dilute acid hydrolysis-based pretreatment technology. In future, technologies based on robust optimization tools for dilute acid hydrolysis, finding the ideal acid or mixed dilute acids considering the basic parameters and suitable reactors would be the ultimate choice. Application of nano-particles-based membrane systems and their implication in hemicellulose hydrolysis may also provide better results in short times with fewer by-products.

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Chapter 3
Detoxification Strategies Applied to Lignocellulosic Hydrolysates for Improved Xylitol Production

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Abstract Hemicellulose is the second most abundant renewable biomass after cellulose on the planet. It is one of the three heterogenous polymers, derived from lignocellulose biomass which yields individual sugars, mainly xylose after dilute acid or hydrothermal pretreatment. Among the microbial co-products generated from hemicellulose sugars, xylitol is the most abundant and holds the most valued potential in numerous medical and non-medical applications. During the hemicellulose hydrolysis, in addition to the production of sugars, a number of plant cell wall derived inhibitors are generated as byproducts of the process. It is essential to apply a detoxification strategy to remove the toxic inhibitors from hemicellulosic hydrolysates. This allows for a satisfactory xylitol yield and productive microbial fermentation. During detoxification, several methods such as calcium hydroxide overliming, activated charcoal, and ion-exchange are routinely used to overcome the inhibitors. More recently, biological applications (laccase, direct implication of microorganisms having the affinity towards inhibitors) and systems biology-based approaches have gained significant attraction for the development of microbial traits to counteract the effects of inhibitors while simultaneously fermenting the xylose sugar solution into xylitol. This chapter aims to discuss the various strategies used in the detoxification of lignocellulose hydrolysates for the fermentative production of xylitol. Particular emphasis is placed on the biological applications used for clarification of hemicellulosic syrups with future perspectives.

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3.1 Introduction

Lignocellulose biomass account for the majority of the total agricultural, forest and industrial biomass present in the biosphere. There are three types of polymers found in lignocellulose biomass that create a complex hydrogen-bonding network: hemicellulose, cellulose and lignin (Fang et al. 2010). Hemicellulose is comprised of D-glucose, D-galactose, D-mannose, D-xylose, D-glucuronic acid and D-arabinose and accounts for 19–34 % of xylose-rich lignocellulosic biomass (Chen et al. 2010). Hemicellulose is the second most abundant polysaccharide in nature, proving to be a good source for xylitol production (Chandel et al. 2010; Chen et al. 2010). It is also a promising renewable carbohydrate source to support sustainable biofuel production (Chandel et al. 2010). Progressive depletion in the world fossil fuel resources and the world's increased energy consumption has intensified the development of alternative forms of energy, which have been extensively researched in hopes to implement biofuel from lignocellulosic biomass as a viable alternative to petroleum.

Xylitol, comprised of five carbon atoms, typically occurs in oats, corn cobs, straw, sugar cane bagasse and certain hardwoods such as birch and beech (Mosier et al. 2005; Watanabe et al. 2011). Xylitol is also referred to as pentiol because five carbon and hydroxyl groups have the capacity to form complexes with certain cations such as Cu^{2+} and Fe^{2+} (Chen et al. 2010). The new trend in biorefinery includes the production of profitable co-products such as xylitol from hemicellulose along with cellulosic ethanol (Huang et al. 2011). Although xylitol can be found in certain fruits and vegetables such as lettuce, berries, mushrooms, cauliflower and plums, it has limited contents to extract in large amounts (Chen et al. 2010). There is a strong market for the development of a cost-efficient method for the production of xylitol from hemicellulosic fraction of lignocellulosic biomass, and if production is improved this can make the process of biomass utilization commercially feasible (Silva and Roberto 2001). This sugar has gained a lot of global attention due to its lower calorie sweetener properties, similar to that of sucrose (2.4 vs. 4.0 cal/g), as well as its non-cariogenic properties that can inhibit the growth of bacteria that causes tooth decay, thus preventing acute otitis, dental caries, and promoting oral health as well as benefiting pregnant and nursing women (Huang et al. 2011; Prakasham et al. 2009; Chen et al. 2010; Zhuang et al. 2012). Xylitol has been employed as a sugar substitute in diabetics as it metabolizes through an insulin-independent pathway in the human body (Huang et al. 2011; Zhuang et al. 2012). In addition, there is also an increasing demand for xylitol in the food industry as it is a cheap, widespread and renewable sugar source (Silva and Roberto 2001). In 2010, the global market value for xylitol was about

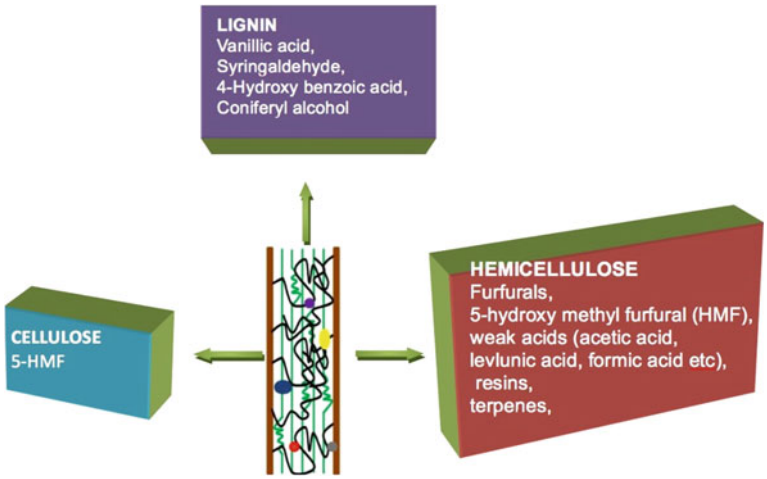

Fig. 3.1 Toxic inhibitors generated during lignocellulose pre-treatment aiming at hemicellulose depolymerisation

$340 million/year and continues to grow (Chen et al. 2010). Many bakery products use xylitol as a sucrose substitute because xylitol has a better taste and outward appearance in comparison to baking sugar (Vieira and Roberto 2010).

Depolymerisation of the hemicellulosic fraction catalyzed by dilute acid, hot water or steam explosion yields appreciable amounts of xylose (five carbon sugar), in addition to significant amounts of cell wall derived inhibitors (sugar-derived furfurals, 5-hydroxy methyl furfurals, weak acids, lignin derived phenolic compounds and other specific compounds due to cell wall structure) (Mussatto and Roberto 2004; Parawira and Tekere 2011; Chandel et al. 2011a). Figure 3.1 shows the various inhibitors derived from lignocellulosic biomass during hemicellulose destruction. It is essential to eliminate these inhibitors from lignocellulose hydrolysates in order to obtain satisfactory yields of xylitol during fermentative reaction.

There are several physical and physico-chemical (evaporation, steam stripping, solvent extraction, membrane based separations), chemical (neutralization, calcium hydroxide overliming, activated charcoal, ion-exchange resins) and biological methods (enzyme-laccase, peroxidases applications, direct implication of microorganisms) in practice to overcome these inhibitors (Mussatto and Roberto 2004; Parawira and Tekere 2011; Chandel et al. 2011a). Each method has pros and cons. Each method has their special affinity towards a particular class of inhibitors. Therefore, combined strategies employing more than one method is required to remove the inhibitors from hydrolysates. In recent years, the biological pretreatment of lignocellulosic hydrolysates prior to hydrolysis was geared toward increasing the sugars yield with minimum amount of inhibitors. Among the various detoxification strategies, biological methods have gained significant momentum because of their simplicity, high effectivity, economics and eco-friendliness (Parawira and Tekere 2011). This chapter aims to discuss the current industrial methods used to produce

xylitol and the limiting factors in this process, and to understand the role inhibitors play in the detoxification process. Various detoxification methods applied to lignocellulose hydrolysates have been discussed. Lastly, future perspectives with a projected outlook in regards to improving the sustainable and economically sound approaches for xylitol production have been discussed.

3.2 Hemicellulose Hydrolysis and Generation of Plant Cell Wall Derived Inhibitors

When lignocellulosic materials are hydrolyzed via chemical-based methods, the result is sugars such as D-glucose, D-galactose, D-arabinose, D-mannose and D-xylose, of which D-xylose is the most abundant sugar found in the hemicellulose fraction (Parajo et al. 1997; Chen et al. 2010; Chandel et al. 2007). The major drawback in the hydrolysis of hemicelluloses for xylitol production is the lack of cost efficiency and requirement of further purification and separation due to the introduction of bi-products toxic to fermenting microorganisms (Mosier et al. 2005; Chen et al. 2010; Huang et al. 2011; Buhner and Agblevor 2004). Currently, commercial production of xylitol begins with chemical hydrogenation of the xylan-rich lignocellulosic biomass such as corn fiber, rice straw, spruce, corncob, sugarcane bagasse and birchwood (Buhner and Agblevor 2004). Figure 3.2 demonstrates the overall view of xylitol production from lignocellulosic materials.

As represented in Fig. 3.2, the lignocellulosic biomass is composed of varying amount of cellulose, hemicellulose, lignin, pectin, proteins, and smaller amounts of extractives and ash. The D-xylose fraction of hemicellulose can be converted into xylitol by chemical reduction and/or by microorganisms. There has been significant progress made in reducing the effect of inhibitors to improve the fermentation of lignocellulosic, thus also improving xylitol yield (Prakasham et al. 2009).

In order to extract xylitol, the lignocellulosic biomass is depolymerized through a pre-treatment phase using a number of different methods such as alkali treatment, steam explosion, acid and the use of a metal catalyst, such as nickel, under high temperature and pressure conditions (Zhuang et al. 2012; Mosier et al. 2005; Converti et al. 1999, 2000). Among many pretreatment methods, formic acid is a novel pathway to convert lignocellulosic biomass to fermentable sugars but is a toxic inhibitory to the fermentation of D-xylose (Zhuang et al. 2012). As briefed in Fig. 3.1, the release of toxic inhibitors generated during lignocellulose pre-treatment is considered to be an unavoidable step in the production of xylitol because this alters the biomass size, structure and sub-microscopic chemical composition, thus allowing for rapid hydrolysis and greater yields of xylitol (Mosier et al. 2005).

The types of inhibitors are determined by factors such as the type of lignocellulosic material, cell wall composition and the thermochemical conditions of the process such as temperature, pH, etc. (Palmqvist and Hahn-Hagerdal 2000a, b; Mussatto and Roberto 2004). These toxic compounds inhibit the growth of fermenting microbes, which result in the decrease in the rate of product formation due to

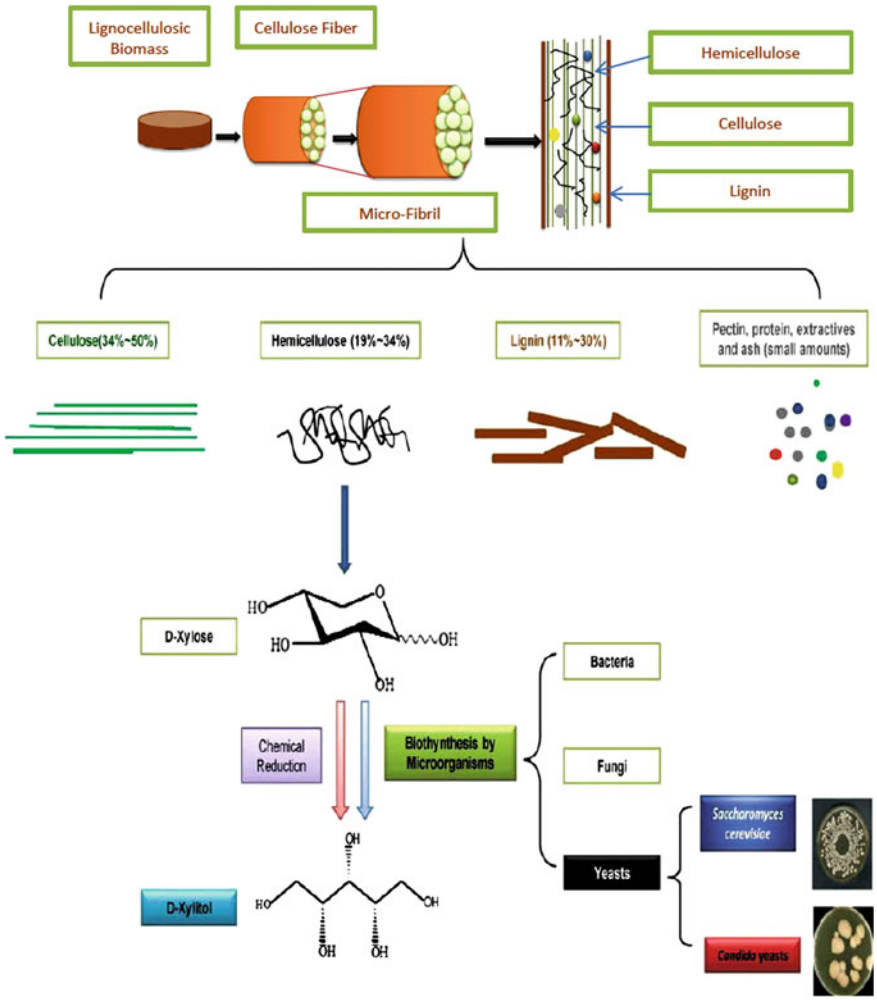

Fig. 3.2 Xylitol production from lignocellulosic biomass with the use of either chemical reduction method or biosynthesis of microorganisms (adapted and modified from source: Chen et al. 2010)

the altered rate of sugar uptake (Mussatto and Roberto 2004). In order to complete fermentation of xylose rich hydrolysate and to obtain suitable xylitol yields and productivities during microbial fermentation, inhibitors have to be removed from the lignocellulose hydrolysates (Zhuang et al. 2012). The inhibitors include weak acids, e.g. formic acids and acetic acids, furan derivatives, e.g. 5-hydroxyl methyl furfural (5-HMF), phenolic compounds such as acid, and aldehyde, e.g. syringic acid, ferulic acid and guaiacol and heavy metal ions, e.g. nickel and copper (Silva and Roberto 2001; Palmqvist and Hahn-Hagerdal 2000a, b; Mussatto and Roberto 2004). Table 3.1 summarizes major plant cell wall derived inhibitors during re-treatment.

Furan derivatives are formed typically at high temperatures and pressures when products of pentose and hexose are undergoing degradation (Mussatto and Roberto 2004). Phenolic compounds such as vanillin are lignin-derived inhibitors. Small concentrations of phenolic compound have been found to be lethal (Rodrigues et al. 2001). The lower their molecular weight is, the more lethal to microorganisms they are. These inhibitors affect cell growth and glucose assimilation by damaging the selectively permeable membrane (Mills et al. 2009). The inhibitory effects on the growth and xylitol production of xylitol producing microorganisms were found to be the most affected by vanillin (Vieira and Roberto 2010). The process of converting xylose to xylitol by *Candida guilliermondii* was strongly dependent on the presence of aldehydes and phenolic compounds in the fermentation medium, and when vanillin was converted to alcohol vanillyl, this increased the detoxification of the medium, thus improving xylitol production (Vieira and Roberto 2010).

3.3 Detoxification Strategies of Lignocellulose Hydrolysates for Xylitol Production

Detoxification strategies to overcome the inhibitors from lignocellulose hydrolysates can be summarized into four categories: physical methods, chemical methods, biological methods and integrated methods. Figure 3.3 summarizes the various detoxification strategies explored to overcome the fermentation inhibitors. Each method has its own specificity towards the removal of cell wall derived inhibitors. Table 3.2 represents the various detoxification methods used to eliminate the plant cell derived inhibitors from lignocellulose hydrolysate and xylitol production. It is difficult to compare the performance of each detoxification method corresponding to xylitol production because of the variation in degree of inhibition, microorganisms and fermentation strategies.

3.3.1 Physical Methods

3.3.1.1 Evaporation

Vacuum evaporation of lignocellulose hydrolysates aids in the removal of volatile compounds such as acetic acid, furfural and vanillin. Non-volatile compounds present in hydrolysates remain unaltered after vacuum evaporation. Rodrigues et al. (2001) reported 98 % removal of furfural during vacuum evaporation from sugarcane bagasse acid hydrolysate before or after activated charcoal treatment. The amount of sugars can be increased in the hydrolysates after vacuum evaporation. Activated charcoal treatment before or after the vacuum evaporation

Table 3.1 Plant cell wall derived inhibitors production

Lignocellulosic material	Hydrolytic conditions used	Plant cell wall derived inhibitors (g/l)	Sugars recovery (g/l) or (g/g)	References
Corn stover	2.13 % H_2SO_4, 180 min, 121 °C, S:L = 1:10	Acetic acid, 1.48; Furans, 0.56; Phenolics, 0.08	Xylose, 9.09; Glucose, 2.13; Arabinose, 1.01	Cao et al. (2009)
Soft wood	2 % HCL + 0.5 % $FeCl_2$ (v/v), 170 °C, 30 min	Acetic acid, 5.3; Furfural, 2.2	Monomeric sugars, 37.5 g/l	Qian et al. (2006)
Rice straw	1.5 % H_2SO_4, 130 °C, 30 min, S:L = 1:10	Acetate, 1.43; HMF, 0.15; Furfural, 0.25	Xylose, 9.09; Glucose, 2.13; Arabinose, 1.01	Baek and Kwon (2007)
Eucalyptus globulus	H_2SO_4 (0.65 %), 157 °C, 20 min, S:L = 8.6:1	Furfural, 0.26; 5-HMF, 0.07; Acetic acid, 3.41	Xylose, 12.34; Glucose, 0.63; Arabinose,	Villareal et al. (2006)
Saccharum spontaneum	160 °C, 15 min and 1.5 % sulfuric acid, S:L = 1:10	Total reducing sugars, 32.15 ± 0.25	Furfurals, 1.54 ± 0.04; Phenolics, 2.01 ± 0.08	Chandel et al. (2011b)
Poplar	AFEX treatment + acid hydrolysis	2-furoic acid, 0.3 microgram/g; 3,4-HBA, 2.5; Salicylic acid, 56; Syringaldehyde, 6.0; Ferulic acid, 4.7	Glucose, 2.0; Xylose, 11.36; Arabinose, 0.49	Balan et al. (2009)
Spruce	Sulfur dioxide (1 kg), 40 kg wood chips, 203 °C, 5 min + Enzymatic hydrolysis	Phenolics, 0.44 ± 0.05; Furfural, 1.0 ± 0.1; HMF,3.3 ± 0.2; Acetic acid, 5.0 ± 0.4; Levulinic acid, 0.2 ± 0.1; Formic acid, 0.7 ± 0.1	Glucoe, 82.4 ± 2.9; Xylose, 9.2 ± 0.1; Galactose, 3.8 ± 0.3; Mannose, 26.4 ± 0.7; Arabinose, 2.9 ± 0.2	Alriksson et al. (2011)

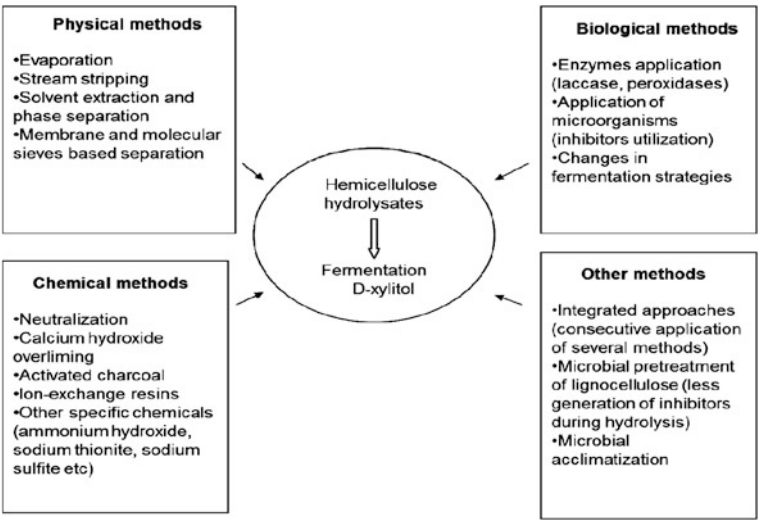

Fig. 3.3 Summarization of various detoxification strategies applied to lignocellulose hydrolysates for xylitol production

favored the removal of both acetic acid and phenolic compounds from hydrolysates. Sugar and inhibitor levels were compared in vacuum evaporated hydrolysates of Spruce by Dehkhoda et al. (2008). More than 96 % removal of furfurals along with the partial removal of formic and acetic acid from Spruce wood acid hydrolysates was recorded after vacuum evaporation. Zhu et al. (2009) observed the removal of 59.89 and 77.72 % formic acid and acetic acid, respectively, from corn stover acid hydrolysates. Wilson et al. (1989) found a decrease in the concentration of acetic acid, furfural and vanillin by 54, 100 and 29 %, respectively, compared with the concentrations in the hydrolysate. Table 3.2 shows the examples of evaporation-based detoxification strategies to overcome the fermentation inhibitors.

3.3.1.2 Stream Stripping

Steam stripping so called steam distillation has been used for the removal of inhibitors from the lignocellulose hydrolysates. This method was used to eliminate the organic compounds from the waste-water streams of industries (Wang and Feng 2010). In this method, dry steam is passed through with the lignocellulose hydrolysates and during this inhibitory compounds such as furfural and weak acids are removed. This method is more effective towards the removal of volatile phenolics from hydrolysates (Wang and Feng 2010). Steam stripping followed by activated charcoal led to removal of fermentation inhibitors from *Pinus radiata* hydrolysates in addition to 30 % sugars loss (Maddox and Murray 1983). Zhu et al. (2009) used steam stripping for the detoxification of corn stover hydrolysates

Table 3.2 Various detoxification strategies explored for detoxification of lignocellulosic hydrolysates for xylitol production

Lignocellulosic material	Method used for detoxification	Effect of method used for detoxification	Microorganism used for xylitol production	References
Eucoleptus globulus	Steam explosion + over + activated charcoal	Removal of acetic acid and furfural, high yields of xylitol	*Pachysolen tannophilus* NRRL Y-7426	Converti et al. (2000)
Wheat straw	Ion-exchange + over liming	Removal of acetic acid, phenolics and furfurals	*Candida tropicalis*	Zhuang et al. (2012)
Sugarcane bagasse	Neutralization + activated charcoal	Increased xylitol production, lowered HMF and furfural inhibitors	*C. guilliermondii* FTI 20037	Carvalho et al. (2004)
Wheat straw	Fresh D311 resins and NaOH	Removal of formic acid and acetic acid, phenolics and furfurals	*C. tropicalis*	Zhuang et al. (2012)
Brewery's spent grain (BSG)	Adjustment of pH by calcium oxide to 5.5 + activated charcoal	Removal of formic acid, furfural, HMF	No tolerance of *Debaryomyces hansenii* CCM 941 towards inhibitors and monosaccharides	Carvalheiro et al. (2006)
Corn fiber	Partial neutralization + activated charcoal	Removal of formic acid and acetic acid, phenolics and furfurals	*C. tropicalis* ATCC96745	Buhnwe and Agblevor, (2004)
Wheat straw	Organic solvent extraction using Ether and ethyl acetate (1:1) + over liming	Removal of acetic acid, phenolics and furfurals	*C. tropicalis*	Zhuang et al. (2012)
Corn fiber	Over liming with $CaOH_2$ + activated charcoal	Removal of acetic acid, phenolics and furfurals	*C. tropicalis* ATCC96745	Buhnwe and Agblevor (2004)
Wheat straw	Organic solvent extraction using ether + over liming	Removal of acetic acid, furfural and phenols	*C. tropicalis*	Zhuang et al. (2012)
Corn cobs	Over liming	Removal of furfural, acetic acid and phenolics	*C. tropicalis* W102	Cheng et al. (2008)
Sugarcane bagasse	Vacuum evaporation + activated charcoal	Removal of acetic acid, furfurals and vanillin	*C. guilliermondii* FTI 20037	Rodrigues et al. (2001)

and observed 58.79 % formic acid and 80.83 % acetic acid removal from the hydrolysates treated with steam stripping for 120 min.

3.3.1.3 Solvent Extraction and Phase Separation

Solvent extraction is generally used to remove the particular compounds from hydrolysates into some immiscible liquid solvent like di-ethyl ether. Parajo et al. (1997) found the removal of some volatile compounds from diethyl ether treated detoxified Eucalyptus wood hydrolysates. Converti et al. (1999) found a removal of phenolics (65 %) from hard wood hemicellulosic hydrolysates as compared to overliming plus charcoal treatment (more than 90 %), which eventually showed high xylitol productivity (19.2 g/L) by *Pachysolen tannophilus*. Supercritical fluid extraction of lignocellulose hydrolysates eliminated furnans, phenolics and aliphatic acids from the hydrolysates, which showed improved fermentation by *Saccharomyces cerevisiae* (Persson et al. 2002).

Griffin and Shu (2004) used boronic acid extractants (phenylboronic acid, 3,5-dimethylphenylboronic acid, 4-tert-butylphenylboronic acid, trans-β-styreneboronic acid or naphthalene-2-boronic acid) dissolved in an organic diluent (Shellsol 2046 or Exxal 10) containing the quaternary amine Aliquat336 to purify and concentrate the sugars present in sugarcane bagasse hemicellulosic hydrolysates. Naphthalene-2-boronic acid mediated extracted sugar solution followed by stripping increased seven-fold xylose concentration in conjunction with the 90 % reduction of acid soluble lignin from the acid hydrolysates. Zautsen et al. (2009) studied the solvent–solvent extraction for the lignocellulosic hydrolysates using decanol, oleyl alcohol, and oleic acid and observed two-fold ethanol productivity in the medium containing 6 g/L vanillin with 30 % volume oleyl alcohol. Due to considerable loss in fermentable sugars in hydrolysates, a modified phase separation system, namely, an aqueous two-phase system is in practice in place of solvent extraction. In this method, two polymers or one polymer with salt are used to eliminate inhibitory compounds from lignocellulose hydrolysates. This extraction is being employed at mild temperature, which does not affect sugar concentration and is safe to fermenting microorganisms. However, high cost of polymers and poor selectivity are the important concerns (Wang and Feng 2010).

3.3.1.4 Membrane and Molecular Sieves Based Separation of Inhibitors

Membrane assisted separation of inhibitors from the lignocellulose hydrolysates is also possible. This process has several advantages such as reducing the entrainment of organic phase in the aqueous phase. The organic phase is toxic to the fermenting microorganisms, therefore it is important to keep a separate organic phase with the aqueous phase through the membrane. Grzenia et al. (2010) investigated the use of membrane extraction to detoxify the corn stover hydrolysates pretreated using dilute sulphuric acid. Extraction of sulfuric, acetic, formic and levulinic acid was evaluated

due to their distribution in the octanol and oleyl-based organic phase solvents, and Alamine 336 was the reactive amine extract. Wickramasinghe and Grzenia (2008) observed better results for removal of acetic acid from biomass hydrolysates with anion exchange membranes (Sartobind Q) than ion exchange resin (Amberlyst A21) with minimal sugar loss. Qi et al. (2011) investigated the potential of commercial nanofiltration membranes (NF 90 and NF 270) to eliminate the furfurals from lignocellulosic hydrolysates in consequence with the concentration of sugars. The NF 90 mediated separation process significantly increased the sugars concentrations, simultaneously reducing the furfural concentration. Sugar maple hemicellulose hydrolysate was detoxified by two-stage membrane filtration (200 and 100 Da molecular weight membranes) and found the significant removal of acetic acid, furfural and HMF (Stoutenburg et al. 2008), which eventually increased the fermentation efficiency of *Pichia stipitis* NRRL Y-7124.

To detoxify the lignocellulose hydrolysates, molecular sieves have also been used as adsorbents. These have very small pores of uniform size. Inhibitory compounds from hydrolysates, which have larger size than these pores, will be retained. Tran and Chambers (1986) observed the removal of acetic acid (40 %) and furfural (82 %) along with xylose loss (10 %) from oak hydrolysates after passing through the hydrolysate with a molecular sieve.

3.3.2 Chemical Detoxification Methods

Using of various alkaline chemicals such as sodium hydroxide, calcium hydroxide, ammonium hydroxide, sodium sulfite, and sodium di-thionite have been used to eliminate the fermentation inhibitors from the lignocellulose hydrolysates (Mussatto and Roberto 2004; Palmqvist and Hahn-Hagerdal 2000a, b; Chandel et al. 2011a; Parawira and Tekere 2011). These alkaline chemicals have specific affinity towards the inhibitors present in lignocellulose hydrolysates. In addition to alkaline-based approaches, materials like activated charcoal and ion-exchange resins have been profoundly used for detoxification of lignocellulose hydrolysates (Mussatto and Roberto 2004; Palmqvist and Hahn-Hagerdal 2000a, b; Chandel et al. 2011a; Parawira and Tekere 2011).

3.3.2.1 Neutralization of Lignocellulose Hydrolysates

Neutralization of lignocellulose hydrolysates is an inevitable process for using them as fermentation substrates. The nature of lignocellulose acid hydrolysates is highly acidic. The most preferred chemicals used for neutralization are alkaline chemicals like sodium hydroxide and calcium hydroxide. These chemicals are added into the hydrolysates to attain the fermentable pH (5.5–6.0). During this process, many inhibitors precipitate and could be separated by filtration. In general, a neutralization step alone is not enough to get rid of the inhibitors

necessitating the requirement of other detoxification processes in order to obtain the desired yields of xylitol during fermentation. Table 3.2 shows the effect of neutralization on the removal of inhibitors from lignocellulose hydrolysates.

3.3.2.2 Calcium Hydroxide Overliming

Calcium hydroxide over liming is one of the most common methods employed for detoxification of lignocellulose hydrolysates for their subsequent conversion into xylitol by microbial fermentation. In this method, dried calcium oxide or calcium hydroxide is added to the hydrolysates under fast stirring at room temperature to attain the alkaline pH (10.5–11) followed by 30 min reaction. Thereafter, the slurry is filtered and adjusted to pH 6.0 by 6 N sulfuric acid and filtered again. During this process, substantial removal of volatile inhibitory compounds such as furfural, hydroxymethyl furfural (HMF), and phenolics has been observed from the hydrolysate additionally causing a sugar loss (almost 10 %) by adsorption (Chandel et al. 2007, 2011b; Martinez et al. 2000). After the filtration, gypsum is recovered which can be used as plaster of Paris which has several commercial applications. This method is promising in order to remove most of the inhibitors from the hydrolysate to an extent and the resultant sugar solution can directly be used in a fermentation reaction for xylitol production. Table 3.2 shows the potential of over liming for removal of inhibitors from lignocellulose hydrolysates.

3.3.2.3 Activated Charcoal

Activated charcoal treatment of lignocellulose hydrolysates is an effective method for detoxification. It is a cost-effective method with good capacity to absorb inhibitory compounds without sugars loss (Mussatto et al. 2004; Canilha et al. 2008; Sene et al. 2011). Factors such as activated charcoal concentration, temperature, contact time and pH are very important for the efficiency of activated charcoal treatment towards the extermination of inhibitory compounds from the lignocellulose hydrolysates (Mussatto et al. 2004). Activated charcoal specifically removes phenolics, furfurals, 5-HMF from the lignocellulose hemicellulosic hydrolysates and thus aids to improve the fermentability of those hydrolysates in order to achieve the desired xylitol production (Marton et al. 2006). Activated charcoal treatment is invariable to pH of hydrolysate and has minimum affinity towards sugars present in lignocellulose hydrolysate. However, charcoal after treatment with lignocellulose hydrolysate is difficult to regenerate (Parawira and Tekere 2011). Table 3.2 shows the effect of activated charcoal on the removal of inhibitors from lignocellulose hydrolysates for fermentative production of xylitol. Miyafuji et al. (2003) prepared wood derived charcoals at different temperature for their application in removal of inhibitors from the hydrolysates. They found the charcoal prepared at high temperature more effective for the removal of furans and phenolics without reducing sugars content and eventually showing high fermentability.

3.3.2.4 Ion-Exchange Resins

The ion-exchange resins based detoxification of lignocellulosic hydrolysates is the most effective method of inhibitor separation, and it is cost effective (Lee et al. 1999). Ion-exchange treatment of lignocellulose hydrolysate effectively removes lignin derived phenolics and acetic acid from lignocellulose hydrolysates by changing the ionic equilibrium, ultimately increasing the fermentability of hydrolysates for xylitol production (Wickramasinghe and Grzenia 2008; Chandel et al. 2011a). Ranjan et al. (2009) observed that anion exchange resins effectively act as inhibitor removal (i.e. levulinic, acetic, formic acids, and furfural and 5-HMF) from the hydrolysate at pH 10 (Ranjan et al. 2009). Ion-exchange resins were found more efficient than activated charcoal to remove all four major groups of inhibitory compounds without sugar loss. Treatment of lignocellulose hydrolysate with ion-exchange treatment revealed the increase in fermentability of the hydrolysate for xylitol production. A maximum of xylitol production (32.7 g/L, 0.57 g/g yield) was achieved after 48 h fermentation (Villarreal et al. 2006).

Nilvebrant et al. (2001) used three different resins: an anion exchanger, a cation exchanger, and a resin without charged groups (XAD-8) for detoxification of Spruce acid hydrolysate. An anion exchanger was found to be maximum effective followed by neutral charged resin and cation exchanger. Anionic and uncharged inhibitors were greatly removed at pH 10.0 by the anion exchanger by hydrophobic interactions. XAD-8 efficiently reduced the concentration of model compounds, such as guaiacol and furfural. Sugarcane bagasse hemicellulosic hydrolysate was treated with four different ion-exchange resins to overcome the inhibitors followed by xylitol production by *C. guilliermondii* FTI 20037. A removal of 82.1 % furfural, 66.5 % hydroxyl methyl furfural, 61.9 % phenolic compounds, 100 % chromium, 46.1 % zinc, 28.5 % iron, 14.7 % sodium and 3.5 % nickel was observed without any significant change in acetic acid concentration. Maximum xylitol yield (0.62 g/g) was obtained from detoxified hydrolysate (Carvalho et al. 2004). Table 3.2 summarizes the effect of ion-exchange treatment on the removal of inhibitors from lignocellulose hydrolysates for fermentative production of xylitol.

3.3.2.5 Other Specific Chemicals

Apart from the routinely applied chemicals, new chemicals have been tried recently for elimination of fermentation inhibitors from lignocellulosic hydrolysates. For example, sodium dithionite has been evaluated as in situ for the detoxification of Spruce acid hydrolysates in conjunction with the fermentation reaction. This approach showed improvement in fermentability of hydrolysates without affecting sugars level (Alriksson et al. 2011). Björklund et al. (2002) used lignin residue for the removal of inhibitors from Spruce dilute-acid hydrolysates prior to fermentation with *S. cerevisiae* for ethanol production. Removal of 53 % phenolics and 68 % furan aldehydes was observed after treating the hydrolysate

with lignin residue. Diatomaceous earth has also been used for the removal of inhibitors from Eucalyptus hemicellulosic hydrolysate (Carvalho et al. 2006). Alriksson et al. (2005) investigated the use of ammonium hydroxide for simultaneous detoxification and a nitrogen source to Spruce hydrolysates for ethanol production by *S. cerevisiae*. A substantial decrease in furfurals and hydroxymethyl furfural was observed after the treatment. Persson et al. (2002) used potassium hydroxide for elimination of the inhibitors from lignocellulosic hydrolysates. Telli-Okur and Eken-Saraçoğlu (2008) detoxified sunflower hemicellulosic hydrolysate by addition of sodium sulfite with $Ca(OH)_2$ for ethanol production by *P. stipitis* NRRL-Y-7124. More recently, Cavka et al. (2011) used sulfur oxyanions such as hydrogen sulfite for in situ detoxification of hydrolysates due to their capability to react with and sulfonate inhibitory aromatic compounds and furan aldehydes at low temperature and slightly acidic pH (such as 25 °C and pH 5.5).

3.3.3 Biological Methods

The more environmentally friendly alternative strategy involves the utilization of microorganisms to convert lignocellulosic hydrolysates to xylitol (Prakasham et al. 2009). In addition, this process reduces environmental pollution. The use of biotechnological applications as a means of detoxification requires the application of cellular enzymes or microorganisms to lignocellulose hydrolysates (Silva and Roberto 2001). The advantage of utilizing microbial processes instead of the chemical approach is the microbial process requires less energy (Sampaio et al. 2003). This process is performed under mild pressure and temperature conditions and the bioconversion process is highly substrate specific, resulting in higher xylitol yield thus lowering costs that go into xylitol separation and purification (Prakasham et al. 2009). When employing microbial species to the hydrolysate, the ideal xylitol-producing microbes should have a high tolerance to toxins and be able to withstand other potential inhibitors and extreme conditions to allow for efficient xylitol production (Mussatto et al. 2004). Very few microbial strains such as *Saccharomyces pombe*, *S. cerevisiae*, *amucae* and *Kluveromyces lactis* can break down xylitol but in relatively low yields (Prakasham et al. 2009). Other strategies include utilizing xylose-fermenting yeast to convert D-xylose to xylitol such as from the genus *Candida* (Guo et al. 2006). Other methods include the extraction of xylitol from D-xylose with the use of recombinant *Bacillus subtillis* and *Candida maltose* to detoxify the D-xylose. HMF and furfural inhibitors were removed by *C. maltose* (Cheng et al. 2011).

3.3.3.1 Enzyme Mediated Approaches

Enzymatic mediated detoxification of lignocellulose hydrolysates is considered a "green and sustainable" approach which has shown to be a promising alternative

to conventional chemical-based methods (Parawira and Tekere 2011; Chandel et al. 2011a). Laccases and peroxidases have shown properties to polymerise the lignin derived phenolics from lignocellulose hydrolysates and thus remove phenolics without affecting sugars level. White rot fungi such as *Trametes versicolor*, *Phenorochete chrysosporium*, *Cythus bulleri, Cythus stercoreus*, and *Pycnoporous cinnabarinus* are known laccase producers. Laccase was used to detoxify the steam-exploded wheat straw hydrolysate for ethanol production. Laccases efficiently removed phenolics from hydrolysate without affecting weak acids, and furan derivates eventually improved ethanol production (Moreno et al. 2012). Kolb et al. (2012) investigated the effect of laccasse from *T. versicolor* on the removal of phenolics from liquid hot water wheat straw hydrolysates. Laccase significantly reduced content of phenolic compounds. Sugarcane bagasse hydrolysate was detoxified by overliming and laccase treatment which showed 80 % removal of phenolics (Martin et al. 2002). Treatment of willow hydrolysate with laccases and peroxidases from *T. versicolor* improved ethanol production (Jonsson et al. 1998). Chandel et al. (2007) evaluated the potential of laccase from *C. stercoreus* for the phenolics removal (77 %) from sugarcane bagasse hemicellulosic hydrolysate. Cho et al. (2009) observed phenolics removal (100 %) after treatment with peroxidase enzyme from *Coprinus cinereus* IFO 8371. There are several reports on enzymatic clarification of lignocellulose hydrolysates, which have been used for ethanol production. Table 3.2 presents the effect of laccase treatment on the removal of inhibitors from lignocellulose hydrolysates for fermentative production of xylitol.

However, there is no report on xylitol production from laccase or peroxidase detoxified lignocellulose hydrolysates. In future, immobilization of laccases and peroxidases on robust supports like Sepa beads, acrylic based resins and Eupergit C may provide cost-effective technological breakthroughs for the detoxification of lignocellulose hydrolysates.

3.4 Systems Biology and Detoxification

Routinely applied physical and chemical detoxification strategies may have capabilities to remove fermentation inhibitors; however, sugar loss during detoxification, environmental and economic concerns have called for system biology-based approaches to develop inhibitor tolerant strains which can cope with inhibitors and xylitol production simultaneously. Development of microbial strains to withstand the inhibitor stress, maintain energy and redox balance, and complete active metabolism for microbial fermentation are important features for the sustainable production of xylitol in large-scale operations (Liu 2011). Functional genomics- and proteomics-based approaches for understanding the mechanism of microbial tolerance and in situ detoxification are in need of time for the commercial xylitol production via microbial fermentation. Following the leads from strain development approaches for ethanol production from lignocellulosic

hydrolysates, these strategies could be employed for xylitol production. Laccase from *T. versicolor* was expressed under control of the PGK1 promoter in *S. cerevisiae* to increase its resistance to phenolic inhibitors in lignocellulose hydrolysates and ethanol production simultaneously (Larsson et al. 2001). Almeida et al. (2008) developed *S. cerevisiae* TMB 3400 from *P. stipitis* for xylose bioconversion into ethanol alongside showing increased tolerance (three-fold higher tolerances to HMF and furfural reduction) than the control strain. Parawira and Tekere (2011) competently reviewed the various metabolic engineering approaches for strain development to tolerate the inhibitors. Bajwa et al. (2011) developed *Scheffersomyces* (*Pichia*) *stipitis* with improved tolerance to lignocellulosic hydrolysates after optimization of genome shuffling methodology. "Omics" based technologies and systems biology for engineering yeast strains for inhibitors tolerance and simultaneous production of xylitol and ethanol surely will pave the way for economic xylitol production. As yet, metabolic engineering approaches have been studied to develop strains for ethanol production; however, reports have to come for strain development for inhibitor tolerance and xylitol production.

3.5 Future Perspective and Conclusions

Detoxification of lignocellulose hydrolysates is an inevitable process for the fermentative production of xylitol. Looking on the research appraisals, conventional physical and chemical methods like neutralization, $Ca(OH)_2$ overliming, activated charcoal, and ion exchange, etc. have been employed more for detoxification of lignocellulose hydrolysates for their further application in fermentative production of xylitol. Biotechnologically-based methods such as microorganisms based detoxification, enzymes (laccase or peroxidase) mediated clarification, in situ microbial delignification (ISMD) or systems biology based approaches to develop inhibitor tolerant strains have not been employed for detoxification of sugar syrups for xylitol production. These methods are in vogue for detoxification of sugar syrups for bioethanol fermentation. On the contrary, fewer developments have been observed in regards to xylitol production. Chemical or physico-chemical methods are expensive and not eco-friendly in addition to causing significant amount of sugar loss eventually showing poor yield of xylitol. Biological mediated detoxification strategies should be in more practice for the economization of the xylitol production process. In the next 5 years, research should be focused to develop system biology based approaches for designing superior strains which can tolerant the inhibitors for their further implication in "in situ" detoxification and simultaneous xylitol production, eliminating the extra step as detoxification.

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Part II
Microorganisms, Strain Improvement, Fermentation and Biochemical Methods for Xylitol Production

Chapter 4
Molecular Mechanism of D-Xylitol Production in Yeasts: Focus on Molecular Transportation, Catabolic Sensing and Stress Response

Jyosthna Khanna Goli, Smita Hasini Panda and Venkateswar Rao Linga

Abstract Xylitol is a naturally occurring non fermentable sugar alcohol. It can be produced by the microbial fermentation of xylose extracted from hemicellulose of lignocellulosic substrates like corn fiber, corn husk, sugarcane bagasse and birch wood. In last few decades, xylitol gained significant importance due to its applications in food and pharmaceutical industries. Sustainable production of xylitol from renewable sources is possible by fermentation process using xylose assimilating microbes. As chemical production of xylitol involves high temperature, pressure and expensive purification steps, highly efficient biotechnological production of xylitol using microorganisms is gaining more interest over chemical processes. For the economic production of xylitol, microorganisms with high osmotolerance, inhibitor resistance, fast conversion rates, and stress tolerance are required in the fermentation process. As xylose uptake might be a limiting factor for xylose fermentation, the study of xylose uptake with respect to xylose transporting proteins and improvement of utilization of sugar mixtures is necessary. This review is to provide an overall view of xylitol production by yeast strains under sugar, saline and different nutritive stress conditions. In addition this review emphasizes the role of molecular changes (genes) and pathways involved in the utilization and transport of sugars for increased xylitol production.

Keywords Xylitol · Stress response · Yeasts · Catabolic sensing · Molecular transport

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4.1 Introduction

Xylitol is a naturally occurring sweetener with one-third fewer calories than sucrose (Granström et al. 2007a). It is a high value polyalcohol produced by the reduction of D-xylose derived from the hemicellulose fraction of lignocelluloses. Xylitol can serve as a building block for organic synthesis and was listed as one of the top 12 value added materials produced from biomass (Werpy et al. 2004; Prakash et al. 2011). Xylitol is naturally found in fruits and vegetables like berries, oats, lettuce and cauliflower. It can be produced fermentatively from xylose extracted from the hemicellulosic fraction of lignocellulosic substrates like corn fiber, corn husk, sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switch grass, straws, stems, stalks, leaves, husks, shells and peels from fruits and cereals like rice, wheat, corn, sorghum and barley (Prakasham et al. 2009; Weber et al. 2010; Xu et al. 2011). The hemicellulosic fraction of lignocellulosic substrates is hydrolyzed into simple monomeric sugars (hexoses and pentoses) by chemical or enzymatic methods. During the hemicellulose hydrolysis by chemical methods, a number of sugars, mainly xylose, is derived in addition to various inhibitors (Chandel et al. 2010, 2011) which need to be eliminated prior to the fermentation process of xylitol production.

Industrial production of most sugar alcohols is performed by chemical hydrogenation of xylose in the presence of a metal catalyst, Raney nickel, with hydrogen gas at high temperature and pressure (Granström et al. 2007a) for which highly pure sugar substrates and costly chromatographic purification steps are required. In contrast, fermentative production of xylitol by microorganisms is becoming a more attractive sustainable alternative (Saha 2003; Lin et al. 2010) as it does not require pure xylose syrup at the starting point of the fermentation process, as the chemical synthesis does. This could be of high economical interest since low-cost hemicellulosic hydrolysates may become potential substrates (Milessi et al. 2011). Biotechnological production of xylitol is potentially attractive as it occurs under much milder process conditions with low purification costs, and it is relatively easy and environmentally safe (Rodrigues et al. 2011).

In the last two decades, bacteria, fungi and yeasts capable of producing xylitol have been identified (Table 4.1) (Cheng et al. 2010). Among the microorganisms, yeasts are considered to be the better xylitol producers (Singh and Mishra 1995; Guo et al. 2006). Some well-known xylitol-producing yeasts are *Candida guilliermondii*, *Debaromyces hansenii*, *Candida tropicalis*, *Candida boidinii*, *Pachysolen tannophilus* and *Pichia stipitis* (Prakasham et al. 2009; Chandel et al. 2011). Recent studies have indicated that insect guts harbor a vast diversity of yeasts belonging to genera *Arxula, Candida, Pichia, Cryptococcus, Debaryomyces*, *Dipodascus,* and *Endomyces*. Some of these insect gut yeast strains, such as *Spathospora passalidarum, C. jeffrisii,* and *P. stipitis* are known for xylitol production. Yeasts such as *C. tropicalis, Candida mogii, P. stipitis, Candida guillermondii* and *D. hansenii* are known to be good xylitol producers under oxygen-limited conditions (Granström et al. 2007b; Ko et al. 2008). Xylose can be converted to xylitol by recombinant

Table 4.1 Microorganisms screened for xylitol production

Yeast	Fungi	Bacteria
Petromyces albertensis	*Aspergillus niger*	*Corynebacterium sp.*
Candida boidinii	*Penicillium brevicompactum*	*Enterobacter liquifaciens*
C. maltosa	*P. citrinum*	*Mycobacterium smegmatis*
C. mogii	*P. expansum*	
C. tropicalis HXP2	*P. griseoroseum*	
Debaromyces hansenii UFV-170	*P. roqueforti*	
Hansenula polymorpha	*P. purpurogenum*	
Pachysolen tannophilus	*P. janthinellum*	
Pichia caribica	*P. chrysogenum*	
C. guilliermondii FTI-20037118	*P. italicum*	
C. intermedia	*P. crustosum*	

Sources Chen et al. (2010), Sampio et al. (2003), Ghindea et al. (2010)

microbes like *Escherichia coli*, *Bacillus*, *Saccharomyces cerevisiae* and also *Candida* (Jeon et al. 2011).

Xylitol is known to be metabolized through insulin-independent pathways in the human body; hence, it can be used as a sugar substitute for diabetics (Arrizon et al. 2011). It is used in foods like chewing gum, bakery products and chocolate as a sweetener with anticariogenic properties. It is also used in medicines as a sugar substitute for people with erythrocytic glucose-6-phosphate dehydrogenase deficiency (Kinterinwa et al. 2008). In rats, xylitol has been found to increase the activity of neutrophils, the white blood cells involved in fighting many bacteria (Renko et al. 2008). Xylitol has also shown potential to be used for the treatment of osteoporosis. As xylitol is highly beneficial to health, it has gained significant market demands corresponding to the current annual global value of $340 million per year and priced at $4–5 kg^{-1} (Kadam et al. 2008; Prakasham et al. 2009) with an annual commercial production of 40,000 tons (Granström et al. 2007a).

4.2 Metabolism of D-Xylose in Yeast

In the majority of yeasts and fungi, the conversion of D-xylose to D-xylulose needs two steps, a reduction step followed by an oxidation step. In yeasts and fungi, D-xylose is first reduced to D-xylitol by either NADH or NADPH-dependent xylose reductase (aldose reductase EC 1.1.1.21) (XR); the resulting D-xylitol was either secreted or further oxidized to D-xylulose by NAD or NADP-dependent xylitol dehydrogenase (EC 1.1.1.9) (XDH) (Chen et al. 2010). These two reactions were considered to be the rate-limiting steps in D-xylose fermentation and D-xylitol production. Some strains of yeast could metabolize D-xylulose to xylulose-5-phosphate by xylulokinase (EC 2.7.1.17) (XK). Xylulose-5-phosphate can subsequently enter the pentose phosphate pathway (Lachke and Jeffries 1986; Chen et al. 2010). This pathway consists of an oxidative phase that converts hexose

phosphates to pentose phosphates providing NADPH needed in biosynthetic pathways and a non-oxidative phase in which the pentose phosphates are converted into hexose and triose phosphates (Jeffries 1983). The pentose phosphate pathway also yields ribose-5-phosphate used for the synthesis of nucleic acids and histidine and of erythrose-4-phosphate which are necessary for the synthesis of aromatic amino acids. The phosphoketolase pathway presents an alternative route for the utilization of xylulose-5-phosphate (Prior et al. 1989) by conversion of xylulose-5-phosphate into glyceraldehyde-3-phosphate and acetyl phosphate. The process of xylitol formation cannot be stopped after the first step, when D-xylose is converted to xylitol. Cell growth depends on some of the above metabolic products and it is also necessary that the cofactors have to be regenerated through different steps in the metabolic pathway. Therefore, to obtain good yields of xylitol, the amount of xylose being converted to xylitol and the amount of xylitol which is available for further metabolism have to be well balanced.

4.3 Co-Enzyme Specificity

The first two enzymes, D-xylose reductase and xylitol dehydrogenase are the key enzymes in xylitol production by yeasts. They both require pyridine nucleotide cofactors exhibiting different cofactor specificity in different yeasts. Under anaerobic or oxygen-limited conditions, the difference in the cofactor requirements of these enzymes causes a redox imbalance which influences xylitol accumulation in yeasts (Bruinenberg et al. 1984). Xylitol formation is favored under oxygen-limited conditions, because of the NADH accumulation and subsequent inhibition of NAD-linked xylitol dehydrogenase. This phenomenon, known as the Custer effect, results from the incapability of the yeasts to compensate for excess NADH as they have no transhydrogenase activity (Van dijken and Scheffers 1986).

The variation in the ratio of NADH- to NADPH-linked D-xylose reductase activity with aeration conditions was first found in *P. tannophilus*; this infers that there was more than one form of n-xylose reductase in the yeast, which was further confirmed by Verdiyn et al. (1985). The same variations were also seen in the yeasts *Candida parapsilosis* ATCC 28474 and *C. boidinii* NRRL Y-17213. Under oxygen limitation, in contrast to all other D-xylose-fermenting and xylitol-producing yeasts, *C. boidinii* exhibits a NADH/NADPH ratio higher than 1 (Table 4.2). Referring to this, Vongsuvanlert and Tani (1988) found that in *C. boidinii* xylitol could be formed by two metabolic pathways: (1) D-xylose is directly reduced to xylitol and (2) D-xylose is initially isomerized by D-xylose isomerase to D-xylulose that is subsequently reduced to xylitol. In both reductions, NADPH was also active as a reductant but with less efficiency.

In most yeast cell-free extracts, D-xylose reductase has a higher preference for NADPH (Table 4.2). Though xylose reductase of *C. parapsilosis* ATCC 28474, *Candida shehatae* ATCC 22984, C. *shehatae* CBS 5813 and *P. stipitis* CBS 5773 is capable of using both NADPH and NADH, its affinity towards NADPH is very

Table 4.2 Enzyme activity of D-xylose reductase and xylitol dehydrogenase in various yeast with D-xylose as a substrate

Specific activity (U/g protein)				
Microorganism	D-Xylose reductase		Xylitol dehydrogenase	
	NADPH	NADH	NAD	NADP
C. boidinii (*Kloeckera* sp.) no. 2201	0.055	0.288	0.272	0.096
C. boidinii NRRL Y-17213	0.019	0.112	0.060	0.003
C. guilliermondii NRC 5578	0.521	0.050	n.r	n.r
C. guilliermondii NRC 5578	1.191	n.d	n.r	n.r
C.mogii ATCC 18364	0.160	0.060	0.220	n.r
C. parapsilosis ATCC 28474	0.416	0.161	n.r	n.r
C.shehatae ATCC 22984	0.333	0.100	0.240	n.r
C. shehatae CBS 5813	0.480	0.210	n.r	n.r
C. tropicalis IFO 0618	10.640	1.720	20.160	0.120
C.utilis CBS 621	0.075	n.d	0.280	n.d
Debaryomyces hansenii DTIA-77	0.091	n.r	0.047	n.r
P. tannophilus CBS 4044	0.220	0.009	0.910	0.070
P. tannophilus NRRL Y-2460	0.033	0.008	0.049	n.d
P. tannophilus U-U-27 (mutant)	0.173	0.118	0.160	n.d
P. stipitis CBS 5773	0.600	0.310	0.720	0.075

Source Winkelhausen and Kuzmanova (1998)
n.d not detected, *n.r.* not relevant

high. The lower NADH/NADPH ratio in *C. guilliermondii* NRC 5578 (0.1) compared to that in *C. parapsilosis* ATCC 28474 (0.4) explains the higher D-xylose reductase activity in *C. guilliermondii.*

4.4 Osmotic Stress in Yeast

Osmo-regulation is a basic biological process which controls cellular water content and turgor pressure. Accumulation of compatible solutes is a well conserved strategy in osmoregulation, though the solute accumulated differs between organisms (Yancey et al. 1982). The yeast *S. cerevisiae* employs glycerol, whose production and transmembrane flux are tightly controlled by osmotic changes (Hohmann 2002). Although the molecular changes and pathways involved in the response to saline or sorbitol stress are widely understood, less is known about how cells respond to high sugar concentrations (Hohmann and Mager 2003). Glucose (or catabolite) repression has been identified in many microorganisms, including budding yeast (Gancedo 2008; Santangelo 2006). According to this phenomenon, the transcription of genes whose products are essential for the catabolism of slowly fermentable or completely non fermentable carbon sources is repressed in the presence of a rapidly fermentable carbon source, such as glucose. This occurs with the genes involved in respiration (Krebs cycle and electron

transport chain), gluconeogenesis, the glyoxylate cycle, and utilization of alternative carbon sources such as galactose. The main glucose repression pathway involves the Snf1p kinase complex (Carlson et al. 1981) which, under glucose limitation conditions, inactivates Mig1p, thus preventing its interaction with co-repressor Cyc8p-Tup1p. When an excess of glucose is present, the Snf1p kinase is inactive (McCartney and Schmidt 2001) thus non phosphorylated Mig1p located in the nucleus exerts its repressing effects (Kaniak et al. 2004; Papamichos-Chronakis et al. 2004). The MAPK HOG (high osmolarity glycerol) pathway is the most prominent signaling pathway that allows *S. cerevisiae* cells to quickly respond to altered osmolarity (Capaldi et al. 2008; Jiménez-Martí et al. 2011). The activity of the HOG pathway can be known by immunological analysis of the level of phosphorylated Hog1 mitogen- activated protein kinase or by the mRNA level of target genes such as GRE2. Using such markers of HOG pathway activity, it has been found that a hyperosmotic shock leads to transient activation of the pathway. The HOG pathway controls glycerol production at a minimum of two levels. First, Hog1 activates the enzyme phosphofructo-2-kinase, leading to stimulation of glycolytic flux and enhanced glycerol production (Dihazi et al. 2004). In addition, Hog1 mediates enhanced expression of the genes GPD1 and GPP2 (Albertyn et al. 1994; Norbeck et al. 1996; Hirayama et al. 1995) and hence increased capacity to produce glycerol.

Under hyperosmotic stress conditions, Pbs2p phosphorylates Hog1p in the cytosol promotes its rapid nuclear accumulation (Ferrigno et al. 1998; Reiser et al. 1999), which is followed by an increase in the mRNA levels of osmo-induced genes (Posas et al. 2000; Rep et al. 1999a, b, 2000).

When 2 % (w/v) glucose was added to cells growing in raffinose, Hog1 phosphorylation was induced to the same extent as when treated with 110 mM NaCl. Further increase in the concentration of glucose led to the higher levels of Hog1p phosphorylation (Tomás-Cobos et al. 2004). Even though there is enough glucose available for growth, the cell has to counteract with the osmotic stress when exposed to high glucose concentration. Several reports have focused on the sugar stress condition by conducting global or particular transcriptomic analyses. In a microarray analysis of cells from the PSY316 strain exposed to 20 % w/v glucose (Kaeberlein et al. 2002), an up-regulation of glycerol and trehalose biosynthetic genes was found. Erasmus et al. (2003) reported the response of wine yeast Vin13 to 40 % w/v sugars compared to 22 % w/v sugars in Riesling grape juice. According to their studies, high sugar stress up-regulated the genes of the glycolytic and the pentose phosphate pathway and those involved in the formation of acetic acid from acetaldehyde, while genes involved in de novo biosynthesis of purines, pyrimidines, histidine and lysine were down-regulated. Proteomic characterization of *S. cerevisiae* response to high glucose concentrations has revealed that most of the proteins involved in glycolysis and pentose phosphate pathways are up-regulated under these conditions (Pham and Wright 2008). In a recent study by Guidi et al. (2010) when *S. cerevisiae* was exposed to 20 % w/v glucose, peroxiredoxin, a protein involved in protection against oxidative stress was found to be down regulated. Proteomic analysis of the mutant deletion strain

(Δyhr087w), YHR087W (RTC3) gene and its encoded protein reveals lower levels of several yeast Hsp proteins, which establishes a link between this protein and the response to several forms of stress. The relevance of YHR087W for the response to high sugar and other stress conditions and the relationship of the encoded protein with several Hsp proteins suggest applications of this gene in biotechnological processes in which response to stress is important (Jiménez-Martí et al. 2011).

Osmotic stress triggers a set of cellular responses that enables the yeast to adapt to the changes in the environment. For example, when ascomycetous yeasts are exposed to osmotic stress, polyols such as glycerol accumulate in the cytoplasm to counteract cell dehydration (Van Eck et al. 1993; Albertyn et al. 1994). The compounds that yeast cells produce and accumulate are also known as compatible solutes or osmolytes and their function is to increase the internal osmolarity after hyperosmotic shock. The cell regulates the concentration of intracellular osmolytes by either metabolic processes or by activating membrane transporters by controlling the inflow or the efflux of solutes and ions together with changes in the cell volume (Kayingo et al. 2001; Tamas and Hohmann 2003).

There are a number of yeast genera that survive in high sugar environments (Tokuoka 1993). These yeasts are termed osmophilic or osmotolerant and almost all species studied belong to the ascomycetous group (Tokuoka 1993; Van Eck et al. 1993). Basidiomycetous yeasts are mainly salt tolerant. In contrast, ascomycetous yeasts were more glucose tolerant than salt tolerant (Van Eck et al. 1993; Tekolo et al. 2010). Basidiomycetous yeasts accumulated glycerol, mannitol and arabitol intracellularly when exposed to $0.96a_w$. These polyols are the main solutes accumulated in other yeasts when subjected to osmotic stress (Spencer and Spencer 1978). Hounsa et al. (1998) found that trehalose is important for the survival of *S. cerevisiae*. When genes involved in trehalose synthesis were deleted, it was found that the survival of this mutant strain was reduced when grown in the presence of 3M NaCl as compared with wild-type strain. However, hyper accumulation of trehalose did not improve the survival rates. Furthermore, the work of Hounsa et al. (1998) indicated that trehalose does not act at any stage as a reserve for glycerol synthesis in *S. cerevisiae* when exposed to osmotic stress. The increase in intracellular sodium concentrations and decrease in potassium concentrations when grown in the presence of NaCl suggest that sodium might replace potassium as an intracellular cation under these conditions and may even be accumulated as was reported previously for the salt-tolerant ascomycetes *D. hansenii* (Prista et al. 1997; Gonzalez-Hernandez et al. 2004).

Due to the versatility of *S. cerevisiae,* it is used to study different stress responses, such as heat shock, osmotic and oxidative stresses, nutrient starvation, etc. (Mager and Ferreira 1993; Serrano 1996). In *S. cerevisiae* the existence of at least four different DNA elements that respond to stress signals and participate in the transcriptional regulation of different genes has been found. Among the best characterized is the heat shock element, which interacts with heat shock transcription factor (Parsell and Lindquist 1994). Other elements participate in the response to multiple stress conditions, such as the post-diauxic shift (PDS) element

that mediates gene activation during diauxic growth and in the stationary phase, and under heat shock stress (Boorstein and Craig 1990a, b; Werner-Washburne et al. 1993). The stress response element (STRE) contains a sequence that is activated by various stress conditions, such as nitrogen starvation, osmotic and oxidative stress, low external pH, weak organic acids, etc. (Belazzi et al. 1991; Marchler et al. 1993; Ruis and Schuller 1995). Although the transcription factor binding to STRE has not yet been identified, recent findings show that the transcription activators Msn2p and Msn4p are not only required for a high level of expression of genes activated via STRE, but also bind specifically to these elements in vitro (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). Finally, the AP-1 responsive elements have also been identified in the promoter region of genes typically involved in the response to oxidative stress (Gounalaki and Thireos 1994; Ruis and Schuller 1995).

One of the first responses, in organisms living under hyperosmotic conditions, was the synthesis of osmolytes or compatible solutes (Yancey et al. 1982). In *S. cerevisiae,* glycerol is the major osmolyte synthesized under hyperosmotic stress (Blomberg and Adler 1989). The enzyme responsible for the production of this osmolyte is glycerol-3-phosphate dehydrogenase, NAD+ (GPD). The GPD1 gene is induced under osmotic stress and the lack of its product renders the yeast osmosensitive (Albertyn et al. 1994). A second gene encoding a GPD isozyme, GPD2, has been described as being important for glycerol production, although it is not induced by osmotic stress conditions (Ansell et al. 1997).

In *S. cerevisiae*, glycerol is produced by the signal transduction pathway (Maeda et al. 1994). This transduction pathway consists of a MAP kinase cascade (MAPKKK, Ssk2/22p; MAPKK, Pbs2p; MAPK, Hog1p) (Brewster et al. 1993; Maeda et al. 1994) that results in the induction of several genes, including GPD1 (Albertyn et al. 1994; Schuffler et al. 1994). The kinase cascade is activated by the product of two different genes that encode osmosensors on the membrane of yeast cells: SLN1, which forms the two-component system with YPD1–SSK1 and SHO1, which acts on PBS2 through STE11 (Maeda et al. 1994, 1995; Posas et al. 1996; Posas and Saito 1997). This regulatory system is known as the high osmolarity glycerol (HOG) pathway as it was initially characterized as the regulatory mechanism for glycerol production during yeast growth in a high osmolarity medium (Brewster et al. 1993).

As in other yeasts, active glycerol uptake from the environment is observed (Lages et al. 1999), but does not contribute to osmoadaptation in *S. cerevisiae* (Holst et al. 2000). Rather, intracellular glycerol levels are controlled by passive glycerol export, which is mediated by Fps1 (Hohmann 2002; Luyten et al. 1995; Oliveir et al. 2003). Upon a hyperosmotic shock the transport capacity of Fps1 is rapidly diminished to ensure that glycerol is maintained inside the cell (Hohmann 2002; Luyten et al. 1995). A specific domain within the N-terminal extension of Fps1 is needed to control glycerol transport and deletion of this domain which makes Fps1 hyperactive (Tamas et al. 1999, 2003). Yeast cells that express this hyperactive Fps1, fail to retain glycerol and hence are sensitive to high external osmolarity. On hypo-osmotic shock Fps1 rapidly releases glycerol to prevent

excessive cell swelling. Therefore, mutants lacking Fps1 are sensitive to hypo-osmotic shock (Tamas et al. 1999). Fps1 is a member of the aquaglyceroporin subgroup of MIP channel proteins (Hohmann et al. 2001) and hence can mediate passive glycerol flux in both directions.

4.5 Salt Stress

Micro-organisms, like many types of yeast (Tokuoka 1993) that can tolerate high saline environments, develop systems to counteract the deleterious effects of the two components of salt stress: (1) intracellular ion toxicity and (2) osmotic stress (turgor and water loss). In yeast, defense responses to salt stress are based on osmotic adjustment by osmolyte synthesis and cation transport systems for sodium exclusion. Polyols, and especially glycerol, are the major osmolytes produced by yeast (Blomberg and Adler 1992, 1993). Both a Na+-ATPase and an H+/Na+ anti-porter have been used for sodium extrusion.

To determine maximum tolerance to salt stress, a study was performed on 42 different yeast species and found that *C. halophila* CBS 4019 proved to be the only yeast able to grow on glucose in the presence of a maximum of 4.5 and 5 M NaCl, respectively, without and with inoculum pre-adaptation to salt. Further, in comparison to other very salt-tolerant yeasts, it has shown considerably less reduction in cell volume as a response to sudden salt shock (Lages et al. 1999). Like other halophilic micro-organisms, *C. halophila* and *D. hansenii* grow better in the presence of a certain amount of salt than in its absence (Prista et al. 1997). Unlike halophillic bacteria, neither *C. halophila* nor *D. hansenii* actually depend on the presence of salt to survive.

To exhibit salt stress response, *C. halophila* either (1) produces a higher amount of osmolyte than other less tolerant yeasts (Blomberg and Adler 1989), (2) possesses efficient ion extrusion transporters or pumps (Ramos 1999) and has active transport systems able to cope efficiently with osmolyte retention needs under stress, i.e., when cells should be demonstrating increasing difficulty in maintaining a membrane potential favorable for active nutrient transporters, (3) possesses channels able to regulate osmolyte retention in response to sudden changes in the environment (Tamas et al. 1999) or (4) has a membrane lipid composition that does not allow leakage of osmolytes or any other lipid-soluble compounds, thus maintaining integrity of the intracellular environment (Yoshikawa et al. 1995). Finally, the control of metabolism through an efficient redox regulation could also contribute to such a high tolerance.

When *C. tropicalis* and *S. cerevisiae* were grown in the absence and presence of 1 M NaCl, *C. tropicalis* was able to grow much faster than *S. cerevisiae* due to its respiratory metabolism (Gancedo and Serrano 1989). In the absence of NaCl the growth rate of *C. tropicalis* was 0.43 h^{-1}, whereas that of *S. cerevisiae* was 0–26 h^{-1}. In the presence of 1 M NaCl, *C. tropicalis* showed a 30 % reduction in growth rate while in the case of *S. cerevisiae* the reduction of growth rate was

much greater, i.e., a 70 % decrease and also the lag phase was increased (to 40 h) in the presence of 1 M NaCl. These results suggest that *C. tropicalis* grows better than *S. cerevisiae* at high NaCl concentrations, not only because of its more vigorous respiratory metabolism but also because of its higher salt tolerance.

When sensitivities of both yeast species to different chloride salts (NaCl, KCl, LiCl) and to sorbitol were studied, all these solutes produced an increase in both the generation time and the lag phase of yeast cultures. It was found that lithium is much more toxic than sodium while KCl is not toxic and only poses osmotic stress similar to sorbitol. KCl was the least inhibitory solute for both *S. cerevisiae* and *C. tropicalis.* Taking KCl as a reference, sorbitol was strongly inhibitory for *C. tropicalis* but much better tolerated by S. *cerevisiae*. NaCl and LiCl were much better tolerated by *C. tropicalis* than by *S. cerevisiae.* The above results indicate that these yeast species differ in their relative sensitivities to osmotic stress (sorbitol) and cation toxicity (sodium and lithium). The low relative toxicity of KCl indicates that chloride posses no toxicity problem for either yeast species. The fact that KCl is much less inhibitory for *C. tropicalis* than sorbitol could be explained if K+ uptake could be utilized for osmotic adjustment in this species. The difference between KCl and sorbitol toxicities is much less apparent in *S. cerevisiae*.

4.6 Dehydration Stress

The osmotolerant yeast *D. hansenii* is highly resistant to dehydration stress and this tolerance was higher for cells taken from the exponential growth phase than from the stationary phase. Growth of *D. hansenii* in medium containing 10 % (w/v) NaCl resulted in an additional increase in cellular resistance to dehydration, which was most prominent for stationary phase cells. Mechanisms of yeast cell responses to changes in external osmolarity were found to have similarities with exposure to dehydration–rehydration. Such similarities are particularly evident during the early stages of the response to these stress treatments. There are important species and even strain-dependent differences in stress response and stress tolerance. Since such differences might prove useful in revealing new mechanisms of tolerance, comparison was made between the sensitivity to dehydration–rehydration of cells of the extremely osmotolerant yeast *D. hansenii* with that of *S. cerevisiae*.

When the viability of dehydrated cells of *D. hansenii* were grown in basal medium and the same medium containing 10 % NaCl, it is found that growth at high salinity improves dehydration tolerance. This fact is applicable to both log phase cells and stationary phase cells, although the effect of NaCl addition was only marginal for cells taken from the exponential phase. The above results indicate that intracellular glycerol accumulation does not play any role in the tolerance to dehydration stress by *D. hansenii*. Exponential cells that accumulate intracellular glycerol show little improvement in stress tolerance to high levels of salinity (Adler and Gustafsson 1980; Larsson et al. 1990), whereas stationary

phase cells lack glycerol but instead accumulate high concentrations of arabinitol and showed improvement in dehydration resistance upon exposure to saline growth conditions. The arabinitol content of *D. hansenii* cultured in basal medium is higher in mid-exponential cells than in stationary phase cells (Adler and Gustafsson 1980) which seems to indicate that arabinitol, but not glycerol, might have a protective function under dehydration conditions. Further investigation of anhydrobiosis of *D. hansenii* might reveal new effective mechanisms by which eukaryotic cells can maintain viability under strongly unfavorable dehydration conditions, specifically mechanisms for dehydration resistance of exponentially growing cells as well as of their capability of tolerating extreme dehydration. It is well known that the characteristic features of yeasts growing in exponential growth phase have enhanced content of nucleic acids and proteins; this could be one of the main reasons which would determine the high sensitivity of the yeast cell to dehydration (Beker and Rapoport 1987). This hypothesis was substantiated by the idea that for the maintenance of the conformation and integrity of these molecules it would be necessary to retain high quantities of the bound water. This means that on dehydration down to relative humidity of about 8–10 %, cells loose much of their bound water, which finally results in irreversible changes in the structure and conformation of membranes and macromolecules.

In the experiments described by Khroustalyova et al. (2001), it was possible to reach not only the humidity level required for the anhydrobiotic state of yeast (that is, about 8–10 %) but to go significantly below it (down to about 5 %), without experiencing a decrease in the viability of the population of dehydrated cells. The above phenomenon can be explained in two ways: (1) the existence of protective molecules in the dehydrated cells which are able to substitute for the water released during drying (Beker and Rapoport 1987; Blomberg and Adler 1992) and (2) maintenance of the stability of the molecular organization of intracellular membranes which usually determines cell sensitivity to dehydration (Crowe et al. 1989; Hoekstra et al. 1992). The most well known protective substance is trehalose which could be synthesized in yeast cells in the stationary phase of growth (Panek 1995). It is known that trehalose could significantly increase the resistance of cells to dehydration–rehydration (Leslie et al. 1994) as well as to stabilize such biological molecules as proteins. Furthermore, investigations of cell resistance mechanisms to dehydration– rehydration stress should also include other protective compounds such as polyols (Krallish et al. 1997). Therefore, further investigations should be carried out by studying protective substances and high stability of the molecular organization of the yeast's intracellular membranes.

Results from the work of Khroustalyova et al. (2001) indicate that further significant progress in investigations of anhydrobiosis mechanisms have to be linked with a 'return' to a more ecological approach. It is now necessary to investigate in detail those yeasts which exist in nature in conditions like high salinity desert conditions leading at times to transfer to the state of anhydrobiosis. There are several alternative directions for this research; one of these involves construction of dehydration resistant strains by genetic approaches.

4.7 Phosphate Limitation Stress

Phosphate is known to play an important role in yeast metabolism, especially for enzyme activation or repression and for biosynthesis of membrane phospholipids (Teixeira de Mattos and Neijssel 1997; Jones and Gadd 1990) which could lead to alterations in membrane permeability thus allowing xylitol freely to cross the membrane. On the other hand, kinase activation/repression, such as the xylulokinase which catalyses the step of xylulose phosphorylation might play a role in xylitol accumulation under phosphate-limited growth. It was found that for xylose and potassium limitation, fully oxidative metabolism occurred leading to the production of biomass and CO_2 as the only metabolic products. However, potassium-limiting cultivation was the most severe nutritional stress of all tested, showing highest xylose and O_2 specific consumption rates along with the lowest biomass yield of 0.22 g g^{-1} xylose. Carbon was mainly assumed to meet the cellular energy requirements for potassium uptake. For the other limiting nutritional conditions, increasing amounts of extracellular xylitol were found for ammonium, phosphate and oxygen limitation. Although xylitol excretion is not significant for ammonium limitation, but where as for phosphate limitation the xylitol productivity reached 0.10 gl^{-1} h^{-1} about half of that found under oxygen-limiting conditions, 0.21 g gl^{-1} h^{-1}. It is the first evidence that xylitol production by *D. hansenii* might not only be a consequence of a redox imbalance attained under semi-aerobic conditions, but additional physiological mechanisms especially phosphate limitation also must have been involved. Cell yields changed drastically as a function of the limiting nutrient, being 0.22, 0.29, and 0.39 g $g^3$1 xylose for potassium, oxygen and phosphate limitation, respectively, and are a good indicator of the severity of nutritive stress (Tavares et al. 1999).

When effect of different nutritional stress on the growth and metabolic rates of *D. hansenii* grown at constant dilution rate (D) = 0.055 h^{-1}, for different growth-limiting essential nutrients were studied, Surprisingly it was found that carbon-, ammonia- and phosphate-limited cultures led to similar oxygen consumption, xylose consumption and carbon dioxide production rates, indicating that the dilution rate of 0.055 h^{-1}, did not provoke a severe growth stress. The cell yields of carbon- and ammonium-limited continuous cultivation was of the same magnitude, while the phosphate-limited culture was slightly lower. Whereas, oxygen- and potassium-limited chemostat cultivation was found to provoke drastic changes in metabolic rates leading to severe growth stress, with cell yields of 0.29 and 0.22 g g^{-1}, respectively. Moreover, potassium limited chemostat cultivation showed the highest oxygen consumption and CO_2 production rates, being more than three times higher than for carbon-limited cultivation (Tavares et al. 1999).

When *D. hansenii* was grown under both oxygen and phosphate limitation, xylitol was excreted into the culture medium. However, the xylitol yield was two times higher for oxygen-limited growth than for phosphate-limited growth, 0.43 and 0.18 g g^{-1}, respectively. Under oxygen-limited conditions, xylitol accumulation is assumed to occur due to the NADH surplus generated in the second

enzymatic step of xylose metabolism that cannot be reoxidised by the first enzymatic reaction.

The xylitol overproduction by *D. hansenii* under oxygen-sufficient cultivation (phosphate-limited chemostat) is the first evidence that xylitol production by yeasts might not be only a consequence of the intracellular redox unbalance due to NADH surplus under low respiratory capacity because this does not occur for the other oxygen-sufficient *D. hansenii* chemostats. This indicates that other regulatory mechanisms associated with xylitol overproduction are related to low-phosphate growth conditions.

4.8 Sugar Transporters in Efficient Utilization of Mixed Sugar Substrates

Among a variety of issues on utilization of sugar mixtures by the microorganisms, recent studies have started to reveal the importance of sugar transporters in microbial fermentation performance. Since most microorganisms cannot effectively utilize raw cellulose or hemicellulose because of their inability to biodegradation, biological production of fuels and chemicals from lignocellulosic biomass need depolymerization of lignocelluloses by enzymatic or chemical hydrolysis in a saccharification process. The resultant monomeric sugars can be converted to desired product by microbial action. The ability of a host organism to efficiently uptake the saccharified sugars greatly impacts microbial efficiency, whereas transporters for glucose derived from the cellulosic fraction are common among most microbial species. Hexose (e.g. mannose and galactose) and pentose (e.g. xylose and arabinose) that are derived mainly from the hemicellulosic fraction are much less common. Hexose and pentose transport systems in yeasts work by an energy-independent and facilitated diffusion mechanism. Hexose (Hxt) and galactose (Gal2) transporters naturally exhibit different affinities and specificities for their substrates. They are abundant in *S. cerevisiae* but less in other industrial important yeast strains including *Kluveromyces lactis* and *P. stipitis* (Boles and Hollenberg 1997). Besides D-glucose, many Hxt proteins transport D-fructose as well as D-mannose (Leandro et al. 2009), indicating that substrate specificities of Hxt proteins in yeast are generally broad. In *S. cerevisiae* Gal2, Hxt1, Hxt2, Hxt4, Hxt5, and Hxt7 catalyze the uptake of xylose (Hamacher et al. 2002; Saloheimo et al. 2007). However, their affinity for xylose is much lower than that for glucose and the xylose uptake by the transporters is strongly inhibited by glucose (Saloheimo et al. 2007). *P. stipitis* is the best studied xylose-fermenting yeast (Jeffries 2006). Three glucose transporters, encoded by the genes SUT1, SUT2, and SUT3, have been experimentally characterized in *P. stipitis* (Weierstall et al. 1999). Wild-type *P. stipitis* shows biphasic kinetics in the uptake of xylose whereas a SUT1 mutant loses the low-affinity component, suggesting that Sut1 protein achieves xylose uptake using the low affinity component. Few high-affinity

xylose transporters have been identified in yeast despite early evidence of the existence of proton-linked symporters in some strains (Hofer and Misra 1978; Lee et al. 2002). The rapid growth of *Candida intermedia* PYCC4715 on xylose was attributed to glucose/xylose proton-linked symporter (Gardonyi et al. 2003; Leandro et al. 2006). The symporter protein produced in *S. cerevisiae* (Leandro et al. 2008) showed much higher affinity to xylose than xylose transporting uniporters from *S. cerevisiae* and *P. stipitis*. However, affinity of the protein for glucose is still higher than that for xylose.

4.9 Xylose Transport Within the Yeast Cell

The first step in the metabolism of D-xylose is the transport of the sugar across the cell membrane. Under aerobic and oxygen limited conditions the rate of transport can limit the utilization of D-xylose in *P. stipitis* CBS 7126 and C. *shehatae* ATCC 22984 (Kilian and van Uden 1988). Under anaerobic conditions, D-xylose metabolism has not appeared to be transport-limited either in C. *shehatae* or in *P. stipitis.* Instead, the limitation was in the two initial steps of D-xylose metabolism, reduction of D-xylose and subsequent oxidation of xylitol. Studying the oxygen requirement for D-xylose uptake, Skoog and Hahn-Hagerdal (1990) found that oxygen induces or activates a transport system in *P. stipitis* CBS 6054. Starvation induced both proton symport and a facilitated xylose uptake diffusion system in *C. shehatae* CBS 2779. In non-starved cells, D-xylose was transported by a facilitated diffusion system. Kilian and van Uden (1988) reported on a low-affinity and a high-affinity xylose proton symport operating simultaneously in both starved and non-starved cells of *P. stipitis* IGC 4374. From the differences between the kinetic parameters of *C. shehatae* CBS 2779 (*K*m varies from 1 to 125 mM) and *P. stipitis IGC 4374 (K*m varies from 0.06 to 2.26 mM) it is found that transport systems in *P. stipitis* are more efficient. A transport system study in *C. mogii* ATCC 18364 (Sirisansaneeyakul et al. 1995) infers that D-xylose uptake rate followed Michaelis–Menten kinetics which suggested a carrier mediated facilitated diffusion transport system.

It was recently proved that *S. cerevisiae* can grow slowly in the presence of xylose as sole carbon source, in aerobic conditions. Until recently, it was considered that S. *cerevisiae* does not present specific transporters for xylose and that it grows poorly in its presence (Kinterinwa et al. 2008). The initial studies regarding the xylose transport alternatives within the yeast cell involved strains of *P. stipitis, P. heedii, C. shehatae* and *C. intermedia* and proved the existence of two transport systems:

1. A facilitated diffusion system, with low affinity—the genes involved here are, for example, SUT1 (sugar transporter 1) for *P. stipitis* or GXF1 (glucose/xylose facilitator 1) for *C. intermedia*, which are constitutively expressed genes that code the glucose/xylose transporter proteins (Jeffries and Jin 2004).

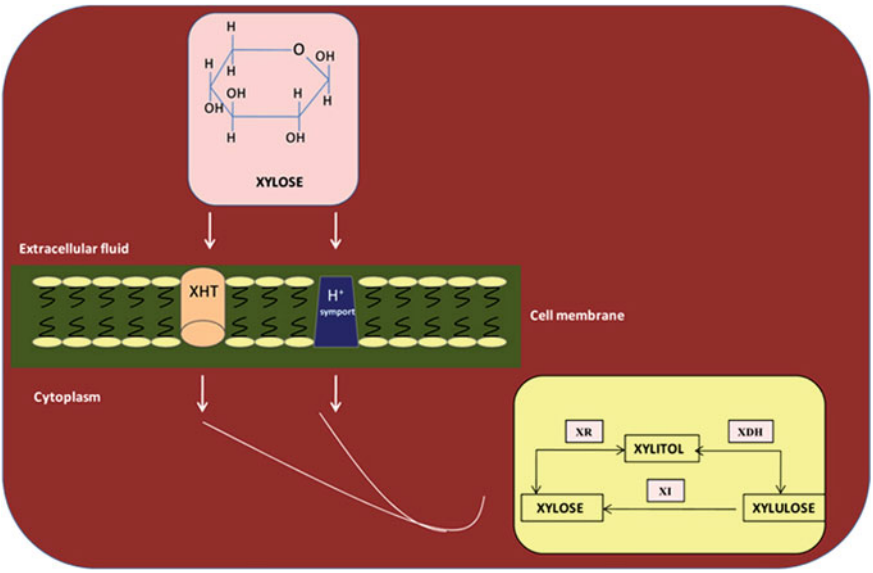

Fig. 4.1 Xylose transport within the yeast cell (*XHT* hexose transpoters, *XR* xylose reductase, *XDH* xylitol-dehydrogenase, *XL* xylose–isomerase (*Source* Ghindea et al. 2010)

2. A symport xylose-proton system, with high affinity—GXS1 (glucose/xylose symporter) that codes proteins involved in the symport monosaccharides-protons transport in various species of yeasts and fungi. In the case of *S. cerevisiae* species, the facilitated diffusion of xylose takes place with the aid of transporter proteins coded by HXT (hexose transporter) genes (Fig. 4.1). The xylose transport in the *S. cerevisiae* cell is, however, less efficient than glucose transport, the transport proteins (code by the genes XHT2, XHT6, XHT7) showing higher affinity for glucose.

4.10 Transport and Pentose Utilization in Yeast

Transport of sugar across the membrane does not limit the endogenous metabolism of sugars, but it may limit exogenous pentose metabolism. Without any genetic modifications, *S. cerevisiae* will transport pentoses across the cell membrane through native hexose transport proteins: Hxt7p, Hxt5p, Hxt4p, Hxt2p, Hxt1p and Gal2p (Becker and Boles 2003; Hamacher et al. 2002; Saloheimo et al. 2007). However, these proteins have a much higher affinity for their native hexose substrates, which may create unfavourable competitive inhibition and lead to diauxic growth in a hexose-pentose co-fermentation. Hence, dedicated pentose

transport may help to improve the simultaneous use of hexoses and pentoses. Gardonyi et al. (2003) reported a flux control coefficient of 0.2 for xylose transport in *S. cerevisiae* TMB 3001 (CEN.PK-2 XR/XDH/XKS) irrespective of xylose concentration, and rising to 0.5 in *S. cerevisiae* TMB 3206 (TMB 3001 with over expressed XR) at xylose concentrations below 0.6 g/l (Gardonyi et al. 2003). An evolutionary engineering experiment performed recently (aided by continuous culturing in xylose), using an optimized *S. cerevisiae* strain over-expressing six key xylose metabolic enzymes (including XI, XKS, TAL and TKL) (Kuyper et al. 2005), accumulated mutations over time resulted in greatly altered xylose transport kinetics, doubling V_{max} (15.8–32 mmol per dry weight per h) and reducing *K*m by 25 % (132–99 mmol/lM). This result infers that xylose transport is a limiting step, especially in metabolically optimized strains with higher downstream flux capacity. Experiment with recombinant *S. cerevisiae* found an increase in expression of the hexose transporter gene HXT5 (Wahlbom et al. 2003) showing that cells choose to increase transport activity when selected on xylose.

Heterologous transport proteins from plant, bacteria and other yeasts have been cloned and expressed in recombinant *S. cerevisiae*. However, only the class of transporters native to yeast, the major facilitator super family (MFS) (Pao et al. 1998) has been investigated. Other classes, such as the ATP binding cassette (ABC) transporters and the bacterial phosphoenolpyruvate (PEP) dependent transporters, were ineffective, because of expression difficulty and high relative energy requirements (Leandro et al. 2009). Xylose uptake in *S. cerevisiae* cells have shown two kinetically distinct uptake components with *K*m values of about 0.19 and 1.5 M, demonstrating that the monosaccharide transport in *S. cerevisiae* has nearly a 200-fold lower affinity for xylose than for glucose (Kotter and Ciriacy 1993). Isolation of transport protein with xylose as its highest affinity substrate would greatly enhance the prospects of a glucose-xylose co-fermentation. The most successful improvement of xylose transport to date is the expression of *C. intermedia* PYCC 4715 transport proteins (Leandro et al. 2006). Both *C. intermedia* GXF1 and GXS1 conferred significantly improved growth phenotypes in recombinant *S. cerevisiae* when glucose and xylose were used as sole carbon sources. Subsequent work has evaluated co-expression of the two proteins (Leandro et al. 2008) as well as more in-depth fermentation analysis (Runquist et al. 2009). Both of these transporters are efficient xylose transporters, yet also have a high affinity for glucose. Therefore, they remain primarily hexose transporters, despite the excellent xylose transport characteristics.

Heterologous xylose transport phenotypes were observed in yeast expressing *Arabidopsis thaliana* At5g59250 and At5g17010 (Hector et al. 2008). Where this was not supported was in a second study in which At5g59250 expression did not confer growth on xylose (Hamacher et al. 2002). In the first study with At5g59250, a standard strain of yeast was used whereas in the second, a strain lacking the HXT family of proteins was used. In this regard, the *A. thaliana* proteins may act as sensors or activators of HXT family transporters. These results show the importance of genotype on transporter characterization studies. Moreover, they emphasize the need for simultaneous optimization of both transport processes and

metabolic pathways. Further investigation is needed, as *P. stipitis* is known to have an excellent xylose transport system, yet to date no high-affinity xylose transporters have been isolated.

4.11 Conclusion and Future Recommendations

Xylitol producing yeast should have inherent characteristics of high osmotolerance, salt tolerance and inhibitor tolerance for the economization of xylitol production. All the above studies make up work concerning heterologous transport protein expression in *S. cerevisiae*, and motivate for further dedicated xylose transporters. However, future studies in this area must proceed with caution. Owing to the native transport characteristics of yeast, efficient transporters may need to be discovered through the use of HXT family knockout strains. It is also unknown how transporter proteins interact with each other to produce certain phenotypes. Naturally, as the internal metabolic pathway is improved, pentose transport will become a greater limitation. Therefore, novel tools and approaches must accomplish simultaneous optimization of transport and metabolism. Metabolic engineering approaches must be adapted to address the challenges for pathway and global cellular optimization that currently limit the construction of an integrated, efficient pentose pathway. Although much progress has been made towards lignocellulosic biomass conversion by yeasts, the approach of identifying limiting steps, and optimizing, must accelerate. More emphasis on host genome and regulatory structure must occur in future projects to understand the full effect of biological complexity on a pathway. In this regard, classic approaches combined with next-generation technologies may allow simultaneous optimization of all steps in a pathway. Although steady progress has been made in improvement of mixed-sugar utilization by microorganisms, more work is required for utilization of lignocellulosic biomass resources by microorganisms to make the process economically viable. Another hurdle to be cleared for efficient microbial utilization of mixed sugar substrates is the absence of a versatile method for over production of membrane proteins. In this respect, more efforts to explore and characterize sugar transporters should be expended. Hopefully, attempts to alter substrate specificities of sugar transporters by protein engineering will become more successful in order to improve utilization of pentoses. Finally, new methods that enable fast and simple assay of sugar uptake will help to facilitate finding and engineering of sugar transporters.

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Chapter 5
Microorganisms for Xylitol Production: Focus on Strain Improvement

Miho Sasaki, Masayuki Inui and Hideaki Yukawa

Abstract Xylitol, a five-carbon sugar alcohol, is widely used as a functional sweetener in the food and confectionary industry because of a number of advantageous properties. Although xylitol is industrially produced by chemical reduction of D-xylose derived from hemicellulose hydrolysates, this production method is uneconomical because of the requirement for pure D-xylose, high temperature, and pressure. Therefore, xylitol production by microorganisms has attracted focus as an economical and environment-friendly method. A variety of compounds have been used as substrates (D-xylose, D-glucose, D-arabitol, and L-arabinose) or co-substrates (D-glucose, ethanol, and glycerol) during microbial production of xylitol. In order to improve the biological production of xylitol, both natural xylitol-producing and nonproducing strains of microorganisms have been subjected to genetic modification strategies. This chapter describes recent advances made in metabolic engineering efforts aimed at improving production of xylitol by fungi, yeasts, and bacteria.

Keywords Xylitol · Recombinant · Microorganisms · Cofactor · Regeneration · Co-substrate

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5.1 Introduction

Xylitol is a five-carbon sugar alcohol as sweet as sucrose but with 1/3 fewer calories. It consequently finds uses as a dietetic sucrose replacement because when ingested it does not cause acid formation and prevents dental caries (Akinterinwa et al. 2008). Such beneficial health properties have resulted in a rapid expansion in xylitol consumption to the current annual global value of $340 million (Kadam et al. 2008). Moreover, being a valuable synthetic building block (Granström et al. 2007b), it recently joined the top 12 value-added materials produced from biomass, thereby serving as a key economic driver for the biorefinery concept (Werpy and Petersen 2004). Industrial xylitol is produced by reducing pure D-xylose derived from hardwood or corncob hydrolysates in the presence of a Raney nickel catalyst (Nigam and Singh 1995). This high temperature, high pressure system has the additional shortcoming of requiring pure D-xylose as substrate (Granström et al. 2007a). Biotechnological xylitol production is a potentially attractive replacement insofar as it occurs under much milder process conditions and can be based on sugar mixtures such as lignocellulose hydrolysates to save on energy and substrate purification costs. Microorganisms employed for biotechnological production of xylitol include fungi, yeast, and bacteria (Winkelhausen and Kuzmanova 1998; Chen et al. 2010). Some of the bioprocesses have enabled high product yields with high volumetric productivities. After xylitol production by recombinant *Saccharomyces cerevisiae* was reported for the first time (Hallborn et al. 1991), various microorganisms have been developed to produce xylitol.

5.2 Xylitol Production by Recombinant Fungi

In most cases, fungi metabolize D-xylose into D-xylulose in two steps (Fig. 5.1) (Chen et al. 2010). D-Xylose is first reduced to xylitol by either NADH- or NADPH-dependent D-xylose reductase (XR). The resulting xylitol is either secreted or further oxidized to D-xylulose by NAD^+-dependent D-xylitol dehydrogenase (XDH). Improvement of xylitol production by recombinant fungi has been reported so far only in *Trichoderma reesei* (Wang et al. 2005). The partial inhibition of XDH (*xdh1* gene) activity in *T. reesei* by antisense RNA enhanced xylitol productivity from 0.008 to 0.020 g l^{-1} h^{-1} in 2 % D-xylose medium (Wang et al. 2005).

5.3 Xylitol Production by Recombinant Yeast

Yeasts are generally considered to be more efficient producers of xylitol than bacteria or fungi. Yeasts naturally produce xylitol as an intermediate during D-xylose metabolism. XR is typically an NADPH-dependent enzyme, while XDH

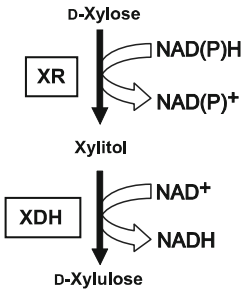

Fig. 5.1 Schematic representation of the pathway from D-xylose to D-xylulose in fungi. *XR* NAD(P)H-dependent D-xylose reductase, *XDH* NAD(P)-dependent D-xylitol dehydrogenase

requires NAD^+ (Granström et al. 2007a). Cofactor imbalance results in the secretion of xylitol as a D-xylose fermentation by-product.

5.3.1 *Candida Tropicalis*

Among the yeast strains that naturally produce xylitol, *Candida* sp. have been extensively studied with regards to their biotechnological application in the production xylitol (Granström et al. 2007b). Although its application is limited in the food industry because of the opportunistic pathogenic nature of some *Candida* spp., *Candida* yeasts are considered better potential candidates than metabolically engineered *S. cerevisiae* due to their being natural D-xylose consumers and their ability to maintain reduction-oxidation balance during D-xylitol accumulation. High xylitol productivities and yields from D-xylose have been frequently reported in these yeasts and *C. tropicalis* produced 12 g l^{-1} h^{-1} xylitol from D-xylose with D-glucose as co-substrate using a fed-batch submerged membrane bioreactor with cell-recycle system (Kwon et al. 2006b). This is among the highest productivities reported. Because this system cannot increase the yield of xylitol and requires additional equipment such as membrane filtration, metabolic engineering of *C. tropicalis* has received recent attention for system simplicity and high yields of xylitol possible.

C. tropicalis was treated with ultraviolet rays and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine to improve xylitol production resulting in 1.2-fold increased XR activity and increased xylitol yield (from 0.77 to 0.85 g g of D-$xylose^{-1}$) (Rao et al. 2006). A maximum improvement in xylitol yield (0.87 g g of D-$xylose^{-1}$) by the mutant strain was observed when medium was supplemented with urea as nitrogen source, but no improvement in biomass was observed. Jeon et al. (2011) carried out chemical mutagenesis treatment using nitrosoguanidine to isolate xylitol assimilation-deficient mutants of *C. tropicalis.* Resultant mutants produced 220 g l^{-1} xylitol at 3.3 g l^{-1} h^{-1} and overall yield of 0.93 g g of D-$xylose^{-1}$ under fully aerobic conditions, with fed-batch fermentation and repeated addition of glycerol and D-xylose.

Ko et al. (2006a, b) produced xylitol aerobically from D-xylose with glycerol as a co-substrate using a batch culture, achieving a volumetric productivity of 3.2 g l^{-1} h^{-1} and a yield of 0.96 mol xylitol·(mol D-xylose)$^{-1}$ in a XDH gene (*XYL2*)-disrupted mutant of *C. tropicalis* (strain BSXDH-3). This mutant required a large amount of glycerol as a co-substrate. Therefore, D-glucose might seem more desirable than glycerol as a co-substrate for xylitol production, because D-glucose and D-xylose are the primary products of industrial biomass hydrolysis. However, formation of XR in *C. tropicalis* is significantly repressed in cells grown on medium containing D-glucose (Ikeuchi et al. 2000). In order to overcome repression of XR formation by D-glucose, the codon-optimized *NcXR* gene (XR from the ascomycete *Neurospora crassa*) was expressed in the strain BSXDH-3 using the glyceraldehyde-3-phosphate (GAPDH) promoter (Jeon et al. 2012). In xylitol fermentation using D-glucose as a co-substrate with D-xylose, this recombinant showed xylitol productivity of 1.44 g l^{-1} h^{-1} and xylitol yield of 96 %, which were 73 and 62 %, respectively, higher than corresponding values for strain BSXDH-3. To construct recombinant *C. tropicalis* strains that produce a high yield of xylitol without adding a co-substrate, XDH activity was attenuated with only one copy of the XDH gene (strain XDH-5) or with site-directed mutations of the XDH gene (strain ARSdR-16), respectively (Ko et al. 2011). The wild-type, XDH-5, and ARSdR-16 strains each produced xylitol using D-xylose as a substrate, with conversion yields of 62, 64, and 75 %, respectively, and volumetric productivities of 0.52, 0.54, and 0.62 g l^{-1} h^{-1}, respectively. To improve *C. tropicalis* xylitol productivity, an NADH-preferring XR gene (*XYL1*) from *Candida parapsilosis* was expressed in *C. tropicalis* (Lee et al. 2003). The resulting recombinant showed high concentration (275 g l^{-1}), production yield (91.6 %, wt wt^{-1}) and productivity (5.09 g l^{-1} h^{-1}) without co-substrate.

Hemicellulose also contains a significant amount of L-arabinose, which is reduced to arabitol by non-specific XRs (Fernandes et al. 1999). Arabitol is difficult to remove during xylitol production and interferes with xylitol crystallization. To minimize the flux from L-arabinose to arabitol, the *XYL1* genes encoding L-arabinose-preferring endogenous XR were disrupted completely in strain BSXDH-3, and a functional D-xylose-preferring heterologous XR originating from *N. crassa* was expressed, yielding strain KNV (Yoon et al. 2011). Then, L-arabinose isomerase (*araA*) of *Bacillus licheniformis* and L-ribulokinase (*araB*) and L-ribulose-5-phosphate 4-epimerase (*araD*) of *Escherichia coli* were codon-optimized and expressed for the efficient assimilation of L-arabinose, yielding strain JY (Fig. 5.2). During xylitol fermentation, strains BSXDH-3 and KNV converted 9.9 g L-arabinose l^{-1} into 9.5 and 8.3 g arabitol l^{-1}, respectively, whereas the recombinant strain JY consumed 10.5 g L-arabinose l^{-1} for cell growth without forming arabitol. Moreover, strain JY produced xylitol (0.64 g l^{-1} h^{-1}) with 42 and 16 % higher productivity than BSXDH-3 and KNV, respectively.

The oxidative pentose phosphate pathway (PPP) is thought to be a major source of NADPH biosynthesis in yeast (Bruinenberg et al. 1983). NADPH is produced in two steps in PPP including the conversion of D-glucose 6-phosphate to D-glucono-δ-lactone-6-phosphate, catalyzed by D-glucose 6-phosphate dehydrogenase

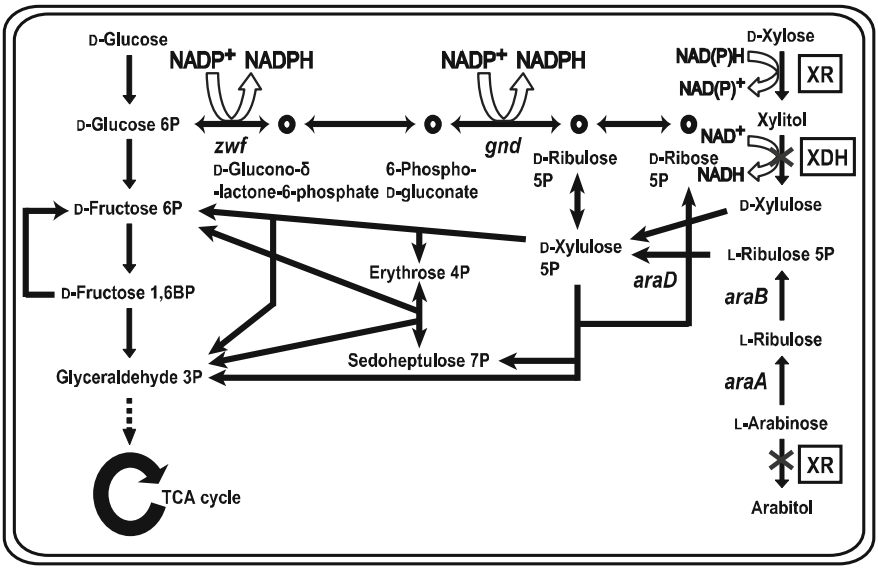

Fig. 5.2 Schematic representation of xylitol production from D-xylose by recombinant *C. tropicalis* strains. *XR* D-xylose reductase, *XDH* D-xylitol dehydrogenase, *araA* L-arabinose isomerase, *araB* L-ribulokinase, *araD* L-ribulose-5-phosphate 4-epimerase, *zwf* D-glucose 6-phosphate dehydrogenase, *gnd* 6-phospho-D-gluconate dehydrogenase

(G6PDH) and the conversion of 6-phospho-D-gluconate to D-ribulose 5-phosphate catalyzed by 6-phospho-D-gluconate dehydrogenase (6-PGDH) (Fig. 5.2). Ahmad et al. (2012) reported that strain BSXDH-3 was engineered to co-express *zwf* and *gnd* genes which, respectively, encode endogenous G6PDH and 6-PGDH, under the control of GAPDH promoter. In xylitol fermentation using glycerol as a co-substrate with D-xylose, NADPH-dependent xylitol production was higher in the engineered strain and the volumetric xylitol productivity of 1.25 g l^{-1} h^{-1} at 48 h; this value is 21 % higher than the corresponding rate for strain BSXDH-3.

5.3.2 Pichia Pastoris

Handumrongkul et al. (1998) cloned a D-xylose reductase gene (*xyl1*) from *Candida guilliermondii* and expressed the gene under the control of an alcohol oxidase promoter (*AOX1*) in methylotrophic yeast *P. pastoris*. The resulting strains were able to utilize D-xylose and accumulate xylitol. In particular, when grown in medium containing methanol as an inducer and D-xylose as a carbon source under aerobic conditions, it could produce a maximum amount of xylitol (7.8 g l^{-1}) at 72 h (Handumrongkul et al. 1998).

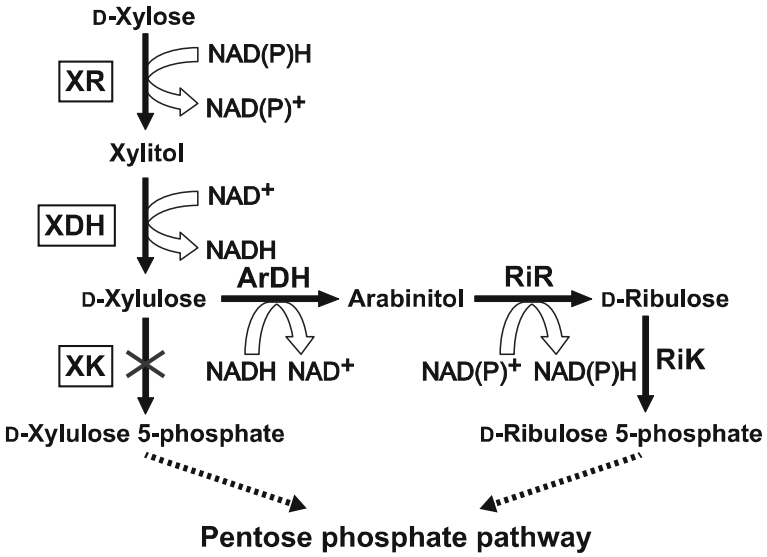

Fig. 5.3 Alternative route for D-xylose assimilation in *P. stipitis* XK gene mutant. *XR* NAD(P)H-dependent D-xylose reductase, *XDH* NAD^+-dependent D-xylitol dehydrogenase, *XK* D-xylulokinase, *ArDH* D-arabinitol dehydrogenase, *RiR* D-ribulose reductase, *RiK* D-ribulokinase

5.3.3 Pichia Stipitis

The D-xylose-fermenting yeast *P. stipitis* is among the few organisms that use both D-xylose and D-glucose and is able to produce ethanol or xylitol from D-xylose (Hahn-Hägerdal et al. 2007). Although xylitol production of *P. stipitis* is naturally very low, three types of mutant strains have been shown to improve xylitol production. First, disruption of two genes coding for isozymes of alcohol dehydrogenase (ADH), designated *PsADH1* and *PsADH2*, resulted in 21 g l^{-1} xylitol at 6 days under oxygen-limited conditions (Cho and Jeffries 1998). The increase of xylitol production has been explained in terms of NADH accumulation and shift of the equilibrium of the XDH-mediated reaction to favor xylitol over D-xylulose formation. Second, a *P. stipitis* mutant defective in *XDH* gene produced 44.8 g l^{-1} xylitol in fed-batch fermentation with 20 g l^{-1} gluconic acid as a co-substrate at 5 days (Kim et al. 2001). This mutant required a co-substrate, which supported growth and regeneration of cofactor, to produce xylitol from D-xylose since its D-xylose metabolic pathway was blocked. Third, a D-xylulokinase gene (*XYL3*) mutant produced 26 g l^{-1} xylitol and exhibited a higher productivity (0.22 g l^{-1} h^{-1}) under aerobic conditions (Jin et al. 2005). In the resulting strain, an alternative metabolic pathway, perhaps via arabinitol and D-ribulose-5-phosphate, bypasses the D-xylulokinase step during D-xylose assimilation (Fig. 5.3). Moreover, Rodrigues et al. (2011) reported that D-xylose converted to xylitol by

P. stipitis XYL3 mutant in corn stover hemicellulosic hydrolysate. The highest xylitol yield (0.61 g g xylose^{-1}) and volumetric productivity (0.18 g l^{-1} h^{-1}) were obtained in the hydrolysate neutralized with phosphoric acid.

5.3.4 Saccharomyces Cerevisiae

Saccharomyces cerevisiae is a GRAS (generally recognized as safe) organism and has strong tolerance to inhibitors present in lignocellulose hydrolysates and thus is a suitable host for production of chemicals used in the food industry. Wild-type *S. cerevisiae* was shown to be a non-D-xylose fermenting yeast because of its lack of the D-xylose metabolic pathway (Hahn-Hägerdal et al. 2007). Hallborn et al. (1991) reported xylitol production by a recombinant microorganism for the first time. The D-xylose reductase gene (*xyl1*) of *P. stipitis* was cloned and expressed in *S. cerevisiae*. This strain converted D-xylose to xylitol at over 95 % ratio. Chung et al. (2002) reported that *xyl1* gene was expressed with chromosomal integration or as a plasmid in *S. cerevisiae*. The xylitol productivity of the genomic DNA-integrated strain was 1.7-fold higher than that of the plasmid-bearing strain (Chung et al. 2002). This genomic DNA-integrated strain produced 116 g l^{-1} xylitol and exhibited 2.34 g l^{-1} h^{-1} in cell-recycle fermentation supplemented with D-xylose and yeast extract solution (Bae et al. 2004). Recently, a recombinant *S. cerevisiae* strain obtained by expressing XR gene from *C. tropicalis* SCTCC 300249 (*xyl1*) produced 93.7 g l^{-1} xylitol and exhibited 2.34 g l^{-1} h^{-1} in batch fermentation using D-glucose as a co-substrate (Zhang et al. 2009).

In order to accelerate NADPH generation through PPP, phosphoglucose isomerase (PGI) which catalyze the isomerization of D-glucose 6-phospate to D-fructose 6-phospate in glycolysis and G6PDH enzyme activities were controlled in NADPH-dependent xylitol production (Know et al. 2006a; Oh et al. 2007). Overexpression of endogenous G6PDH encoded by *ZWF1* to increase NADPH availability in a recombinant strain of *S. cerevisiae* harboring *P. stipitis* XR gene (*XYL1*) in the chromosome (Chung et al. 2002) resulted in six-fold increase in G6PDH activity and increased xylitol concentration (from 71 to 86 g l^{-1}) and xylitol productivity (from 1.6 to 2.0 g l^{-1} h^{-1}) in D-glucose-limited, fed-batch cultivations containing D-xylose (Know et al. 2006a). To further increase NADPH-generating PPP flux, PGI activity encoded by *PGI1* was reduced by replacing the *PGI1* promoter with the *ADHI* (encoding alcohol dehydrogenase I) promoter (Oh et al. 2007). Simultaneous overexpression of G6PDH and attenuation of PGI in a recombinant *S. cerevisiae* strain expressing *P. stipitis XYL1* (Fig. 5.4) resulted in a specific productivity of 0.34 g·(g cdw h)$^{-1}$ in D-glucose-limited, fed-batch cultivations, which was 1.9-fold higher than the specific productivity of the parent strain expressing only *XYL1* (Oh et al. 2007). As another approach to facilitate NAD(P)H supplementation, acetaldehyde dehydrogenase 6 (*ALD6*) and/or acetyl-CoA synthetase 1 (*ACS1*) were overexpressed in recombinant *S. cerevisiae* harboring the *P. stipitis* XR gene (Oh et al. 2012). Among these recombinants, *ACS1* overexpression gave

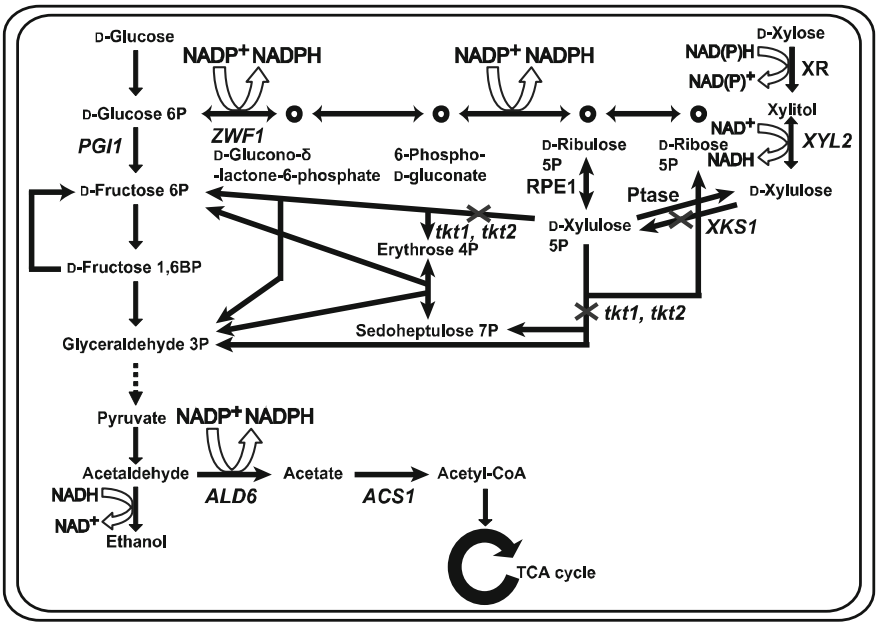

Fig. 5.4 Schematic representation of xylitol production from D-xylose or D-glucose by recombinant *S. cerevisiae* strains. *XR* D-xylose reductase, *RPE1* D-ribulose-phosphate 3-epimerase, *Ptase* sugar phosphate phosphatase, *PGI1* phosphoglucose isomerase, *ALD6* acetaldehyde dehydrogenase 6, *ACS1* acetyl-CoA synthetase 1, *ZWF1* D-glucose 6-phosphate dehydrogenase, *XYL2* D-xylitol dehydrogenase, *XKS1* D-xylulokinase, *tkt1* and *tkt2* transketolase isoenzymes 1 and 2

that best result of xylitol production: 91.3 g l^{-1} xylitol concentration and 1.76 g l^{-1} h^{-1} xylitol productivity in D-glucose-limited, fed-batch cultivation.

Although D-xylose is widely found in nature as a constituent of hemicellulose, economically viable sources of pure D-xylose suitable for the manufacture of xylitol are limited. This problem could be solved if an efficient technology for conversion of a raw material of virtually unlimited availability such as D-glucose into xylitol could be developed. Xylitol production from D-glucose was also demonstrated using engineered *S. cerevisiae* (Fig. 5.4) (Toivari et al. 2007). The strain design included deleting transketolase genes (*tkt1* and *tkt2*) followed by overexpressing XDH from *P. stipitis* (*XYL2*) and deleting *XKS1* encoding D-xylulokinase (Toivari et al. 2007). The recombinant *S. cerevisiae* produced 0.3 g l^{-1} xylitol at 100 h with 20 g l^{-1} D-glucose.

5.4 Xylitol Production by Recombinant Bacteria

Although wild-type bacterial species such as *Corynebacterium* sp., *Enterobacter liquaefaciens*, and *Mycobacterium smegmatis* produce xylitol, their productivities are inferior to those of metabolically engineered strains of bacteria such as *E. coli* and

Lactococcus lactis (Akinterinwa et al. 2008; Chen et al. 2010). Xylitol production by genetically engineered bacteria has increased in recent decades.

5.4.1 Bacillus Subtilis

In 1969, Onishi and Suzuki (Onishi and Suzuki 1969) described a three-step fermentation process for conversion of D-glucose to xylitol. First, D-glucose was converted to D-arabitol by an osmophilic yeast strain *Debaryomyces hansenii*. D-Arabitol was then converted to D-xylulose by *Acetobacter suboxydans* and finally *C. guilliermondii* converted D-xylulose to xylitol. Recently, Povelainen and Miasnikov (2007) reported one-step xylitol production from D-glucose in engineered strains of *B. subtilis*. Expression of xylitol phosphate dehydrogenase (XPDH) from *Lactobacillus rhamnosus* or *Clostridium difficile* in a pentulose-producing mutant of *B. subtilis* with the two genes (*rpi* and *tktA*) coding for D-ribose 5-phosphate isomerase and transketolase disrupted, resulted in xylitol production with a yield of 0.26–0.27 mol xylitol·(mol D-glucose)$^{-1}$ in rich medium containing 10 % D-glucose. Because XPDH had two enzyme (D-xylulose 5-phosphate reductase and D-ribulose 5-phosphate reductase) reactions, ribitol production also increased slightly during xylitol fermentation (Fig. 5.5).

Dephosphorylation of pentitol phosphates was presumably owing to an intracellular or membrane-associated sugar phosphate phosphatase (Povelainen and Miasnikov 2007).

5.4.2 Corynebacterium Glutamicum

Corynebacterium glutamicum has been widely used for the industrial production of various amino acids and nucleic acids (Kinoshita 1985; Terasawa and Yukawa 1993). The recently developed *C. glutamicum* recombinant able to co-utilize D-xylose and D-glucose efficiently and simultaneously (Sasaki et al. 2009) is an exceedingly attractive platform strain for xylitol production given the general characteristics of *C. glutamicum*. Upon oxygen deprivation, it can produce significant amounts of lactic, succinic and acetic acids (Inui et al. 2004, 2007). Since cell growth is arrested under oxygen deprivation, energy is primarily channeled to product formation, facilitating high cell densities, high yields, high volumetric productivity, and low by-product formation. Moreover, sugar metabolism of the growth-arrested cells is highly resistant to the phenols, furans, and other fermentation inhibitors derived from lignocellulose pretreatments (Sakai et al. 2007). Some *Corynebacterium* species are able to produce xylitol, albeit at very low productivities (Rangaswamy and Agblevor 2002; Yoshitake et al. 1973). Wild-type *C. glutamicum* produced 0.56 g l^{-1} xylitol from D-xylose at a productivity of 0.01 g l^{-1} h^{-1} under oxygen deprivation using 30 g l^{-1} D-xylose as a sole carbon

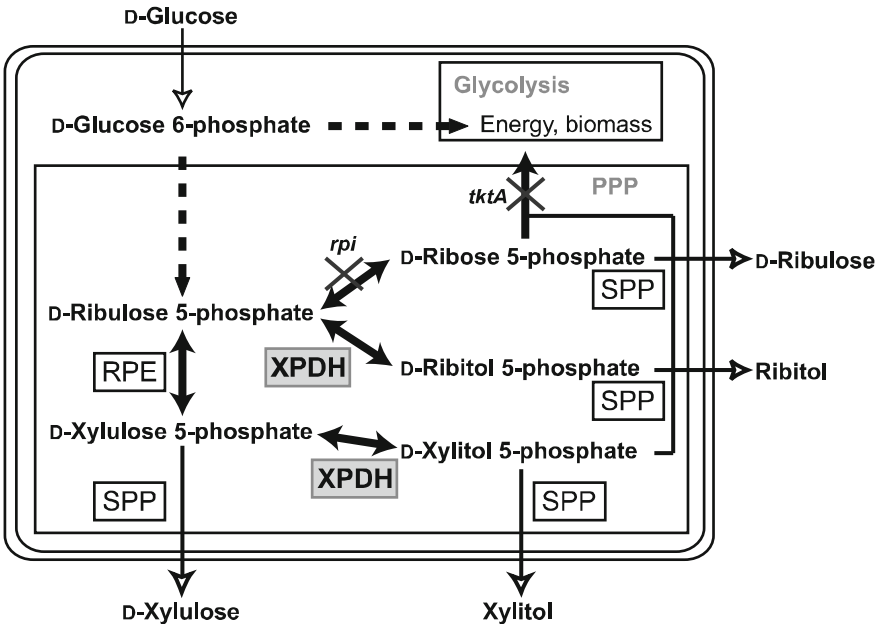

Fig. 5.5 Schematic representation of xylitol production from D-glucose by recombinant *B. subtilis*. *RPE* D-ribulose 5-phospate 3-epimerase, *XPDH* xylitol phosphate dehydrogenase, *SPP* presumptive intracellular (or membraneassociated) sugar phosphate phosphatase, *rpi* D-ribose 5-phosphate isomerase, *tktA* transketolase, *PPP* pentose phosphate pathway

source, although it was incapable of xylitol production under aerobic conditions (Sasaki et al. 2010).

Recombinant *C. glutamicum* strains were able to produce xylitol from D-xylose with D-glucose as a co-substrate (Kim et al. 2010; Sasaki et al. 2010). The biosynthetic pathway of xylitol in the recombinant *C. glutamicum* is shown Fig. 5.6. A recombinant *C. glutamicum* strain expressing *P. stipitis XYL1* achieved a specific productivity of 0.092 g xylitol·(g cdw h)$^{-1}$ and 34.4 g l^{-1} xylitol using a fed-batch culture under D-glucose- and oxygen-limited conditions (Kim et al. 2010). To improve *C. glutamicum* xylitol productivity, heterologous D-xylose reductase (XR) gene from *Candida tenuis* which has dual (NADH and NADPH) coenzyme specificity (Lee et al. 2003) was expressed in *C. glutamicum*, yielding strain CtXR1. Xylitol concentration of strain CtXR1 under oxygen-deprived conditions using 30 g l^{-1} D-xylose as a sole carbon substrate was 1.6 g l^{-1}. To investigate whether the NADH generated at the glyceraldehyde 3-phosphate dehydrogenase (*gapA*)-catalyzed reaction under oxygen deprivation can drive xylitol production by strain CtXR1, cells were cultured in a 30 g l^{-1} D-xylose and 36 g l^{-1} D-glucose mixture. The xylitol concentration (3.5 g l^{-1}) realized in the presence of D-glucose co-substrate was higher than that with D-xylose alone, implying a role for the generated NADH in xylitol production.

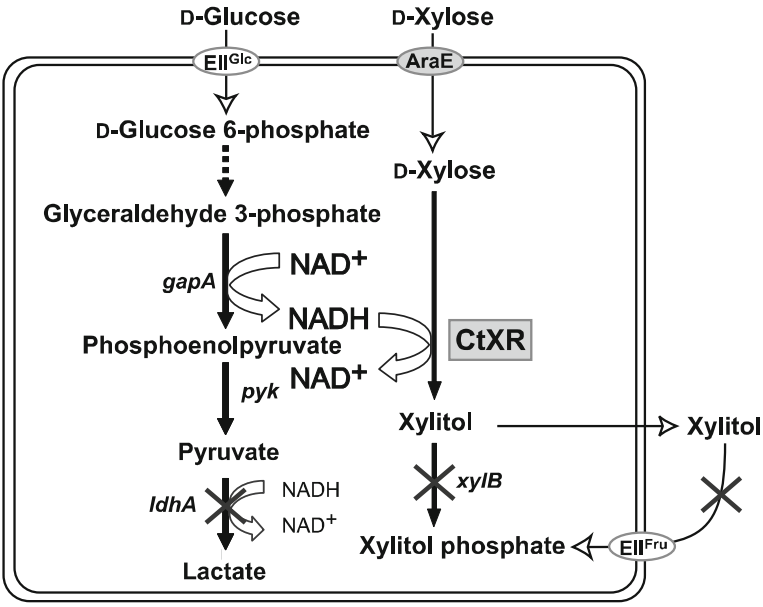

Fig. 5.6 Schematic representation of xylitol production from D-xylose with D-glucose as a co-substrate by recombinant *C. glutamicum* strains. *XR* D-xylose reductase, *AraE* arabinose transporter, *EIIGlc* glucose enzyme II, *EIIFru* fructose enzyme II (*ptsF*), *gapA* glyceraldehyde 3-phosphate dehydrogenase, *pyk* pyruvate kinase, l*dhA* lactate dehydrogenase, *xylB* xylulokinase. *Shadow box* presents the introduced genes. *Source* Adapted from Sasaki et al. (2010)

As expected, strain CtXR1 D-xylose consumption rate (1.5 g l^{-1} h^{-1}) was lower than that of D-glucose (4.1 g l^{-1} h^{-1}), an observation that would mean suboptimal xylitol productivity. To improve D-xylose consumption, the *araE* gene encoding a pentose transporter derived from *C. glutamicum* ATCC31831 that catalyzes the uptake of D-xylose (Sasaki et al. 2009) was integrated in the chromosomal DNA of strain CtXR1, yielding strain CtXR2. Strain CtXR2 showed a significant increase in D-xylose consumption (3.5 g l^{-1} h^{-1}), resulting in a 13-fold improvement in xylitol productivity (2.6 g l^{-1} h^{-1}) over that of strain CtXR1.

Nevertheless, further increase in xylitol productivity of strain CtXR2 should be possible if consumption of NADH formed at the pyruvic acid to lactic acid stage is eliminated. To this end, the lactate dehydrogenase (*ldhA*) gene of strain CtXR2 was deleted, yielding strain CtXR3. Although strains CtXR3 D-glucose consumption (1.6 g l^{-1} h^{-1}) reduced to about 50 % that of CtXR2, the xylitol productivity hardly changed (2.7 g l^{-1} h^{-1}), suggesting that the supply of NADH is for XR activity.

Even further improvement in xylitol productivity is possible through improved coenzyme specificity (Petschacher et al. 2005). Expression of single-site mutant XR from *C. tenuis* (*CtXR* (K274R)) exhibiting higher XR activities with NADH and NADPH resulted in recombinant *C. glutamicum* strain CtXR4 that produced 26.5 g l^{-1} xylitol at 3.1 g l^{-1} h^{-1}.

Xylitol inhibits the growth of many bacteria (London and Hausman 1982; Reiner 1977; Trahan 1995). Because *C. glutamicum* R is not able to metabolize xylitol, its growth was examined in mineral salts medium containing D-glucose with xylitol to ascertain any inhibitory effects of xylitol. The specific growth rates decreased with the increase of xyltiol concentration (Sasaki et al. 2010). It was recently reported that xylulokinase (XylB) catalyzes the conversion of xylitol to xylitol-phosphate in *E. coli*, and xylitol production is improved significantly upon deletion of *xylB* gene (Akinterinwa and Cirino 2009). To eliminate possible formation of toxic intracellular xylitol phosphate, the *xylB* gene was disrupted. Deletion of the *xylB* gene of strain CtXR4 yielded strain CtXR5 which consumed D-xylose and D-glucose better than strain CtXR4 with concomitantly higher xylitol productivity (3.5 g l^{-1} h^{-1}). In *C. glutamicum*, xylitol is transported by a phosphoenolpyruvate-dependent fructose phosphotransferase system (PTS^{fru}) and is probably phosphorylated via the same system (Dominguez and Lindley 1996). To evaluate the effect of *ptsF* gene deletion on xyltiol production, strain CtXR6 was constructed by deletion of the *ptsF* gene of strain CtXR4. The xylitol productivity of strain CtXR6 increased significantly (4.6 g l^{-1} h^{-1}). At the same time, strain CtXR7 exhibiting deleted *xylB* and *ptsF* genes showed further increased D-xylose consumption rate and xylitol productivity (5.0 g l^{-1} h^{-1}). Using strain CtXR7, xylitol production was measured in a fed-batch culture under oxygen deprivation using D-xylose and D-glucose sugar mixture. The xylitol concentration and productivity at 21 h were 166 g l^{-1} and 7.9 g l^{-1} h^{-1}, respectively, representing the highest bacterial xylitol productivity reported to date.

5.4.3 *Escherichia Coli*

Escherichia coli is an ideal organism for industrial production of chemicals due to its ability to assimilate both hexose and pentose sugars, rapid growth rates, clear genetic background, ease of manipulation, and inexpensive growth medium requirements, as evidenced by its industrial implementation for production of value-added products such as a succinic acid, lactic acid, and 1,3-propanediol (Yu et al. 2011). Because *E. coli* does not have the native capability to synthesize xylitol, many research efforts have focused on metabolically engineering *E. coli* for xylitol production (Fig. 5.7).

Two major pathways are used for xylitol production from D-xylose in recombinant *E. coli*: either direct D-xylose reduction (via expression of heterologous XR) or conversion of D-xylose to D-xylulose (via the native D-xylose isomerase) followed by reduction to xylitol (via expression of heterologous XDH) (Fig. 5.7). In 1999, expression of XR gene (*xyrA*) from *C. tropicalis* in *E. coli* resulted in 13.3 g l^{-1} xylitol from D-xylose and D-glucose during 20 h of cultivation (Suzuki et al. 1999). In order to enable co-utilization of D-glucose and D-xylose, a native cyclic AMP receptor protein gene (*crp*) was replaced with a catabolite repression mutant (*crp**) (Cirino et al. 2006). To prevent D-xylose metabolism, *xylB* was

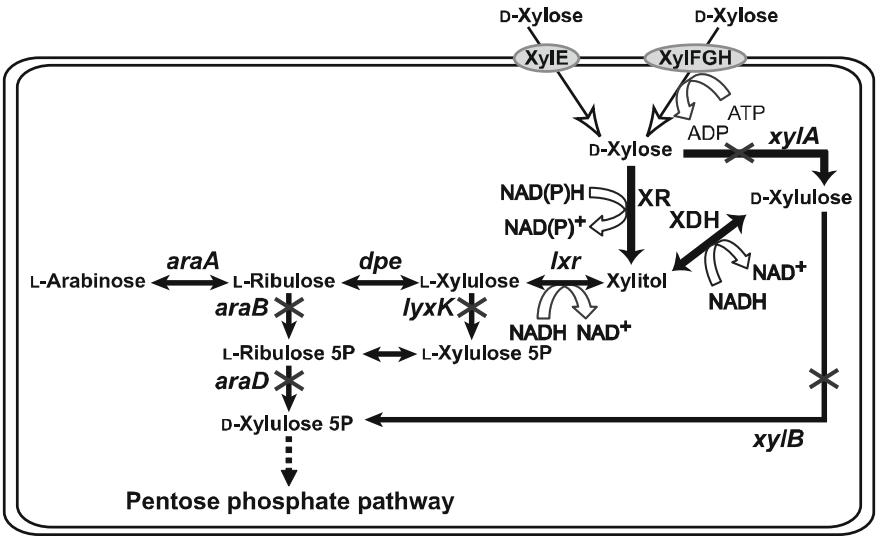

Fig. 5.7 Schematic representation of xylitol production from D-xylose or L-arabinose by recombinant *E. coli* strains. *XR* D-xylose reductase, *XDH* D-xylitol dehydrogenase, *XylE* D-xylose-proton symporter, *XylFGH* ATP-dependent D-xylose transporter, *araA* L-arabinose isomerase, *araB* L-ribulokinase, *araD* L-ribulose- 5-phosphate 4-epimerase, *xylA* D-xylose isomerase, *xylB* xylulokinase, *dpe* D-psicose 3-epimerase, *lxr* L-xylulose reductase, *lyxK* L-xylulose 5-phosphate 3-epimerase

deleted from *crp** mutant, yielding strain PC09. Several XRs and XDHs were screened in strain PC09, and the NADPH-dependent XR from *Candida boidinii* (CbXR) allowed for the highest levels of xylitol production from D-xylose and D-glucose in batch cultures (38 g l^{-1} in 96 h using minimal medium) (Cirino et al. 2006). An alternative to the use of *crp** in *E. coli* is plasmid-based overexpression of D-xylose transporters genes *xylE* (D-xylose-proton symporter) or *xylFGH* (ATP-dependent D-xylose transporter) (Fig. 5.7) (Khankal et al. 2008a). Overexpressing the XylFGH transport system from *E. coli* resulted in an average specific xylitol productivity of 0.33 g·(g cdw h)$^{-1}$ and 56 g l^{-1} xylitol in 46 h fed-batch cultivation using mineral salts medium (Khankal et al. 2008a). Alternate approaches to xylitol production from D-glucose and D-xylose mixture (expression of *crp**, *xylE*, or *xylFGH*) were compared in three common *E. coli* host strains (K-12 strains W3110 and MG1655, and wild-type *E. coli* B), and differences in host strain genetic background were found to significantly impact metabolic engineering strategies. Xylitol production in fed-batch cultivation was similar for the (*crp**, Δ*xylB*)-derivatives of W113 and MG1655 expressing *CbXR* [average specific productivities of 0.43 g·(g cdw h)$^{-1}$] (Khankal et al. 2008b).

An analysis of mutant strains lacking various genes in strain PC09 central metabolism (e.g., *pgi*, *zwf*, *pntA* pyridine nucleotide transhydrogenase, *sthA* soluble pyridine nucleotide transhydrogenase, *sucC* succinyl-CoA synthetase, *ndh* NADH dehydrogenase II, *pfkA* phosphofructokinase) revealed the importance of

direct NADPH supply from $NADP^+$-reducing pathways to improve xylitol yields (Chin et al. 2009; Chin and Cirino 2011). NADPH availability limited xylitol production in resting cells, and was increased by increasing flux through PPP during D-glucose metabolism. Excess reducing equivalents in the form of NADH (resulting from D-glucose oxidation) did not translate into available NADPH for xylitol production (Chin et al. 2009).

To reveal a factor capable of increasing NADPH supply for xylitol production, transcriptome analysis was performed using a xylitol-producing *E. coli* strain with deletion of D-xylose isomerase (*xylA*) and chromosomal insertion of NADPH-dependent XR from *Kluyveromyces lactis* (*XYL1*) and the *E. coli xylE* under the control of an IPTG-inducible promoter (Hibi et al. 2007). Transcriptome analysis of xylitol-producing and nonproducing conditions for the recombinant revealed that xylitol production down-regulated 56 genes, which were considered as factors related to reduced NADPH supply. The 56 individual gene deletions were studied and a *yhbC*-deficient strain showing the highest improvements in xylitol production, increasing xylitol concentration (from 43.4 to 51.7 g l^{-1}) and xylitol productivity (from 0.68 to 0.81 g xylitol l^{-1} h^{-1}) in D-xylose and D-glucose mixture. YhbC is uncharacterized, but is potentially a regulatory factor. Akinterinwa and Cirino (2011) reported anaerobic xylitol production from a D-xylose and D-glucose mixture in engineered *E. coli*. Fermentation pathways were eliminated so that NADPH-dependent D-xylose reduction for xylitol production serves as the sole means of regenerating NAD^+ and maintaining redox balance. With a mutant of *E. coli* incapable of fermentation, the membrane-bound transhydrogenase (PntAB) is required to achieve the maximum theoretical yield of 4 mol xylitol per mole of D-glucose under anaerobic conditions.

XylB of *E. coli* was shown to phosphorylate xylitol to xylitol phosphate, resulting in inhibited growth on D-xylose and poor xylitol production (Akinterinwa and Cirino 2009). To overcome this inhibition, endogenous *xylB* was disrupted and heterologous xylulokinase (*Xyl3*) from *P. stipitis* was expressed in the recombinant *E. coli* expressing *CbXR*. Replacement of *xylB* with *Xyl3* resulted in significantly improved xylitol concentration (from 0.15 to 19.8 g l^{-1}) and xylitol productivity (from 0.002 to 2.74 g l^{-1} h^{-1}) during growth on D-xylose as a source of carbon and energy.

To efficiently produce xylitol from D-xylose in the presence of L-arabinose with minimal production of L-arabinitol by product, expression of mutant *NcXR* gene (50-fold lower catalytic efficiency toward L-arabinose) was combined with deletion of *xylAB*, replacement of *crp**, and expression of *xylE* (Nair and Zhao 2010). The resulting strain was able to eliminate L-arabinitol production and produce xylitol at near 100 % purity from a mixture of L-arabinose, D-glucose, and D-xylose. Cheng et al. (2009) cloned the NAD^+-dependent D-xylulose-forming D-arabitol dehydrogenase gene (*aArDH*) from an acetic acid bacterium, *Acetobacter suboxydans,* and heterogeneously expressed it in *E. coli* for the bio-conversion of D-arabitol to xyltiol.

Sakakibara et al. (2009) reported xylitol production from L-arabinose by introducing three genes in *E. coli* deleting *araA*, *araB*, *araD*, and L-xylulose

5-phosphate 3-epimerase (*lyxK*) genes (Fig. 5.7). Expressed genes were *araA* from *E. coli*, *dpe* encoding D-psicose 3-epimerase (which converts L-ribulose to L-xylulose) from *Rhizobium radiobacter*, and *lxr* encoding L-xylulose reductase (which converts L-xylulose to xylitol) from *Ambrosiozyma monospora*. The resulting strains produced 14.5 g l^{-1} xylitol from 15.2 g l^{-1} L-arabinose with a xylitol yield of 0.95 g g^{-1} L-arabinose under medium containing glycerol without L-arabitol formation.

5.4.4 *Gluconobacter Oxydans*

Suzuki et al. (2002) described that *G. oxydans* was able to produce xylitol from D-arabitol. Because D-arabitol can be produced efficiently from D-glucose by fermentation with osmophilic yeasts (Onishi and Suzuki 1969; Saha et al. 2007), the development of an efficient method for xylitol production from D-arabitol, thereby lowering manufacturing costs by allowing xylitol to be produced from D-glucose, is an attractive proposition. Conversion of D-arabitol to xylitol with *G. oxydans* is an alternative process that is independent from D-xylose, involving oxidation of D-arabitol to D-xylulose by a membrane-bound D-arabitol dehydrogenase (AraDH), followed by reduction of D-xylulose to xylitol by a NAD^+-dependent D-xylitol dehydrogenase (XDH) (Fig. 5.8). A disruptant of *adhB*, which encodes the cytochrome c-553 subunit of membrane-bound alcohol dehydrogenase (ADH), produced 47.1 g l^{-1} xylitol from 100 g l^{-1} D-arabitol in the presence of ethanol, whereas the wild-type strain produced 31.9 g l^{-1} xylitol (Suzuki et al. 2002). The increase in xylitol yield may be as a result of loss of unfavorable consumption of NADH via respiratory electron transport chains of *G. oxydans*. Overexpression of endogenous *XDH* resulted in 11-fold increase of XDH activity and increase of xylitol concentration from 225 g l^{-1} D-arabitol (from 27 to 57 g l^{-1} xylitol) under controlled aeration and around pH 5.0–6.0 in the presence of ethanol (Sugiyama et al. 2003).

5.4.5 *Lactococcus Lactis*

Lactic acid bacteria (LAB) such as *L. lactis* are widely used in industrial food fermentations and are receiving increased attention for use as cell factories for the production of food and pharmaceutical products (De Vos and Hugenholtz 2004). *L. lactis* has a small genome, genetic accessibility and a simple and controllable metabolism, which combine to make the strain an attractive target for metabolic engineering. Nyyssölä et al. (2005) reported xylitol production by recombinant *L. lactis* (Nyyssölä et al. 2005). Using D-glucose as the energy source, xylitol was produced from D-xylose in a recombinant *L. lactis* strain by expressing the XR gene from *P. stipitis* (*XYL1*). Xylitol production by this recombinant was

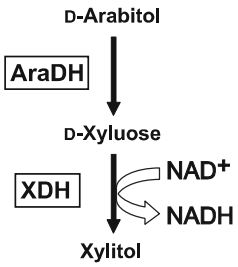

Fig. 5.8 Schematic representation of xylitol production from D-arabitol by recombinant *G. oxydans* strains. *AraDH* membrane-bound D-arabitol dehydrogenase, *XDH* soluble NAD^+-dependent D-xylitol dehydrogenase

investigated using non-growing cells at high cell-density under microaerobic conditions in the presence of D-xylose and D-glucose. Co-expression of the D-xylose-H^+ transporter *xylT* gene from *Lactobacillus brevis* with *XYL1* did not significantly increase the efficiency of xylitol production. A D-glucose-limited, fed-batch fermentation produced 2.5 mol xylitol·(mol D-glucose)$^{-1}$ and 1.0 mol xylitol·(mol D-xylose)$^{-1}$ (D-xylose was not metabolized) at the rate of 2.72 g xylitol l^{-1} h^{-1} at 20 h.

5.5 Conclusions and Future Recommendations

The xylitol production characteristics of recombinant microorganisms discussed in this chapter are summarized in Table 5.1. The genetic engineering avenues explored in order to obtain improved xylitol production are threefold, namely, modification of metabolic pathways, adjustment of redox balance, and/or increase of sugar import. It is noteworthy that the xylitol productivity by recombinant *C. glutamicum* (7.9 g l^{-1} h^{-1}) is the highest reported among xylitol-producing recombinant microorganisms to date. This can be attributed mainly to the unique growth-arrested process that is possible with this microorganism. In general, however, the productivity and maximum concentration of xylitol by recombinant yeasts tend to be higher than those by other recombinant fungi and bacteria. In particular, a recombinant strain of the yeast, *C. tropicalis*, permitted impressive figures (5.09 g l^{-1} h^{-1} productivity and 275 g l^{-1} yield) despite lacking the obvious benefit of a co-substrate. The demand and applications of xylitol would grow further as biorefinery technology develops. Because some microbial-based processes for sugar alcohols such as mannitol and erythritol are already commercially viable (Akinterinwa et al. 2008), xylitol production by engineered microorganisms, by following the paths of these commercial processes, has great potential to become commercially viable in the very near future. Approaches other than genetic modification attempted so far are necessary for further improvements in productivity. For instance, not much is known about the xylitol export system of xylitol producing microorganisms except that the *FPS1* gene responsible for the facilitated diffusion of glycerol in *S. cerevisiae* is involved in xylitol export (Hahn-Hägerdal et al. 2001). Moreover, industrial strains for xylitol production are

Table 5.1 Summary of xylitol production characteristics by recombinant microorganisms in this chapter

Organism	Genetic modifications	Yield and/or productivity	Maximum concentration (g l^{-1})	Substrate and co-substrate	Reference
Fungi					
T. reesei	Partial mutation of *xdh1*	0.020 g l^{-1} h^{-1}	2.37	D-Xylose	Wang et al. (2005)
Yeast					
C. tropicalis	Expressed *C. parapsilosis XYL1*	5.09 g l^{-1} h^{-1}	275	D-Xylose	Lee et al. (2003)
C. tropicalis	Disrupted *XYL2*	3.23 g l^{-1} h^{-1} 0.96 mol·(mol D-xylose)$^{-1}$	48.6	D-Xylose + Glycerol	Ko et al. (2006a)
C. tropicalis	Expressed *N. crassa NcXR*, disrupted *XYL2*	1.44 g l^{-1} h^{-1}	63.4	D-Xylose + D-Glucose	Jeon et al. (2012)
C. tropicalis	Expressed *zwf* and *gnd*, disrupted *XYL2*	1.25 g $l^{-1}h^{-1}$	60	D-Xylose + Glycerol	Ahmad et al. (2012)
P. pastroris	Expressed *C. guilliermondii xyl1*	0.11 g l^{-1} h^{-1}	7.8	D-Xylose	Handumrongkul et al. (1998)
P. stipitis	Disrupted *XYL3*	0.22 g l^{-1} h^{-1}	26	D-Xylose	Jin et al. (2005)
S. cerevisiae	Expressed *P. stipitis xyl1*	2.34 g $l^{-1}h^{-1}$	116	D-Xylose + Yeast extract	Bae et al. (2004)
S. cerevisiae	Overexpressed *ZWF1*, expressed *P. stipitis XYL1*, attenuated PGI activity	1.49 g l^{-1} h^{-1} 0.34 g·(g cdw h)$^{-1}$	103	D-Xylose + D-Glucose	Oh et al. (2007)

(continued)

Table 5.1 (continued)

Organism	Genetic modifications	Yield and/or productivity	Maximum concentration (g l^{-1})	Substrate and co-substrate	Reference
S. cerevisiae	Overexpressed *P. stipitis XYL2*, disrupted *tkl1*, *tkl2*, and *XKS1*	0.003 g l^{-1} h^{-1}	0.3	D-Glucose	Toivari et al. (2007)
S. cerevisiae	Expressed *C. tropicalis* SCTCC 300249 *xyl1*	2.34 g l^{-1} h^{-1}	93.7	D-Xylose + D-Glucose	Zhang et al. (2009)
S. cerevisiae	Overexpressed *ACS1*, Expressed *P. stipitis XYL1*	1.76 g l^{-1} h^{-1}	91.3	D-Xylose + D-Glucose	Oh et al. (2012)
Bacteria					
B. subtilis	Expressed XPDH gene, disrupted *rpi* and *tktA*	0.27 mol·(mol D-glucose)$^{-1}$		D-Glucose	Povelainen and Miasnikov (2007)
C. glutamicum	Expressed *P. stipitis XYL1*	0.86 g l^{-1} h^{-1} 0.092 g·(g cdw h)$^{-1}$	34.4	D-Xylose + D-Glucose	Kim et al. (2010)
C. glutamicum	Expressed *araE* and *CtXR*(K274R), disrupted *ldh*, *ptsF*, and *xylB*	7.9 g l^{-1} h^{-1}	166	D-Xylose + D-Glucose	Sasaki et al. (2010)
E. coli	Expressed *C. tropicalis xyrA*	0.67 g l^{-1} h^{-1}	13.3	D-Xylose + D-Glucose	Suzuki et al. (1999)
E. coli	Expressed *C. boidinii CbXR*, replaced *crp* with *crp**, disrupted *xylB*	0.79 g l^{-1} h^{-1}	38	D-Xylose + D-Glucose	Cirino et al. (2006)

(continued)

Table 5.1 (continued)

Organism	Genetic modifications	Yield and/or productivity	Maximum concentration ($g\ l^{-1}$)	Substrate and co-substrate	Reference
E. coli	Expressed *xylE* and *K. lactis XYL1*, disrupted *xylA* and *yhbC*	0.81 $g\ l^{-1}\ h^{-1}$	51.7	D-Xylose + D-Glucose	Hibi et al. (2007)
E. coli	Overexpressed *xylFGH*, expressed *C. boidinii CbXR*, disrupted *xylB*	0.58 $g\ l^{-1}\ h^{-1}$ 0.33 $g\cdot(g\ cdw\ h)^{-1}$	56	D-Xylose + D-Glucose	Khankal et al. (2008a)
E. coli	Expressed *C. boidinii CbXR* and *P. stipitis Xyl3*, disrupted *xylB*	0.27 $g\ l^{-1}\ h^{-1}$	19.8	D-Xylose	Akinterinwa and Cirino (2009)
E. coli	Expressed *E. coli araA*, *R. radiobacter dpe*, and *A. monospara lxr*, disrupted *araA*, *araB*, *araD*, and *lyxK*	0.48 $g\ l^{-1}\ h^{-1}$	14.5	L-Arabinose + Glycerol	Sakakibara et al. (2009)
G. oxydans	Disrupted *adhB*	0.98 $g\ l^{-1}\ h^{-1}$	47.1	D-Arabitol + Ethanol	Suzuki et al. (2002)
G. oxydans	Expressed *XDH*	1.12 $g\ l^{-1}\ h^{-1}$	57	D-Arabitol + Ethanol	Sugiyama et al. (2003)
L. lactis	Expressed *P. stipitis XYL1* and *L. brevis xylT*	2.72 $g\ l^{-1}\ h^{-1}$ 2.5 $mol\cdot(mol\ D\text{-glucose})^{-1}$ 1.0 $mol\cdot(mol\ D\text{-xylose})^{-1}$	75.0	D-Xylose + D-Glucose	Nyyssölä et al. (2005)

adversely affected by the presence of fermentation inhibitors inevitably present in lignocellulose hydrolysates (Hahn-Hägerdal et al. 2007). Moreover, the strains more often than not preferentially utilize D-glucose over any other sugars due to carbon catabolite repression or allosteric competition of the sugars in sugar transport (Dien et al. 2003; Hahn-Hägerdal et al. 2007). Nevertheless, subject to solution of these obstacles, effective microbial production of xylitol from hemicellulose hydrolysates of agricultural residues is a tremendously attractive way of containing xylitol production costs. This is a goal that can only be attained if high-yielding recombinant strains able to utilize the entire spectrum of sugars present in hemicellulose hydrolysates can be developed. With such strains in hand, increased xylitol export, tolerance to fermentation inhibitors, further expansion of the range of sugar utilization, and removal of D-glucose repression are some of the ways in which even more improvements in xylitol production can be attained.

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Chapter 6
Statistical Approaches for the Optimization of Parameters for Biotechnological Production of Xylitol

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Abstract Statistics is a fundamental tool in the analysis of any process data where there is variability. There are many ways to approach the problem of optimization and design of a process, which can be handled quickly using a number of statistical techniques. Statistical design of experiments is a mechanism of data collection appropriate to study the biotechnological process, like xylitol production. Several fermentation processes have been optimized using response surface methodology. However, one of the major problems to the researcher is identifying the independent variables that influence the study in order to explain the model which best represents the process. The upstream independent variables studied in the statistical design for fermentation processes are aeration rate, temperature, phosphate level, back pressure, carbon source, pH, power input, agitation rate, carbon/nitrogen ratio, nitrogen source and dissolved oxygen level. The statistical approach for biotechnological production of xylitol from lignocellulosic materials also could be helpful to optimize pretreatment of lignocellulosic biomass, conditioning of

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hemicellulosic hydrolysates and xylitol recovery from fermented hydrolysates. This chapter will provide an overview on the state of knowledge in these areas focus on statistical approaches.

Keywords Statistical approaches · Optimization · Lignocellulosic materials · Xylitol bio-production

6.1 Introduction

Most of the chemical products used today in our society originate from fossil sources through refinery operations. The continual price increase of fossil resources, their uncertain availability, and the environmental concerns of their exploitation have led to a demand for the elaboration of alternative chemical production patterns based on renewable sources (Cherubini and Strømman 2011). Besides fossils, the only resource available for producing a wide variety of fine and bulk chemicals is biomass (renewable, abundant and economical carbon sources) (Fengel and Wegener 1989; Lynd et al. 1991; Jacobus 2001; Greene et al. 2004; Turner et al. 2007; Mäki-Arvela et al. 2011), and the establishment of biorefinery complexes is increasingly perceived as a promising alternative to oil refineries (Cherubini and Strømman 2011; Mäki-Arvela et al. 2011). The biorefinery concept to produce fuel components, platform chemicals, and fine chemicals from biomass is currently a hot topic, since mankind needs alternative carbon sources for replacing oil. A basic process in biorefinery is hydrolysis of cellulose and of other biomass components to sugars, which can be performed in several ways (Mäki-Arvela et al. 2011).

Biomass feedstocks are constituted mainly of cellulose, hemicellulose and lignin in various proportions (Fengel and Wegener 1989). A pretreatment of biomass refers to a process that disrupts the cellulose, hemicellulose and lignin matrix, making the carbohydrates more accessible to further enzymatic hydrolysis and fermentation. According to Elander et al. (2012), today many pretreatment technology are under evaluation, which are by category: base catalyzed (AFEX/FIBEX, ammonia recycle percolation, lime), not catalyzed (autohydrolysis, steam, hot water, hot water pH neutral), acid catalyzed (carbonic, nitric, sulfuric acids), solvent based (organosolv), and chemical based (wet oxidation CO_2, peroxide, ozone).

Pretreatment methods produce both a soluble and insoluble fraction. The soluble fraction is called hydrolysate and it is composed of some combination of solubilized hemicellulose and hydrolysis products, including xylose oligomers and monomers, glucose and other hexoses sugars, as well as the toxic byproducts (Olsson and Hahn-Hägerdal 1996; Parajó et al. 1998; Klinke et al. 2004). The insoluble fraction is greatly enriched for cellulose and lignin, and is and of itself, non-toxic, though a substantial amount of toxic compounds may be present due to soluble material entrained within its matrix (Berson et al. 2006). Each pretreatment method and each biomass feedstock will generate hydrolysates containing different

sets of toxic compounds, but in general, three classes of toxic compounds can be found (Klinke et al. 2004): furans, furfural and hydroxymethylfurfural (HMF), produced by the degradation of xylose and glucose, respectively; aliphatic acids, especially acetic acid produced by the deacetylation of hemicellulose and lignin, formic acid produced by the degradation of furans, and levulinic acid produced by the degradation of HMF; and phenolic compounds formed by the breakdown of lignin. In addition to these major components, a wide range of aromatic acids, aldehydes and ketones also can be found in varying degrees (Klinke et al. 2004).

Efforts to determine the toxicity of various compounds present in the hydrolysates usually involve differential fermentation performance in the hydrolysate before and after various methods of conditioning with inferences drawn by analytical determination of differences in toxic compounds. Conditioning refers to methods developed to treat hydrolysate to reduce toxicity and make the sugars more accessible to fermentation (Pienkos and Zhang 2009). In general, it would be more advantageous in terms of cost savings to modify the pretreatment processes to reduce formation of toxic compounds rather than add additional cost of a conditioning step, but less toxic pretreatment methods typically release less sugar for fermentations and therefore require additional hydrolytic enzymes (Pienkos and Zhang 2009). There are three categories of conditioning processes: biological (Schneider 1996; Jonsson et al. 1998; Lopez et al. 2004; Nichols et al. 2005; Okuda et al. 2008), physical (Sreenath and Jeffries 2000; Cantarella et al. 2004; Berson et al. 2006; Mancilha and Karim 2003) and chemical (Nilvebrant et al. 2003; Alriksson et al. 2005, 2006; Purwadi et al. 2004). The biological and chemical approaches are designed to convert toxic compounds to less toxic products; the physical approach is designed to remove toxic compounds from the hydrolysate (Pienkos and Zhang 2009).

Sequestration of the toxic compounds, followed by recovery, will provide some insights into hydrolysate fractionation as well as open up possibilities for generation of biorefinery chemical feedstocks. Werpy and Petersen (2004) identify 12 building block chemicals that can be produced from sugars via biological or chemical conversions. The 12 building blocks can be subsequently converted to a number of high-value bio-based chemicals or materials. Building block chemicals, as considered for this analysis, are molecules with multiple functional groups that possess the potential to be transformed into new families of useful molecules (Werpy and Petersen 2004). According to these authors, the xylitol/arabinitol belong to the 12 sugar-based building blocks, of which the others are 1,4-diacids (succinic, fumaric and malic), 2,5-furan dicarboxylic acid, 3-hydroxy propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol and sorbitol. The conversion of xylose to xylitol and subsequent conversion to glycols has been demonstrated and could be a first generation process for the production of propylene glycol and ethylene glycol (Werpy and Petersen 2004).

D-xylitol is a five-carbon polyol (five-carbon sugar alcohol). In industrial scale, D-xylitol is produced by catalytic hydrogenation from pure D-xylose of lignocellulosic materials with high contents of xylan. The hydrogenation of the five carbon

sugars to the sugar alcohols occurs with one of many active hydrogenation catalysts such as nickel, ruthenium and rhodium. If the xylose feedstock is inexpensive then the production of xylitol could be done for very low cost (Pienkos and Zhang 2009). D-xylitol has attracted worldwide interest because of its unique properties and huge potential. It has almost the same sweetness as sucrose, but lower energy value than sucrose (2.4 vs. 4.0 cal/g) (Russo 1977), thus it has been used as a sugar substitute in dietary foods, especially for insulin-deficiency patients. Due to its anticariogenicity, tooth rehardening and remineralization properties, D-xylitol has been widely applied in the odontological industry. It could also prevent ear and upper respiratory infections and benefit pregnant and nursing women. According to the current price of D-xylitol of $4–5/kg (Prakasham et al. 2009), the global market is about $340 million year, and it will definitely grow bigger and bigger.

The statistical approaches for biotechnological production of xylitol from lignocellulosic materials could be helpful to optimize pretreatment of lignocellulosic biomass, conditioning and fermentation of hemicellulosic hydrolysates and xylitol recovery from fermented hydrolysates. This chapter will provide an overview on the state of knowledge in these areas with focus on statistical approaches.

6.2 Statistical Approaches

The experimenter who believes that only one factor at a time should be varied, is amply provided for by using a factorial experiment (Box et al. 2005).

When you are doing *statistics* do not neglect what you and your colleagues know about the subject matter field. Statistical techniques are useless unless combined with appropriate subject matter knowledge and experience. They are an adjunct to, not a replacement for, subject matter expertise (Box et al. 2005). The process optimization is important to improve the systems performance by increasing process yield without increasing their cost. There are many ways to approach the problem of optimization and design of a fermentation process, which can be handled quickly using a number of statistical techniques. Consequently each new problem should be treated on its own merits and with respect (Box et al. 2005). Being too hasty causes mistakes. It is easy to obtain the right answer to the wrong problem (Box et al. 2005). According to Geiger (1997) the traditional approach to the optimization problem is the one-variable-at-a-time method (OVT), which has alternately the technique of evolutionary optimization (EVOP), also known method of *steepest ascent*. However, there are a number of statistical methods which will find an optimum more quickly and more efficiently than these. The best method for process optimization is response surface methodology (RSM), of which are the most extensive applications in the industrial world, particularly in situations where several input variables potentially influence some performance measure or quality characteristic of the product process (Geiger 1997; Myers et al. 2009). This technique is a highly efficient procedure for determining not only the optimum conditions, but also, the data necessary to design the entire process

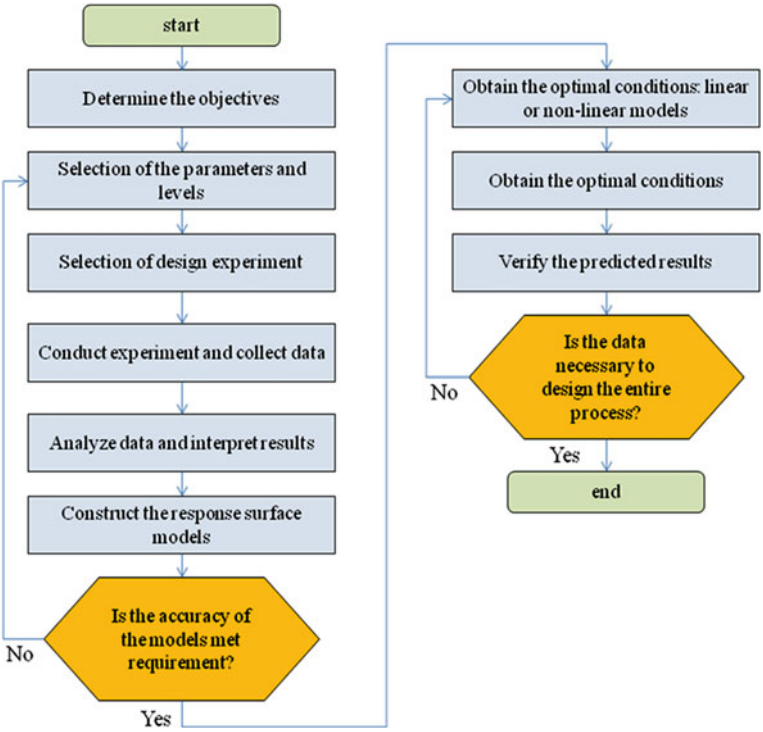

Fig. 6.1 Simplified flowchart of RSM

(Geiger 1997; Myers et al. 2009; Box et al. 2005) (Fig. 6.1). Also, in cases where RSM cannot be applied, EVOP is an alternative method for optimization of a process (Geiger 1997).

In the case of the OVT, one variable is held constant and the optimum level for this variable is determined and, by using this optimum, the second variable's optimum is found (Geiger 1997; Myers et al. 2009; Box et al. 2005). Geiger (1997) states that this statistical methodology works if, and only if, there is no interaction between variables. To make sure the true optimum was reached, it would have been necessary to repeat the one-variable-at-a time process at each step. However, this method approach is both time-consuming and inefficient and is not satisfactory for the fermentation process (Geiger 1997). Also, the major disadvantage of this technique is that it does not include interactive effects among the variables and, eventually, it does not depict the complete effects of the parameters on the process (Bas and Boyaci 2007). The OVT has been used to optimize parameters for xylitol production (Hao et al. 2006; Sampaio et al. 2006b; Villarreal et al. 2006; Baek and Kwon 2007; Martínez et al. 2007).

The evolutionary optimization process (EVOP) is a systematic method of adjusting the variables until an optimum is reached. It is an iterative process in which little needs to be known about the system before beginning the process. In

this case, a simplex figure is generated by running one more experiment than the number of variables to be optimized. A simplex process is designed to find the optimum by ascending the reaction surface along the lines of the steepest slope, i.e., path with greatest increase in yield, cellular growth, and productivity. Also, because of its iterative nature it is a slow process which can require many steps. Another limitation is expressed by providing only limited information about the effects of the variables. However, EVOP is an extremely useful optimization since it is robust, can handle many variables at the same time, and will always lead to an optimum (Geiger 1997).

Response surface methodology is a method of optimization using statistical techniques based upon the special factorial designs of Box and Behenkin and Box and Wilson. It is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing process (Myers et al. 2009). It is a scientific approach to determine optimum conditions which combines special experimental designs with Taylor first and second order equations. The RSM process determines the surface of the Taylor expansion curve which describes the response (yield, impurity level, etc.). A multiple regression technique used by RSM determines the coefficients for the Taylor expansion which best fits the data. However, the RSM does not determine the function which describes the data by the other side as the Taylor equation only approximates the true function. The number of curves available to fit the data is limited by most RSM programs that use only the first and second order terms of the Taylor equation to the data (Geiger 1997). RSM shows the advantages to show the greatest amount of information from experiments, forces you to plan, know how long projects will take, gives information about the interaction between variables, shows multiple responses at the same time and gives information necessary for design and optimization of a process (Geiger 1997; Myers et al. 2009; Box et al. 2005). The RSM disadvantages could be related by telling what happens but not why and have been notoriously poor for predicting outside the range of study (Geiger 1997; Myers et al. 2009; Box et al. 2005). RSM is a very efficient procedure, which utilizes partial factorial designs, such as central composite or star designs, and therefore, the numbers of experimental points required are a minimum. RSM yields the maximum amount of information from the minimum amount of work. To perform a RSM as an optimization technique we must follow three stages. In the first stage, it is necessary to select the parameters that have major effects on the biochemical process. Screening experiments are useful to identify the independent parameters, which may be used factorial designs for this purpose. Also, the direction in which improvements lie is determined and the levels of the parameters are identified. Mistakenly chosen levels result in an unsuccessful optimization (Geiger 1997; Myers et al. 2009; Box et al. 2005). The second stage is the selection of the experimental design and the prediction and verification of the model equation. The last one is obtaining the response surface plot and contour plot of the response as a function of the independent parameters and determination of optimum points (Geiger 1997; Myers et al. 2009; Box et al. 2005). Several processes of xylitol production have been optimized using this methodology (Mayerhoff et al. 1998;

Silva and Roberto 2001; Almeida e Silva et al. 2003; Martinez et al. 2003; Rodrigues et al. 2003, 2012; Carvalho et al. 2004b; De Faveri et al. 2004b; Canilha et al. 2005; Sampaio et al. 2006a; Canettieri et al. 2007; Mussatto and Roberto 2008; Um and Bae 2011; Moutta et al. 2012).

6.3 Statistical Methods for Biotechnological Production of Xylitol

Normally, the factors which are critical for a specific process are known. For example, in the fermentation process, typical variables are aeration rate, temperature, phosphate level, back pressure, carbon source, pH, power input, agitation rate, carbon/nitrogen ratio, magnesium level, sulfur level, nitrogen source and dissolved oxygen level (Geiger 1997). Table 6.1 summarizes some critical parameters that influence all the steps of xylitol production. Table 6.2 shows some examples of statistical optimization studies carried out for xylitol production.

6.4 Acid Pretreatment of Lignocellulosic Materials

Many different pretreatment processes have been developed over the years. They are designed to make the sugars more available for subsequent hydrolysis and fermentation steps through the breakdown of the cell wall, and the degradation of the cellulose, hemicellulose and lignin matrix. A cheap method for producing the sugars (D-glucose, D-galactose, D-manose, D-xylose, and L-arabinose) from lignocellulosic material to xylitol production is the diluted acid biomass hydrolysis, which has been studied intensively (Fengel and Wegener 1989; Alves et al. 1998; Almeida e Silva et al. 1998; Rodrigues et al. 2001; Santos et al. 2005; Canilha et al. 2006, 2008a; Villarreal et al. 2006; Canettieri et al. 2007; Huang et al. 2011). According to Mosier et al. (2005) an "ideal" pretreatment process:

- Produces a high digestible pretreated solid
- Does not significantly degrade pentoses
- Does not significantly inhibit subsequent fermentation steps
- Requires little or no size reduction of biomass
- Can work in reactors of reasonable size and moderate cost
- Produces no solid-waste
- Has a high degree of simplicity
- Is effective at low moisture content.

In addition to these, an ideal pretreatment process would also be sustainable with regard to energy consumption and ecological impact, and would not adversely impact downstream processes. While it is true that the less severe

Table 6.1 Some critical parameters that influence the process of xylitol production

Parameters	Step of xylitol process	Reference
Acid concentration	Acid hydrolysis	Carvalho et al. (2004a), Fogel et al. (2005), Baek and Kwon (2007), Canettieri et al. (2007), Um and Bae (2011), Moutta et al. (2012), Rodrigues et al. (2012)
Temperature	Acid hydrolysis	Carvalho et al. (2004a, b), Baek and Kwon (2007), Canettieri et al. (2007), Um and Bae (2011), Moutta et al. (2012)
Residue/acid solution	Acid hydrolysis	Fogel et al. (2005), Baek and Kwon (2007), Canettieri et al. (2007), Moutta et al. (2012), Rodrigues et al. (2012)
Residence time	Acid hydrolysis	Carvalho et al. (2004a, b), Fogel et al. (2005), Baek and Kwon (2007), Um and Bae (2011), Moutta et al. (2012)
Pressure		Fogel et al. (2005)
Absence or presence of treatment	Conditioning	Rodrigues et al. (2003)
Different types of treatment	Conditioning	Canilha et al. (2004), Villarreal et al. (2006)
Active charcoal concentration	Conditioning	Sampaio et al. (2006b), Villarreal et al. (2006)
Xylose concentration	Conditioning	Carvalho et al. (2004b), Canilha et al. (2005), Mussatto and Roberto (2008)
Phosphate level	Fermentation	Kern et al. (1998), Rao et al. (2004)
Sulfate level	Fermentation	Canilha et al. (2005), Mussatto and Roberto (2008)
Nitrogen level	Fermentation	Kern et al. (1998), Rao et al. (2004), Canilha et al. (2005), Mussatto and Roberto (2008)
Nutrients level	Fermentation	Kern et al. (1998), Canilha et al. (2005), Mussatto and Roberto (2008)
Xylose concentration	Fermentation	Kern et al. (1998), Silva and Roberto (2001), Rodrigues et al. (2003), Rao et al. (2004), Canilha et al. (2005), Sampaio et al. (2006a), Mussatto and Roberto (2008)
pH	Fermentation	Mayerhoff et al. (1998), Martinez et al. (2003), Carvalho et al. (2004b), Rao et al. (2004), Canilha et al. (2005)

(continued)

Table 6.1 (continued)

Parameters	Step of xylitol process	Reference
Cell concentration	Fermentation	Mayerhoff et al. (1998), Silva and Roberto (2001), Carvalho et al. (2004b), Rao et al. (2004), Sampaio et al. (2006a)
Time	Fermentation	Mayerhoff et al. (1998)
Temperature	Fermentation	Rao et al. (2004)
Agitation rate	Fermentation	Mayerhoff et al. (1998), Carvalho et al. (2004b), Rao et al. (2004), Sampaio et al. (2006a)
Dissolved oxygen level	Fermentation	Mayerhoff et al. (1998)
Aeration rate	Fermentation	Carvalho et al. (2004b)
Support	Fermentation	Santos et al. (2005)
Xylitol concentration	Xylitol recovery	De Faveri et al. (2004b), Sampaio et al. (2006b)
Cooling temperature	Xylitol recovery	De Faveri et al. (2004a, b), Sampaio et al. (2006b)
Solvent content	Xylitol recovery	Hao et al. (2006)
Saturation temperature	Xylitol recovery	Hao et al. (2006), Martínez et al. (2007, 2008, 2009)
Cooling rate	Xylitol recovery	Martinez et al. (2007, 2008)

Table 6.2 Some examples of statistical optimization studies carried out for xylitol production

Statistical optimization method	Step of xylitol process	Factor studied	Range	Optimization condition	Did the factor influence the response?	Response	Microorganism	Reference
DOE	Fermentation of sugars from synthetic solution	NH_4NO_3	0–8 g/L	0	Yes	–	*Candida tenuis*	Kern et al. (1998)
PBD		concentration	4–8 g/L	8 g/L	Yes			
		Yeast extract	20–40 g/L	20 g/L	No			
		concentration	4–8 g/L	4 g/L	No			
		Xylose	0–1 mL/L	0	No			
		concentration	0–1 mL/L	0	No			
		Peptone	0–1 g/L	0	No			
		concentration						
		Vitamin solution						
		Trace element solution						
		KH_2PO_4 concentration						
DOE	Fermentation of sugars from rice straw hydrolysate	Aeration level	20–60 mL	60 mL	Yes	$Y_{P/S} = 0.71$ g/g	*Candida mogii*	Mayerhoff et al. (1998)
RSM		pH	4.5–7.0	6.2	Yes			
		Cell concentration	1–4 g/L		No	$Q_P = 0.46$ g/L h		
		Fermentation time	44–72 h		No			
DOE	Fermentation of sugars from rice straw hydrolysate	Xylose	48–132 g/L	82 g/L	Yes	$Y_{P/S} = 0.65$ g/g	*Candida guilliermondii*	Silva and Roberto (2001)
RSM		concentration	0.2–5.8 g/L	3 g/L	Yes			
		Inoculum level						
DOE	Fermentation of sugars from sugarcane bagasse hydrolysate	pH	0.01–0.05 L/h	0.038 L/h	Yes	$Q_P = 0.68$ g/L h	*Candida guilliermondii*	Martinez et al. (2003)
RSM		Dilution rate	4.0–7.0	6.7	Yes			

(continued)

Table 6.2 (continued)

Statistical optimization method	Step of xylitol process	Factor studied	Range	Optimization condition	Did the factor influence the response?	Response	Microorganism	Reference
DOE RSM	Conditioning of sugarcane bagasse hydrolysate	pH Temperature Xylose concentration Treatment with charcoal	0.92–9.50 43–80 °C 34.54–77.25 g/L After or before the vacuum evaporation	0.92–51 g/L Before	Yes No Yes Yes Yes	$Y_{P/S} = 0.54$ g/g	*Candida guilliermondii*	Rodrigues et al. (2003)
DOE	Pretreatment of sugarcane bagasse	H_3PO_4 concentration Temperature Residence time	70–130 mg/g 120–160 °C 20–60 min	70 mg/g 160 °C 60 min	No Yes Yes	Xyl = 17.1 g/L	–	Carvalho et al. (2004a)
DOE RSM	Conditioning and fermentation of sugarcane bagasse hydrolysate	Hydrolysate concentration Air flow rate Agitation speed Cell concentration pH	Three to fivefold 1.30–2.60 L/min 300–500 rpm 0.6–1.4 g/L 4.0–6.0	Fivefold 1.30 L/min 300 rpm 1.4 g/L 6.0	Yes Yes Yes Yes Yes	$Y_{P/S} = 0.81$ g/g $Q_P = 0.40$ g/L h	*Candida guilliermondii*	Carvalho et al. (2004b)
DOE RSM	Fermentation of sugars of rice straw hydrolysate	Starting xylose concentration Oxygen mass flowrate	50–150 g/L 2.5–5.9 mg_{O2}/s	50 g/L 4.2 mg_{O2}/s	Yes Yes	$Y_{P/S} = 0.73$ g/g $Q_P = 0.70$ g/L h	*Debaryomyces hansenii*	De Faveri et al. (2004a)
DOE RSM	Recovery of xylitol from synthetic solution	Xylitol concentration Cooling temperature	582–730 g/L −10 to 0 °C	728 g/L −6 °C	Yes Yes	$Y_c = 0.54$	–	De Faveri et al. (2004b)

(continued)

Table 6.2 (continued)

Statistical optimization method	Step of xylitol process	Factor studied	Range	Optimization condition	Did the factor influence the response?	Response	Microorganism	Reference
DOE TM	Fermentation of sugars from synthetic solution	Temperature pH Agitation Inoculum size Corn steep liquor Xylose concentration Yeast extract concentration KH_2PO_4 concentration	30–33 °C 4.5–5.5 200–300 rpm 6–10 (% v/v) 0.1–2 (% v/v) 2–5 (% w/v) 0.2–0.4 (% w/v) 0.3–0.7 (% w/v)	33 °C 4.5 250 rpm 8 (% v/v) 2 (% v/v) 2 (% w/v) 0.3 (% w/v) 0.5 (% w/v)	Yes No Yes No Yes Yes No Yes	$Y_{P/S} = 0.789$ g/g	*Candida* sp.	Rao et al. (2004)
DOE RSM	Conditioning and Fermentation of wheat straw hydrolysate	Ammonium sulfate concentration Calcium chloride concentration Rice bran extract pH Hydrolysate concentration	1–3 g/L 0–1 g/L 5–20 g/L 4.0–6.0 3–5-fold	1 g/L 0 5 g/L 6.0 threefold	No No Yes Yes Yes	$Y_{P/S} = 0.49$ g/g $Q_P = 0.34$ g/L h	*Candida guilliermondii*	Canilha et al. (2005)
DOE	Pretreatment of sugarcane bagasse	Solid:liquid ratio H_2SO_4 concentration Pressure Time	1:6–1:9 1–7 (%) 0.5–1.5 atm 20–40 min	1:4 3 % 1 atm 40 min	Yes Yes Yes Yes	Xyl = 57.25 g/L	–	Fogel et al. (2005)
DOE	Fermentation of sugarcane bagasse hydrolysate	Treatment of support Average diameter Amount of support (bagasse)	Untreated or treated 0.559–1.080 mm 0.5–1.5 g	Treated 1.080 mm o.5 g	Yes No Yes	$Y_{P/S} = 0.59$ g/g $Q_P = 0.42$ g/L h	*Candida guilliermondii*	Santos et al. (2005)

(continued)

Table 6.2 (continued)

Statistical optimization method	Step of xylitol process	Factor studied	Range	Optimization condition	Did the factor influence the response?	Response	Microorganism	Reference
OVT	Recovery of xylitol from synthetic solution	Solvent content (metanol) Temperature	0–66.7 % 20–55 °C	–	–	–	–	Hao et al. (2006)
DOE RSM	Fermentation of sugars from synthetic solution	Xylose concentration Rotational speed Biomass concentration	55–220 g/L 100–400 rpm 1.4–6.4 g/L	156 g/L 280 rpm 6.4 g/L	Yes Yes Yes	$Y_{P/S} = 0.77$ g/g $Q_P = 1.49$ g/L h	*Debaryomyces hansenii*	Sampaio et al. (2006a)
OVT	Conditioning of synthetic solution and recovery of xylitol	Active charcoal concentration Xylitol concentration Cooling temperature Xylose concentration	5–200 g/L 675–911 g/L −10 to 15 °C Presence or absence	20 g/L 911 g/L −10 °C Presence	–	$Y_c = 0.42$	–	Sampaio et al. (2006b)
OVT	Conditioning and Fermentation of *Eucalyptus* hydrolysate	Active charcoal concentration Time using charcoal Type of treatment pH using resins Flow rate using resins	1 and 5 % 30 and 60 min charcoal or resins 1.8 and 5.5 6 and 10 mL/min	Resins pH = 1.8 10 mL/min	–	$Y_{P/S} = 0.57$ g/g $Q_P = 0.68$ g/L h	*Candida guilliermondii*	Villarreal et al. (2006)

(continued)

Table 6.2 (continued)

Statistical optimization method	Step of xylitol process	Factor studied	Range	Optimization condition	Did the factor influence the response?	Response	Microorganism	Reference
OVT	Pretreatment of rice straw	H_2SO_4 concentration Temperature Residence time Residue/acid solution	0.5–2 % 120–130 °C 10–60 min 1:6–1:10	1.5 % 130 °C 20 min 1:10	–	Xyl = 17.4 g/L	–	Baek and Kwon (2007)
DOE RSM	Pretreatment of *Eucalyptus grandis*	H_2SO_4 concentration Temperature Residue/acid solution	0.18–0.82 % 147–173 °C 1:3.4–1:8.6	0.65 % 157 °C 1:8.6	Yes Yes Yes	Xyl = 13.65 g/L	–	Canettieri et al. (2007)
OVT	Recovery of xylitol from sugarcane bagasse fermented broth	Saturation temperature Cooling rates	30–50 °C 0.10–0.50 °C/min	–	–	–	–	Martinez et al. (2007)
NM	Recovery of xylitol from synthetic solution	Saturation temperature Cooling rates	40–60 °C 0.10–0.50 °C/min	–	–	–	–	Martinez et al. (2008)
DOE RSM	Conditioning and Fermentation of brewer's spent grain hydrolysate	Xylose concentration Calcium chloride concentration Ammonium sulfate concentration Rice bran extract concentration	55–95 g/L Presence or absence Presence or absence Presence or absence	70 g/L Absence Absence Absence	Yes No No No	$Y_{P/S}$ = 0.78 g/g Q_P = 0.58 g/L h	*C. guilliermondii*	Mussatto and Roberto (2008)

(continued)

Table 6.2 (continued)

Statistical optimization method	Step of xylitol process	Factor studied	Range	Optimization condition	Did the factor influence the response?	Response	Microorganism	Reference
DOE RSM	Pretreatment of sugarcane bagasse	H_2SO_4 concentration Reaction time Temperature	0.06–0.34 % 1–22 min 170 e 200 °C	0.24 % 15 min 170 °C	Yes Yes Yes	$Y_{xyl} = 78.9$ %	–	Um and Bae (2011)
DOE RSM	Pretreatment of sugarcane leaves	Residence time Temperature H_2SO_4 concentration Residue/acid solution	10–60 min 110–130 °C 0.5–3.5 % 1:2–1:10	30 min 130 °C 2.9 % 1:4	No Yes Yes No	Xyl = 56.5 g/L $Y_{xyl} = 85.09$ %	–	Moutta et al. (2012)
DOE RSM	Pretreatment of corn stover	Diethyoxalate concentration Corn stover moisture	0–4 % 12–60 %	4 % 60 %	Yes Yes	$Y_{xyl} = 81.2$ %	–	Rodrigues et al. (2012)

DOE design of experiment, *NM* Nyvlt's methodology, *OVT* one variable-at-a-time method, *PBD* Plackett-Burman design, *RSM* response surface methodology, *TM* Taguchi methodology, *Xyl* xylose concentration, Y_c xylitol crystallization yield, $Y_{P/S}$ xylose-to-xylitol bioconversion yield, Y_{xyl} maximum yield for xylose production, Q_P volumetric productivity

pretreatment methods often result in formation of less toxic hydrolysate, they also may result in reduced hydrolysis of hemicellulose to monosaccharides, requiring the use of additional enzymes during the saccharification process, increasing the overall process costs (Pienkos and Zhang 2009). In planning acid hydrolysis conditions, the optimization through central composite design (CCD) and RSM is a common practice (Fogel et al. 2005; Baek and Kwon 2007; Um and Bae 2011; Moutta et al. 2012; Rodrigues et al. 2012). RSM is suitable for multiple factor experiments, sensitive to relationships between factors, and the ability to not only find the most suitable reaction conditions but to also forecast the response (Um and Bae 2011).

Carvalho et al. (2004a) evaluated the effect of temperature, phosphoric acid concentration and residence time on the hydrolysis of sugarcane bagasse hemicellulosic hydrolysate. Assays were carried out in a 25-dm^3 reactor, using a solid-to-liquid ratio of 1:10 (w/v). A 2^3 full factorial design was used to optimize the xylose concentration variable. A statistical analysis of the results showed that the temperature and residence time had significant effects on response variable. The experimental maximum xylose concentration (17.1 g/dm^3) was attained when the bagasse was treated at 160 °C for 60 min, using 70 mg of phosphoric acid per gram of dry bagasse.

Fogel et al. (2005) optimized the dilute-acid hydrolysis process of sugarcane bagasse in an autoclave. The assays were carried out in a three-stage statistical experimental design, where the first two stages consisted of a 2^4-factorial design with five replications of the center point and the third stage of a 3^2 full factorial design with three replications of the two best results. The maximum xylose concentration (57.25 g/L) was obtained using 1:4 solid: liquid ratio, 3 % sulfuric acid, a pressure of 1 atm and 40 min of residence time.

Canettieri et al. (2007) studied the optimization of acid hydrolysis process in a 1.4 L pilot-scale reactor and investigated the effects of the acid concentration, temperature and residue/acid solution ratio on the hemicellulose removal and consequently on the production of sugars (xylose, glucose and arabinose) as well as on the formation of by-products (furfural, 5-hydroxymethylfurfural and acetic acid). This study was based on a model composition corresponding to a 2^3 orthogonal factorial design and employed the RSM to optimize the hydrolysis conditions, aiming to attain maximum xylose extraction from hemicellulose of residue. The considered optimum conditions were: 0.65 % H_2SO_4 concentration, temperature of 157 °C and residue/acid solution ratio of 1:8.6 with a 20 min reaction time. Under these conditions, 79.6 % of the total xylose was removed, and the hydrolysate contained 1.65 g/L glucose, 13.65 g/L xylose, 1.55 g/L arabinose, 3.10 g/L acetic acid, 1.23 g/L furfural and 0.20 g/L HMF.

Um and Bae (2011) optimized the temperature, the sulfuric acid concentration and the residence time to release xylose from the sugarcane bagasse in sealed tubular reactors (volume of 30 cm^3). By using design of experiment (DOE) and RSM, these authors obtained a maximum xylose yield of 78.9 % at 170 °C, 0.24 % acid, for 15 min.

Moutta et al. (2012) obtained a maximum xylose recovered (85.1 %) from the hemicellulosic fraction of sugarcane leaves, which consists of 30.79 % hemicelluloses, 40.84 % cellulose and 25.80 % lignin on dry solid basis, evaluating different conditions using dilute sulfuric acid. Acid hydrolysis experiments were performed according to an experimental design considering the following factors: time, temperature, concentration of the acid solution and solid: liquid ratio. Analysis of variance was performed and it was observed that only acid solution concentration and temperature were significant at 95 % confidence. The optimum conditions found were 130 °C, 2.9 % w/v concentration of sulfuric acid solution, solid: liquid ratio (1:4) and 30 min of residence time.

Recently Rodrigues et al. (2012) using a RSM based on a 2^2 full factorial design, evaluated the moisture effects in recovering xylose by diethyloxalate (DEO) hydrolysis from corn stover. Experimentally, a maximum xylose recovery (81.2 %) was achieved using initial corn stover moisture of 60 % and a DEO concentration of 4 % (w/w). The mathematical statistical model showed that xylose recovery increases during DEO corn stover acid hydrolysis as the corn stover moisture level increases. The corn stover moisture was an important variable to improve xylose recovery by DEO acid hydrolysis.

6.5 Conditioning and Fermentation of Hemicellulosic Hydrolysates

Pretreatment of biomass plays a critical role in producing materials with acceptable enzymatic digestibility and subsequent fermentability for the production of xylitol or other advanced biofuels such as ethanol and butanol also derived from biomass. The various pretreatment processes, in addition to generating suitable substrates for conversion to biofuel, typically produce a range of compounds that inhibit the organisms used for fermentation. These inhibitory components include carboxylic acids, primarily acetic acid, the sugar degradation products furfural and HMF, phenolic compounds, and inorganic salts (Klinke et al. 2004). These components have been shown to inhibit the growth of the fermentation organisms thereby reducing the rate of xylitol production and in some cases, the overall the yield.

Conditioning refers to methods developed to treat hydrolysate to reduce toxicity and make the sugars more accessible to fermentation. In general it would be more advantageous in terms of costs savings to modify the pretreatment process to reduce the formation of toxic compounds rather than add the additional cost of a conditioning step, as less toxic pretreatment methods typically release less sugar for fermentations and therefore require additional hydrolytic enzymes (Pienkos and Zhang 2009).

According to Banerjee et al. (1981), furfural and HMF inhibit glycolysis, especially interfering with the activity of dehydrogenases, causing a reduction in growth rates and cell yields. Phenolics partition into membranes and lead to loss of

integrity, interfering with cell growth and sugar transport (Heipieper et al. 1994). Acids disrupt cellular energy generation by collapsing pH gradients especially at low pH. The relative toxicity is a function of hydrophobicity because this characteristic determines the ability of the compound to pass through the membrane (Zaldivar and Ingram 1999). Aldehyde toxicity is also related to hydrophobicity, but aldehydes do not disrupt membrane integrity or cause a collapse of pH gradient (Zaldivar et al. 1999). Alcohols are generally less toxic than related acids or aldehydes, but their toxicity is also related to hydrophobicity. They appear to cause a breakdown in membrane structure (Zaldivar and Ingram 2000).

Although these toxic materials can be detoxified with, e.g. laccase enzyme, inhibition of the phenolic compounds is limited due to their low solubility in water (Pamqvist and Hahn-Hägerdal 2000; Huang et al. 2011). The furans are probably the most important group of inhibitors, since the fermentability of dilute-acid hydrolyzates is inversely related to the concentration of these compounds (Taherzadeh et al. 1997). Furfural was suggested to be transformed to furfuryl alcohol and furoic acid by using NADH and NAD+, respectively (Taherzadeh et al. 1997). The reduction and oxidation are catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase.

Conditioning of lignocellulosic hydrolyzates with alkali has frequently been employed as a detoxification method to improve the fermentability. This process is mainly used to convert furan derivatives into other, less toxic compounds. Treatment with $Ca(OH)_2$ (overliming) seems to be a good alkali treatment for hydrolyzate detoxification. In this method, Cao or $Ca(OH)_2$ is added to hydrolyzates to increase the pH (up to 7–12) and keep this condition for a period of time (from 15 min up to several days), followed by decreasing the pH to 5 or 5.5 (Alves et al. 1998; Canettieri et al. 2001; Canilha et al. 2004; Villarreal et al. 2006) and recommended doing overliming at elevated temperature in order to avoid high pH, where less lime and acid are used. According to Rodrigues et al. (2003), the composition of the hydrolyzate depends on the vacuum evaporation process variables (pH, temperature, xylose concentration degree and treatment with activated charcoal before or after the process). The influence of these variables on xylitol volumetric productivity by the yeast *Candida guilliermondi* FTI 20037 was measured using a 2^{5-1} fractional factorial CCD followed by a RSM. The model predicted a xylitol volumetric productivity of 0.350 g/L h in hydrolyzate treated with charcoal (pH 0.92) and subsequently evaporated under vacuum to reach a xylose concentration of 51.0 g/L. Under these conditions, the xylose, acetic acid and arabinose consumption levels were 93, 57 and 13.62 %, respectively, and xylitol yield and production were 0.54 g/g and 24.33 g/L, respectively. The final cell concentration was fivefold the initial value (0.278×10^8 cell/mL). The temperature levels (43–80 °C) tested during vacuum evaporation did not influence xylitol production significantly.

The ion-exchange resins are also used to treat the hemicellulosic hydrolysates (Canilha et al. 2004, 2008b; Villarreal et al. 2006; Martínez et al. 2007). Villarreal et al. (2006) studied the influence of detoxification methods (active charcoal or sequence of four different resins) on the removal of sugars and toxic compounds

of *eucalyptus* residue hydrolysate for xylitol production by *Candida guilliermondii* FTI 20037. Ion exchange resins were more efficient than active charcoal adsorption to remove the inhibitory compounds without sugars loss. The resins were used in the following sequence: (1) cation exchanger type Applexion in H^+ form, (2) anion exchanger type A-860S in Cl^- form, (3) cation exchanger type C-150 in H^+ form, and (4) anion exchanger type Applexion in OH^- form. By using the OVT, these authors obtained 32.7 g/L of xylitol after 48 h fermentation, which corresponds to 0.68 g/L h volumetric productivity and a 0.57 g/g xylitol yield factor.

The optimization through CCD and RSM is also common practice in the fermentation process. Mayerhoff et al. (1998) used a multifactorial experimental design to evaluate the influence of aeration level, initial pH, initial cell concentration and fermentation time on the xylitol production from rice straw hemicellulose hydrolysate by *Candida mogii*. A statistical analysis of the results showed that the aeration level and the initial pH had significant effects on yield factor, volumetric productivity, and xylose consumption. For the latter, fermentation time was also a significant variable. Based on the RSM, models for the range investigated were proposed. The maximum values for the yield factor ($Y_{p/s}$) and volumetric productivity (Q_p) were, respectively, 0.71 g/g and 0.46 g/L h.

Rodrigues et al. (1998) evaluated xylitol production by *C. guilliermondii* FTI 20037 from sugar cane bagasse hydrolyzate by the fed-batch process using exponential feeding rate. A factorial design was employed to study the effects of the xylose concentration in the fermenter (S_0) and the xylose concentration in the feeding solution (S_i) upon the xylitol/xylose yield coefficient and on the xylitol concentration. It was found that none of the variables had a significant effect on the xylose-to-xylitol conversion factor. However, their effects on the xylitol concentration were significant. A RSM permitted the authors to obtain a polynomial model for xylitol production (C_P) at 95 % confidence level: $C_P = 15.896 - 1410\ S_0 - 0.487\ S_i - 0.032\ S_0^2$. The best experimental results achieved for xylitol production was 44 g/L, with a xylitol yield coefficient of 0.78 g/g and a volumetric productivity of 0.62 g/L h.

Silva and Roberto (2001), using RSM, combined effects of initial xylose concentration and inoculum level on xylitol production by *C. guilliermondii* in rice straw hydrolysate. A 2^2 full-factorial CCD was employed for experimental design and analysis of the results. The optimum xylose concentration and inoculum level were found to be 82 and 3 g/L, respectively. In these conditions, xylitol concentration of 52 g/L with a production rate of 0.54 g/L and a yield factor of 0.65 g/g was attained.

Martinez et al. (2003) using a 2^2 full-factorial CCD studied the effects of pH and dilution rate (D) on continuous fermentation of sugarcane bagasse hemicellulosic hydrolysate by *C. guilliermondii*. From the response surface and contour lines described by the model it was verified that productivity value is high (0.72 g/L h) when a pH of 6.7 and D = 0.038 L/h were used. A volumetric productivity of 0.68 g/L h, representing 95.8 % of the predicted value was obtained. The oxygen availability (k_La) effect on the volumetric productivity was not significant.

Carvalho et al. (2004b) used a screening design and RSM to determine adequate cultivation conditions for entrapped cells of *C. guilliermondii* FTI 20037 in Ca-alginate beads for xylitol production from sugarcane bagasse hemicellulosic hydrolysate in a stirred tank reactor. Quadratic models were fitted to the experimental data by regression analysis, considering the yield ($Y_{P/S}$) and the productivity (Q_P) of the xylose-to-xylitol bioconversion as dependent variables. The best results (xylitol production of 47.5 g/L after 120 h of fermentation, $Y_{P/S}$ of 0.81 g/g and Q_P of 0.40 g/L h) were obtained using a five-fold concentrated hydrolysate, air flowrate of 1.30 L/min, agitation speed of 300 rpm, initial cell concentration of 1.4 g/L and value 6.0 for the initial pH of the fermentation medium.

De Faveri et al. (2004a) using a 3^2 full-factorial design combined with RSM investigated the simultaneous effects of initial xylose concentration (S_0) and oxygen mass flowrate (qO_2) on xylitol production from rice straw hydrolyzate. Fermentations were performed at 30 °C, using *Debaryomyces hansenii* NRRL Y-7426 as xylitol producer and varying S_0 between 50 and 150 g/L and qO_2 between 2.5 and 5.9 mg O_2/s. At the lowest starting xylose concentration and an oxygen mass flowrate of 4.2 mg O_2/s, volumetric productivity and xylitol yield reached maximum values ($Q_p = 0.70$ g/L h and $Y_{P/S} = 0.73$ g/g, respectively), but xylitol concentration was quite low (35.9 g/L). The results were in close agreement with the model prediction. The statistical model also allowed identifying the optimum operating conditions ($S_0 = 71$ g/L and $qO_2 = 4.1$ mg O_2/s) able to simultaneously maximize volumetric productivity (0.53 g/L h), xylitol yield (0.71 g/g) and final xylitol concentration (42.2 g/L).

Canilha et al. (2005) evaluated the influence of the medium composition (hydrolysate concentration, supplementation with ammonium sulfate, calcium chloride and rice bran extract, and initial pH) on xylitol production from wheat straw hemicellulosic hydrolysate using *C. guilliermondii* FTI 20037. A 2^{5-1} factorial design followed by RSM was used to optimize the xylitol production (P), bioconversion yield ($Y_{P/S}$) and volumetric productivity (Q_p). The statistical analysis showed that neither the initial concentration of ammonium sulfate nor of calcium chloride influences the response variables. The maximum values for P, $Y_{P/S}$ and Q_p (24.17 g/L, 0.49 g/g and 0.34 g/L h, respectively) were obtained using a threefold concentrated hydrolysate, supplemented with 1 g/L ammonium sulfate, 5 g/L rice bran extract, and initial pH adjusted to 6.0.

Santos et al. (2005) tested the yeast *C. guilliermondii* immobilized in sugarcane bagasse to xylose-to-xylitol bioconversion using a factorial design, of which the independent variables were treatment, average diameter, and amount of bagasse used as support for cell immobilization. The authors found that by increasing the amount of support, the xylitol yield decreased, whereas the biomass yield increased. The diameter of the support did not influence xylitol production, and treatment of the bagasse with hexamethylene diamine prior to fermentation resulted in the highest amount of immobilized cells.

Sampaio et al. (2006a) carried out two sets of batch bioconversion tests on synthetic medium by using two types of full factorial designs (3^3 and 3^2) to select the initial xylose concentration (S_0), rotational speed (v) and starting biomass

concentration (X_0) as independent variables and the maximum xylitol concentration (P), xylitol yield on consumed xylose ($Y_{P/S}$), volumetric productivity (Q_P) and specific productivity (q_P) as response variables. They also performed a RSM and overlapped the responses curves under investigation. The point chosen as representative of this optimal area corresponded to $S_0 = 156$ g/L, $v = 280$ rpm and $X_0 = 6.4$ g/L, conditions under which the model predicted $P = 116.25$ g/L, $Y_{P/S} = 0.77$ g/g, $Q_P = 1.49$ g/L h and $q_P = 0.16$ g/g h.

Mayerhoff et al. (2006) used a central composite experimental design leading to a set of 16 experiments with different combinations of pH and temperature to attain the optimal activities of xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes from *Candida mogii* cell extract. Under optimized conditions (pH 6.5 and 38 °C), the XR and XDH activities were found to be 0.48 U/mL and 0.22 U/mL, respectively, resulting in an XR to XDH ratio of 2.2. XR enzyme is responsible for the first step in the xylose metabolism of yeasts. In a reaction catalyzed by this enzyme, xylose is reduced to xylitol that can be oxidized into xylulose by the XDH enzyme or released to the environment, depending on the culture conditions and on the microorganism utilized (Kern et al. 1997; Ho et al. 1990; Bolen et al. 1986). Xylitol is a sugar–alcohol of economic interest due to its sweetening power and its dietetic and anticariogenic properties (Hyvönen and Koivistoinen 1982). Studies on extraction and purification of XR from yeast cells are being conducted aimed at either characterizing the enzyme to improve the fermentative process or obtaining a purified XR solution for direct use in enzymatic conversion of xylose into xylitol (Mayerhoff et al. 2001; Cortez et al. 2001).

Mussatto and Roberto (2008) using a 2^2 full-factorial CCD studied the effects of initial xylose concentration and nutritional supplementation of Brewer's spent grain hydrolysate on xylitol production by *C. guilliermondii*. By using RSM, the best conditions for maximum xylitol production were found, using the non-supplemented hydrolysate containing 70 g/L initial xylose concentration. Under these conditions, a xylitol yield of 0.78 g/g and productivity of 0.58 g/L h were achieved.

Other authors used different methodology to optimized medium to xylitol production in association with full factorial design, such as Kern et al. (1998) who investigated the effects of different medium components on growth of the D-xylose-fermenting yeast *Candida tenuis* CBS 4435 as well as on formation of aldose reductase (ALR, XR) and XDH. By using the Plackett-Burman statistical screening method, important effects for the two N sources yeast extract and NH_4NO_3 were found, while a change in the concentration of the other medium components (D-xylose, peptone from casein, a vitamin solution, a trace element solution, KH_2PO_4) neither significantly affected growth nor enzyme synthesis in *C. tenuis*. The importance of these two N sources was confirmed by subsequent shaken flask experiments planned according to a full factorial design and by bioreactor cultivations. Yeast extract was identified as important for growth as well as for the formation of ALR and XDH. Ammonium (NH_4Cl) was also shown to have a stimulatory effect on the levels of these two enzymes formed in *C. tenuis*, whereas it reduced growth when present in the medium in a concentration of 100 mM.

Many Japanese manufacturers have used the Taguchi approach and improved product and process qualities with unprecedented success. It created significant changes in several industrial organizations in the USA (Rajesham and Cheong 1989; Rajesham et al. 1997; Roy 1990). Rao et al. (2004) studied the optimization of parameters on xylitol production using a newly isolated *Candida* sp by using a Test plan L18, available in the form of an orthogonal array and software for automatic design and analysis of the experiments, both based on the Taguchi approach. Optimal levels of physical parameters and key media components, namely, temperature, pH, agitation, inoculum size, corn steep liquor (CSL), xylose, yeast extract and KH_2PO_4 were determined. Among the physical parameters, temperature and agitation contribute higher influence. Media components CSL, xylose concentration and KH_2PO_4 play an important role in the conversion of xylose to xylitol. The yield of xylitol under these optimal conditions was 78.9 %.

6.6 Xylitol Recovery by Crystallization

Crystallization is a separation process where a solid phase is created from a liquid phase. To induce crystallization from a solution, a drive force is needed. Very soluble substances in solution can be crystallized by either solvent evaporation, to increase the solute concentration, or cooling, to decrease the solute solubility (Nývlt et al. 2001; Martínez et al. 2007). The choice of a crystallization method (cooling, evaporation, precipitation and salting-out) is dependent on the solubility and saturation slopes with the temperature. First, there is a metastable zone where excess solute of the equilibrium concentration is deposited on existing crystals but no new crystal nucleus is formed. Second, there is an intermediate zone where both crystals growth and nucleation occur simultaneously. Third, there is a labile zone where nuclei are formed spontaneously from a clear solution. These three zones are controlled not only by equilibrium, but also by process parameters like agitation, temperature, solution purity and cooling rate. In order to obtain a good product, the cooling rate must maintain the supersaturation inside the metastable zone, thus minimizing the formation of new crystals (Nývlt et al. 2001).

There are few works in the literature about xylitol crystallization using statistical approaches. De Faveri et al. (2004b) used RSM to investigate the effects of initial xylitol supersaturation value (Xyt_s) and cooling temperature (T_c) on xylitol crystallization from synthetic solutions. These authors used a 3^2 full-factorial design for experimental design. By means of response surface analysis, the statistical model identified the operating conditions ($Xyt_s = 728$ g/L and $T_c = -6.0$ °C) under which purity degree (0.97) and xylitol crystallization yield (0.54) were simultaneously optimized. On the other hand, a purity degree close to 1.0 to meet industrial standards is expected at $Xyt_s = 583$ g/L and $T_c = -2.4$ °C, but the crystallization yield would be unsatisfactory (0.25).

Martínez et al. (2008) used the Nyvlt's methodology (NM) coupled with models experiments to determine the kinetic parameters of batch cooling crystallization of

xylitol (52.98, 63.59 and 73.45 g/100 $g_{solution}$) in water–ethanol (50:50 %, w/w) solutions. Models experiments were carried in order to verify the combined effects of saturation temperatures (40, 50 and 60 °C) and cooling rates (0.10, 0.25 and 0.50 °C/min) on the kinetics exponents (apparent order of nucleation $n = 2.44$, order of overall crystal growth kinetics $g = 2.44$, and apparent order of nucleation $m = 3.44$) and the system kinetic constant ($B_N = 4.76 \times 10^{-18}$ kg/$kg_{solvent}$). According to these authors, these parameters made calculation of the mean crystal size possible so that they were able to compare it with experimental values. The fitting between experimental and calculated crystal sizes has 11.3 % mean deviation.

The traditional approach to the optimization problem using the OVT (Geiger 1997) is more used in the literature for xylitol crystallization. Hao et al. (2006) studied the effect of methanol on crystallization kinetics of xylitol in a batch operated crystallizer and observed that the methanol content had an apparent effect on nucleation and growth rates of xylitol. These authors reported lower values of growth rate ($0.02–5 \times 10^{-9}$ m/s) and higher values of nucleation rate ($0.01–9.7 \times 10^{9}$ m^{-3}/s) in the crystallization of xylitol in a methanol–water system.

Sampaio et al. (2006b), in a study on isothermal crystallization of xylitol from synthetic broth by *D. hansenii*, reported that xylitol crystals with purities ranging from 96 to 97.8 % were produced using xylitol concentrations ranging from 675 to 911 g/L at −10 °C.

Martínez et al. (2007), after the first crystallization of rich-xylitol syrup (935.4 g/L xylitol and 13.1 g/L arabinose) from fermentation of sugarcane bagasse hemicellulosic hydrolysate by *C. guilliermondii*, obtained crystals with properties inferior to those of the commercial xylitol. These crystals were used in the second crystallization step, preparing water–ethanol solutions (50–50 %, w/w) in concentrations of 42.63, 52.98 and 63.59 g corresponding to the saturation temperatures 30, 40 and 50 °C, respectively. The tests were carried out using 0.1, 0.25 and 0.50 °C/min cooling rates in each saturation temperature. In the second crystallization step, an increase of the crystals purity from 85 % to 91.20–94.85 % was reported.

According to Martínez et al. (2009), the fermented broth from semi-synthetic medium using *C. guilliermondii* (61.3 g/L xylitol) was centrifuged, treated and concentrated obtaining a syrup (745.3 g/L xylitol) which was crystallizated twice. The first crystallization of xylitol-rich syrup with supersaturation degree equal to 1.17 was carried out using 50 °C saturation temperature, and in the second crystallization stage, the experiments were carried out using xylitol crystals (95 % purity) in the following conditions: saturation temperatures of 30, 40 and 50 C, and cooling rate of 0.25 °C/min and seeding with 0.1 % xylitol crystal. Xylitol crystals with 98.5–99.2 % purity were obtained. The saturation temperature corresponding to 30 °C promoted a higher nucleation rate (4.58×10^{7} m^{-3}/s) and the process was carried out with longer crystallization time (3982.8 s). On the other hand, a higher crystal growth rate (5.15×10^{-8} m/s) and crystal mean size (93.975×10^{-4} m) were obtained at 40 °C saturation temperature.

6.7 Conclusions and Future Recommendations

Statistics is a fundamental tool in the analysis of any process data where there is variability. There are many ways to approach the problem of optimization and design of a fermentation process, which can be handled quickly using a number of statistical techniques. Statistical DOE is a mechanism of data collection appropriate to study the biotechnological xylitol production. Several xylitol fermentation processes have been optimized using RSM. However, one of the major problems to the researcher is identifying the independent variables that influence a study in order to explain the model which best represents the process. The statistical approach for biotechnological xylitol production from lignocellulosic materials could be also helpful to optimize pretreatment of lignocellulosic biomass, conditioning of hemicellulosic hydrolysates and xylitol recovery from fermented hydrolysates. However, an effort needs to be done to improve xylitol yield and productivity aiming at industrial xylitol production. In this case, others techniques such as molecular biology and analysis of cost could be integrated into the statistical design of the experiments. Also, a scaled-up xylitol production needs to be studied more carefully associated with improvement in pretreatment of lignocellulosic biomass, conditioning of hemicellulosic hydrolysates and xylitol recovery from fermented hydrolysates.

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Chapter 7
Fermentation Strategies Explored for Xylitol Production

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Abstract This chapter reviews different fermentation strategies for xylitol production using synthetic media or hemicellulosic hydrolyzates as carbon source. Most of the published works were carried out with free cells in batch operation because of its versatility and easy use in preliminary tests, where the age of inoculum, cell recycling, initial cell concentration, pH, temperature, type and concentration of nutrients in the culture medium, initial xylose concentration, presence of carbon sources other than xylose, and dissolved oxygen level were selected as the main variables. Conversely, continuous fermentation systems were shown to offer additional advantages such as high productivity for long periods of time, elimination of idle time for cleaning and sterilization and simplicity to perform an automated control. The most attractive equipment employed for this purpose included the continuous flow stirred tank, crossflow membrane and submerged membrane bioreactors. The use of cells immobilized by adsorption, entrapment, or covalent binding showed several advantages compared to the free ones, including higher cell density and possible biomass recycling for continuous operation. Repeated-batch fermentations were also investigated to evaluate the durability of immobilized cells with the aim of implementing the technology into a continuous process or scaling up the conversion of xylose to xylitol. Seeking long-term stability, the packed bed and fluidized bed bioreactors proved to be the most

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effective equipment; however, their hydrodynamic characteristics and the influence of aeration rate on fermentation performance still deserve further efforts. Finally, the fed-batch process, mainly with free cells, was also reported as an effective tool to keep the substrate at a suitable level throughout the whole fermentation process.

Keywords Batch systems · Continuous fermentation · Fed-batch systems · Immobilization · Packed bed reactors · Fluidized bed reactors

7.1 Introduction

In the last two decades, considerable efforts have been addressed to produce xylitol by biotechnological means, in spite of the fact that xylitol had been discovered and isolated in 1890 by the German chemist Fischer from beech wood, and produced for the first time in 1930 as an unstable form of xylitol by reduction of purified D-xylose (Mäkinen 2000). These discoveries set up the basis to produce primarily xylitol by microorganisms. Initially, the most outstanding papers were focused on screening of suitable microorganisms and optimum operating conditions (aeration, pH, temperature, age of inoculum, etc.) in batch fermentation. Advances in the applications of xylitol, increased demand of consumers for natural additives and awareness of environmental protection by reducing the release of residues have been stimulating xylitol production by using continuous or fed-batch processes either in traditional or novel systems. Therefore, the aim of this chapter is to review the most outstanding technologies available in literature on the microbial production of xylitol.

7.2 Production Technologies

Due to the shortage of sugar during the Second World War, Finland, Japan, Germany and the Soviet Union promoted research on the production of xylitol, which almost ceased after the war. In 1975 the Finnish company Oy began the xylitol production on a large scale, and in 2004, after several associations and incorporations, with the name of Danisco (Denmark), it accounted for about 95 % of worldwide xylitol production, amounting to 30,000 tons per year (Tran et al. 2004). The rest of production was distributed among International Sells Cerestar (Belgium), Roquette Frères (France) and Hebei Baoshuo Pipe Group Company (China), with Japan being the largest importer (about 10,000 tons per year) (Tran et al. 2004). In 1997, the consumption of xylitol in the European Union was estimated at 1,000 tons.

The scarce production of xylitol is due to its cost of production by chemical means (6.39 €/kg), which is ten times higher than those of other traditional sweeteners (51,000 tons per year) or polyols (75,000 tons per year) (Tran et al. 2004).

Xylitol can be obtained by direct extraction from fruits and vegetables as lettuce, cauliflower, yellow plums, strawberries or raspberries. However, this method is not profitable owing to the small concentrations at which it appears (less than 9 g/kg) (Hyvonen et al. 1982; Pepper and Olinger 1988). Alternatively, xylitol can be produced by chemical synthesis or by fermentation (Saha 2003; Winkelhausen and Kuzmanova 1998).

7.2.1 Chemical Synthesis

Nowadays xylitol is produced commercially by chemical synthesis from xylose. This process implies a prehydrolysis stage of lignocellulosic materials (LCM) rich in xylans (Mikkola et al. 1999; Nigam and Singh 1995). Hydrolyzates are concentrated and subject to physico-chemical treatments (ion exchange, discoloration and chromatography) to obtain solutions of pure xylose, a prerequisite for subsequent hydrogenation (Baudel et al. 2005; Winkelhausen and Kuzmanova 1998). This is carried out in the presence of catalysts such as nickel, palladium or ruthenium (Mikkola et al. 2000) at 80–140 °C and 50 atm, because the presence of other sugars generates unwanted byproducts (arabitol, ribitol, mannitol, sorbitol among others). The resulting solution is concentrated and xylitol is recovered by crystallization, yielding a product with a purity of 99.7 % and a yield of 50–60 % with respect to the initial xylose (Nigam and Singh 1995). The production costs are high due to the complexity of the purification steps needed to remove byproducts from hemicellulose hydrolyzates, low yields and difficult purification of xylitol (Choi et al. 2000).

Alternatively, xylitol can be obtained from substrates other than xylose such as 2-keto-gulonic acid, L-sorbose, glucuronic acid, L-xylonic acid, L-xylulose, L-gulonic acid and L-idonic acid, which are transformed into L-xylose or other compounds, whose hydrogenation leads to xylitol although with low yields (Heikkilä et al. 2003).

7.2.2 Fermentation

Xylitol present in fermentative media was considered for years as an unwanted byproduct obtained during D-xylose fermentation to ethanol (Perego et al. 1990); however, subsequent works showed that xylitol could be obtained with promising conversion yields and productivities (Parajó et al. 1998a, b, c). Xylitol production by fermentation of xylose has certain advantages over chemical means, as it takes

place under milder conditions of pressure and temperature and releases lower amounts of by-products (Nigam and Singh 1995; Saha 2003; Tran et al. 2004; Winkelhausen and Kuzmanova 1998). Several studies have been addressed to the selection of new microorganisms able to overproduce xylitol from commercial xylose, whereas in many others hydrolyzates of LCMs with high content of xylan were employed to produce xylitol by fermentation using bacteria, yeasts or fungi (Parajó et al. 1998a; Saha 2003; van Zyl et al. 1999).

7.2.2.1 Fermentation by Bacteria and Filamentous Fungi

Literature reports scarce information on xylitol production by bacteria, being mostly related to the use of xylose or xylulose solutions. The bacteria capable of producing xylitol are *Corynebacterium* sp. (Rangaswamy and Agblevor 2002; Yoshitake et al. 1973), *Enterobacter liquefaciens* (Yoshitake et al. 1976), *Cellulomonas cellulans*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Serratia marcescens* (Rangaswamy and Agblevor 2002), *Bacillus coagulans* and *Mycobacterium smegmatis* (Izumori and Tuzaki 1988). Although in some cases some interesting yields were obtained, the final concentrations of xylitol were very low. The same can be said for filamentous fungi such as *Fusarium oxysporum* (Suihko 1984), *Petromyces albertensis* (Dahiya 1991), *Penicillium roqueforti*, *Penicillium crustosum*, *Penicillium brevicompactum, Penicillium crysogenum, Penicillium purpurogenum, Penicillium citrinum, Penicillium janthinellum, Penicillium griseorolsum, Penicillium expansum*, *Penicillium italicum* and *Aspergillus niger* (Sampaio et al. 2003).

Alternatively, xylitol production by genetically-modified bacteria carrying the genes responsible for xylitol production in yeasts has also been investigated (Akinterinwa and Cirino 2009; Povelainen and Myasnikov Povelainen and Miasnikov 2007); however, this process appears to be hindered by certain genetic instability and safety problems.

7.2.2.2 Fermentation by Yeasts

Yeasts exhibit a number of advantages in the production of xylitol compared to other microorganisms. Many studies have been directed towards searching for xylitol producers and identifying optimum conditions to obtain high yields ($Y_{P/S}$) and productivities (Q_P). Parajó et al. (1998b) reviewed in detail the published data on xylitol production from xylose solutions by commercial yeasts, which can be summarized as follows.

Barbosa et al. (1988) studied 44 strains of different species, selecting *Candida guillermondii* and *Candida tropicalis* as the best xylitol producers. These authors achieved concentrations of 77 g/L of xylitol from solutions containing 104 g/L of xylose. Conversely, Vandeska et al. (1995a) selected *Debaryomyces hansenii* and *Candida boidinii* as the best producers, while Sirisansaneeyakul et al. (1995)

worked with 11 different yeasts, obtaining a maximum xylitol yield of 0.62 g/g of consumed xylose with *Candida mogii*.

The largest concentrations of xylitol were achieved with different strains of *Candida* sp. (204–210 g/L from 250 g/L of xylose, $Y_{P/S} = 0.84$ g/g) (Chen and Gong 1985; Ikeuchi et al. 1999), *C. guilliermondii* (221 g/L from 300 g/L of xylose, $Y_{P/S} = 0.75$ g/g) (Meyrial et al. 1991) and *D. hansenii* (221 g/L of xylitol) (Domínguez et al. 1997), and the highest volumetric productivities (2.24–4.60 g/L h) with *D. hansenii* (Domínguez et al. 1997; Sampaio et al. 2005).

7.2.2.3 Genetically Modified Yeasts

Metabolic engineering offers opportunities to change the genetic properties of the microorganisms themselves. In the quest for a microorganism able to efficiently convert D-xylose to xylitol, strains of *Saccharomyces cerevisiae* were genetically modified (Winkelhausen and Kuzmanova 1998). Thus, the formation of xylitol by recombinant *S. cerevisiae* expressing the *XylI* gene was investigated by comparing the efficiency of different co-substrates (glucose, ethanol, acetate, and glycerol), oxygenation levels and different ratios of substrate and co-substrate. With both glucose and ethanol, the conversion yields were close to 1 g xylitol per gram of consumed D-xylose (Hallborn et al. 1994). Xylitol production was also investigated in continuous process carried out in a bench-scale packed bed reactor, where two recombinant strains of *S. cerevisiae* (H475 and S641), expressing low and high xylose reductase (XR) activities, respectively, were immobilized by gel entrapment using Ca alginate as the support (Roca et al. 1996). The effect of hydraulic residence time, substrate/co-substrate ratio, recycling ratio and aeration rate (AR) were investigated, and the highest xylitol concentration (15 g/L) was observed at a hydraulic residence time of 8.5 h. However, it still remains to be seen whether these microorganisms can remain sufficiently stable over a relatively long period of time and endure the operational conditions prevailing during the production of xylitol (Winkelhausen and Kuzmanova 1998).

7.3 Fermentation Technologies

Xylitol has been extensively produced using free cells in batch, continuous (chemostat) or fed-batch processes using either synthetic media or hydrolyzates obtained from different agroindustrial residues, although some attempts have also been made with yeasts immobilized in different supports. Some authors (Kim et al. 1999; Preziosi-Belloy et al. 1997) proposed a sequential process of utilization of the sugars present in hemicellulosic hydrolyzates, consisting in a first stage of fermentation performed under aerobic conditions, to promote glucose catabolism into biomass and prevent ethanol production, and a second stage in which the conversion of xylose to xylitol took place. This protocol was mainly developed in

batch culture (Preziosi-Belloy et al. 1997) preceding a chemostat process (Martínez et al. 2003) or during the first stage of fed-batch culture (Vandeska et al. 1996). The following section summarizes these technologies.

7.3.1 Free Cells

Most of the published works using free cells have been carried out batchwise in flasks or lab-scale stirred tank reactors, because these cultures offer versatility and easy use in preliminary tests (Winkelhausen and Kuzmanova 1998), but, comparatively, continuous or fed-batch processes allow increasing both yields and productivities.

7.3.1.1 Batch Systems

Because batch xylitol production by yeasts is strongly influenced by the operating conditions, the following sections take into consideration the variables that mostly impact on this bioprocess.

Age of Inoculum and Cell Recycling

The age of inoculum and cell recycling were shown to influence the metabolic activity as well as the viability of cells (Pfeifer et al. 1996), hence impacting on both productivities and yields. Felipe et al. (1997a) found that a 48 h-inoculum of *C. guilliermondii* resulted in a productivity 36 % smaller than a 24 h-inoculum; however, using the same microorganism, Sene et al. (1998, 2001), remarkably increased both xylitol yield and volumetric productivity using cells recycled four times, demonstrating that the more adapted the cells, the better the yeast ability to reduce xylose to xylitol in hemicellulose hydrolyzates. However, Cunha et al. (2006, 2007) found no significant differences when recycling cells immobilized in polyvinyl alcohol (PVA)-hydrogel, whereas Domínguez (1998) and Sampaio et al. (2005), working with two different strains of *D. hansenii*, observed a decrease in both xylitol yield and productivity. This behavior was justified by Rivas et al. (2003) with the tendency of the yeast, after two adaptations, to address the most significant fraction of the carbon source to respiration and production of ATP.

Initial Cell Concentration

Several studies highlighted that xylitol volumetric productivity increases linearly with the initial biomass level within a relatively wide range (Cao et al. 1994). This behavior was observed in particular with *D. hansenii* (Parajó et al. 1997; Sampaio

et al. 2008) and *Candida parapsilosis* (Oh et al. 1998), in which xylitol concentration varied in the ranges 0.042–32.4 and 8–30 g/L, respectively, while specific productivity remained almost constant or even slightly decreased. Several authors (Gírio et al. 1994; Felipe et al. 1997a; Parajó et al. 1997, 1998c) suggested that it could be the result of a reduction in the toxic effect of some inhibitors present in the culture medium. On the other hand, Domínguez et al. (1997) and Felipe et al. (1997a), working with *D. hansenii* and *C. guillermondii*, respectively, observed such a volumetric productivity increase only up to a maximum cell concentration. Rivas et al. (2003) proposed for the first yeast, that the reduction of xylitol production at high biomass level may result from an increased fraction of xylose required to sustain the large ATP requirements for cell maintenance.

pH

The optimum initial pH of cultivation is strongly species-dependent. It usually lies in the range 5.5–6.5 for *D. hansenii* (Converti and Domínguez 2001; Domínguez et al. 1996, 1997; Sampaio et al. 2006), 4–6 for *Candida* sp. (Cao et al. 1996), 4.5–5.0 for *C. parapsilosis*, 5.5–6.0 for *C. guilliermondii* (Converti et al. 2003; Felipe et al. 1997b; Nolleau et al. 1995), 2.5–6.0 for *Candida peltata* (Saha and Bothast 1999) and *C. tropicalis* (El-Batal and Khalaf 2004; Tamburini et al. 2008) and 6.0–7.5 for *Pachysolen tannophilus* (Converti et al. 1999), while *C. boidinii* (Vandeska et al. 1995a) and *Hansenula polymorpha* (Sánchez et al. 1998) have optimum pH of 7.0 and 5.5, respectively.

Converti and Domínguez (2001) and Converti et al. (2003) using *D. hansenii* and *C. guilliermondii*, respectively, explained the existence of an optimum pH for xylitol production on the basis of the fact that xylose is transported across the cell membrane by a facilitated diffusion system of the proton symport type. At pH above the optimum, this system is limited because H^+ transport must be performed against gradient, favoring respiration. On the other hand, at sub-optimal pH values, xylose transport is favored thus increasing the intracellular pH, which is likely to be restored consuming xylose by respiration to obtain ATP. As a result, both xylitol yields and productivities decrease.

Temperature

Yeasts produce xylitol in the range of 24–45 °C, but the optimal temperature is usually between 28 and 30 °C, depending on the microorganism: 28 °C for *C. guillermondii* (Converti et al. 2003), 28–35 °C for *D. hansenii* (Converti and Domínguez 2001; Domínguez et al. 1997; Sampaio et al. 2006) and 30 °C for *P. tannophilus* (Converti et al. 2001). When fermentations are carried out at temperatures different from the optimal ones, yields and productivities decrease due to reductions in xylitol dehydrogenase (XDH) activity (Winkelhausen and Kuzmanova 1998).

Nutrients of the Culture Medium

The type and concentration of the nitrogen source are key factors in the production of xylitol by fermentation, although the influence of both variables also depends on the yeast. In general, organic nitrogen sources such as urea, yeast extract, rice grain, peptone, etc. allow obtaining higher productivities and yields compared to the inorganic ones, because their presence stimulates the oxidative step of the pentose phosphate pathway in various yeasts (*C. boidinii, C. guillermondii, C. mogii*, etc.) (Barbosa et al. 1988; Kim and Moon 2003; Lu et al. 1995; Preziosi-Belloy et al. 2000; Silva and Roberto 2001; Sirisansaneeyakul et al. 1995; Vandeska et al. 1995a).

Some yeasts like *C. guilliermondii* and *P. tannophilus* require vitamins such as biotin, ascorbic acid, pyridoxine, or choline (Kim and Moon 2003; Lee et al. 1988). Similar effects have been reported for other nutrients; for example, the presence of Mg^{2+} ions can redirect the fermentation of xylose by *Pichia stipitis* towards xylitol production (Mahler and Guebel 1994), while xylitol production by *D. hansenii* can be stimulated, under phosphate-limiting conditions, by some enzyme activities like that of xylulose kinase (Tavares et al. 1999).

Initial Xylose Concentration

The initial xylose concentration has a significant impact on xylitol production by yeasts. Product yields are low when using low initial xylose concentrations, because the carbon source is mainly employed for biomass production. By increasing the concentration of xylose, a greater percentage of substrate is addressed to the production of xylitol, thereby increasing the yield (Converti et al. 2002). This behavior was demonstrated in several yeasts such as *D. hansenii* (Converti et al. 2002), *C. parapsilosis* (Oh et al. 1998) and *C. tropicalis* (Kim et al. 2002; Walther et al. 2001).

However, too high initial concentrations of xylose reduce the yield owing to excess substrate inhibition and high osmotic pressure of the medium (Kim et al. 2002; Silva et al. 1996a). Such an inhibition, which depends on the degree of aeration and the species, appears at 60 g/L of xylose in *P. tannophilus* (Thonart et al. 1987), 100–200 g/L in *C. tropicalis* (Gong et al. 1981; Silva and Afschar 1994), 200 g/L in *C. guilliermondii* (Meyrial et al. 1991; Silva et al. 1996b), 100–150 g/L in *C. boidinii* (Vandeska et al. 1995a) and 175–200 g/L in *D. hansenii* (Converti et al. 2002; Domínguez et al. 1997).

Carbon Sources Other than Xylose

Also the presence of other sugars in LCMs hemicellulose hydrolyzates impacts on xylitol production depending on their concentrations and the microorganism employed.

In some works, glucose was shown to be initially consumed by fermentation exerting an inhibitory effect on xylose uptake (Furlan and Castro 2001; Ikeuchi et al. 2000); once glucose is consumed, xylose consumption starts (Nobre et al. 1999; Preziosi-Belloy et al. 1997). In others, a stimulatory effect of glucose on xylitol production was identified in different yeasts (*C. tropicalis, C. parapsilosis, D. hansenii*) up to an optimal concentration threshold beyond which it behaved as an inhibitor (Kim et al. 1999; Sheu et al. 2004; Tavares et al. 2000; Walther et al. 2001). Other hexoses such as galactose and mannose (Preziosi-Belloy et al. 1997; Silva et al. 1996a; Walther et al. 2001) as well as ethanol (Saha and Bothast 1999; Walther et al. 2001) exert moderate inhibitory effects on the production of xylitol.

In general, other pentoses and glycerol do not exert any inhibitory effect (Saha 2003; Walther et al. 2001). Although Preziosi-Belloy et al. (1997) have suggested an inhibiting effect of arabinose on XDH activity, some yeasts showed higher affinity for xylose that was completely consumed before starting the assimilation of arabinose, with an increase in xylitol yield (Saha and Bothast 1999). The metabolism of arabinose leads to arabitol (Saha 2003), although many researchers believe it may also lead to xylitol (Gírio et al. 2000; Walther et al. 2001) or ethanol (Kim et al. 1999).

Dissolved Oxygen Concentration

The dissolved oxygen concentration is one of the most important parameters to be considered in the production of xylitol (Gírio et al. 1994), because it influences not only the operational aspects of xylitol production (du Preez 1994) but even the yeast physiology. Its effect was deeply investigated either in synthetic media (Granström et al. 2002; Nolleau et al. 1995; Vandeska et al. 1995b; Winkelhausen et al. 1996) or hemicelluloses hydrolyzates (Preziosi-Belloy et al. 2000; Roberto et al. 1999), but different methods were employed to measure it, thus making the results from different studies often not comparable (Skoog and Hahn-Hägerdal 1988).

Using yeasts with NADPH-dependent XR activity, such as *D. hansenii*, the dissolved O_2 level must be controlled at low levels to avoid xylitol consumption by respiration. Optimal values of O_2 concentration for xylitol production usually correspond to micro-aerobic conditions that allow, in addition to NAD^+ regeneration to the minimum extent necessary, producing the ATP and NADPH required to sustain cell growth and XR activity, respectively (Aguiar et al. 2002; Aranda-Barradas et al. 2000; Converti and Domínguez 2001; Faria et al. 2002a; Gírio et al. 2000; Roseiro et al. 1991).

This being so, the oxygen transfer rate (OTR), oxygen transfer coefficient (k_La) and specific rate of oxygen consumption (q_{O2}) are critical parameters to be strictly controlled during xylose-to-xylitol bioconversion (Aguiar et al. 2002; Nobre et al. 2002; Roseiro et al. 1991; Walther et al. 2001; Winkelhausen and Kuzmanova 1998). Optimal values for xylitol production in synthetic medium by *C. parasilopsis* were OTR = 65.7–211.7 mg/L h and k_La = 9.0–36.1 h^{-1}

(Aranda-Barradas et al. 2000; Faria et al. 2002a), by *C. boidinii* $k_La = 26–78\ h^{-1}$ (Winkelhausen et al. 2004) and *D. hansenii* $q_{O2} = 47.9$ mg/gMS L (Sampaio et al. 2004).

7.3.1.2 Continuous Fermentation

Contrary to batch systems, where only a small fraction of the total fermentation time corresponds to the phase of maximum rate of product formation, the use of continuous fermentation systems allows maintaining high productivities for long periods of time (Faria et al. 2002b). When compared with batch systems, the continuous ones show a number of additional advantages, the main of which being the elimination of idle time for cleaning and sterilization, greater steadiness in product synthesis and simplicity to implement automated control (Faria et al. 2002b). Consequently, several bioreactor configurations have been considered for continuous production of xylitol, which are briefly described in the following.

Continuous Stirred Tank Bioreactors

Ideally, the components of the culture broth in a Continuous Stirred Tank Bioreactor (CSTBR) are perfectly mixed, showing the same concentration as in the outflow stream. CSTBR is suitable when substrate costs are not so high and when a stable productivity is essential (Kosseva et al. 2009). This type of bioreactor was successfully employed by Cruz et al. (2000a) for the continuous production of xylitol by *D. hansenii* from barley bran hydrolyzate containing 30 g/L of xylose, varying the dilution rate from 0.008 to 0.088 h^{-1}. A maximum volumetric productivity (Q_P) of 0.60 g/L h and a product yield ($Y_{P/S}$) of 0.66 g/g were achieved at a dilution rate (D) of 0.048 h^{-1}, leaving 11 g/L of xylose in the fermented broth. These results improved those obtained in batch runs (Cruz et al. 2000b) using the same hydrolyzate and microorganism ($Q_P = 0.33$ g/L h; $Y_{P/S} = 0.61$ g/g). Martínez et al. (2003) also carried out continuous experiments using *C. guilliermondii* on a fermentation medium obtained from sugarcane bagasse hydrolyzate containing 51 g/L of xylose. Operating at $D = 0.038\ h^{-1}$, they obtained a xylitol concentration of 18.0 g/L, corresponding to $Q_P = 0.68$ g/L h and $Y_{P/S} = 0.69$ g/g.

Cross Flow Membrane Bioreactor

Increases in volumetric and specific productivities along with product yield are fundamental requisites to make the industrial xylitol bioproduction feasible (Kim et al. 2004; Oh and Kim 1998). Whereas the increase in specific productivity is in the domain of microbial genetics, such as the screening and mutant selection of a high producing strain, cell concentration can be effectively raised using cell-

recycle fermentation (Kwon et al. 2006). The Cross Flow Membrane Bioreactor (CFMBR), a combination of external membrane devices, like those employed for microfiltration or ultrafiltration, and a suspended cell bioreactor, has been proposed with the aim of improving xylitol productivity in a continuous process. Such a bioreactor configuration does in fact allow separating cells outside the bioreactor and recycling them to increase cell concentration, hence enhancing the kinetics and yields of the process as a result of reduced amount of xylose employed for microbial growth.

Silva et al. (1999) reported marked improvement of continuous xylitol production by recycling *C. guilliermondii* cells in such a system. Faria et al. (2002b) reported $Q_P = 1.14$ g/L h and $Y_{P/S} = 0.79$ g/g at $D = 0.030$ h^{-1} in a 2.6 L fermentor using *C. guilliermondii* in a synthetic medium containing 50 g/L of xylose. Q_P increased to 1.4 g/L h when the D was increased to 0.05 h^{-1}, but $Y_{P/S}$ decreased to 0.72 g/g. Likewise, Cruz et al. (2000a), using barley bran hydrolyzate containing 30 g/L of xylose and *D. hansenii* in a 2.0 L fermentor, obtained results ($D = 0.28$ h^{-1}; $Q_P = 2.53$ g/L h; $Y_{P/S} = 0.39$ g/g; residual xylose concentration of 4 g/L) much better than those obtained without membrane devices. Using the CFMBR, Choi et al. (2000) substantially improved batch xylitol production by *C. tropicalis*, obtaining results ($Q_P = 4.94$ g/L h; $Y_{P/S} = 0.82$ g/g; final xylitol concentration of 189 g/L) 1.3–2.2 times higher than without membranes.

Despite these advantages, the CFMBR have some drawbacks such as high power consumption, cell damage resulting from shear stress, and difficulty in controlling fouling (Delgado et al. 2004). In addition, the bioreactors used for cell-recycle fermentation are unsuitable for aerobic fermentation because of the lack of an air supply (Kwon et al. 2006).

7.3.1.3 Long-Term Cell Recycle by Centrifugation

Kim et al. (2004) proposed the long-term recycle of *C. tropicalis* cells by centrifugation to increase biomass concentration within the bioreactor. Using 2 L of complex or chemically defined media in a 7 L jar fermenter considerably improved the performance of batch fermentations, as a result of the reduction in the fermentation time and increase in the substrate consumption rate. After 14 rounds of fermentation, corresponding to a fermentation time of 284–333 h, the average xylitol concentration reached 105–110 g/L, and Q_P (4.4–5.4 g/L h) and $Y_{P/S}$ (0.78–0.81 g/g) were about twice and 4–7 % higher, respectively, than those of simple fermentations. However, application of centrifugation to large-scale production would not be economically viable, due to high complexity, inability to ensure long-term aseptic conditions and high cost of equipment (Kwon et al. 2006).

7.3.1.4 Submerged Membrane Bioreactor

Kwon et al. (2006) developed a new membrane-based cell recycling system, called Submerged Membrane Bioreactor (SMBR), consisting of a SMBR with suction and air sparging. According to these authors, the SMBR is more effective than the CFMBR in terms of power consumption, membrane filtration, fouling control, cell recycling and cultivation of aerobes. Operating ten recycle rounds, they achieved productivity ($Q_P = 12$ g/L h) and maximum xylitol concentration ($P = 182.0$ g/L) 3.4 and 11.0 times higher than in batch fermentation, respectively.

7.3.2 Immobilized Cells

The most common methods of cell immobilization employed in bioprocesses are adsorption, entrapment and covalent binding (Kosseva et al. 2009). According to Choi et al. (2000), calcium alginate, polyacrylamide and non-woven fabric are among the most effective entrapping materials for cell immobilization. The use of immobilized cells has several advantages compared to suspended cells, including higher cell density within the bioreactor, higher productivity, improved stability, cell reutilization, continuous operation, and no need to separate cells from substrate and products at the end of the process (Kosseva et al. 2009). However, cell immobilization techniques are characterized by lower mass transfer rate of substrates from the bulk to cells and leakage of cells to the medium (Audet et al. 1991). Immobilized cells have been studied in both batch and continuous systems. As an example, Fig. 7.1 shows a micrograph by scanning electron microscopy of the yeast *D. hansenii* immobilized in calcium alginate, while Table 7.1 summarizes the information concerning the microorganisms, immobilization supports, carbon sources and bioreactors most commonly employed.

7.3.2.1 Batch Systems

The behavior of immobilized cells can be significantly different from that of free cells. For example, conditions ensuring micro-aerobiosis to free cells may correspond to anaerobic conditions with immobilized cells due to mass transfer (diffusion) limitation. Therefore, application of low ARs in immobilized cell cultures may cause drastic reductions in productivity (Hinfray et al. 1995).

Repeated-batch fermentations were investigated by different authors to evaluate the long-term stability of immobilization with the perspective to implement the technology into a continuous process or to scale up the process from flasks to stirred tank bioreactors. According to Domínguez (1998), immobilization is a technique that allows maintaining the functionality of the fermenting microorganisms used in xylitol production processes. When cells of *D. hansenii* immobilized in calcium alginate beads were reused to ferment synthetic media in

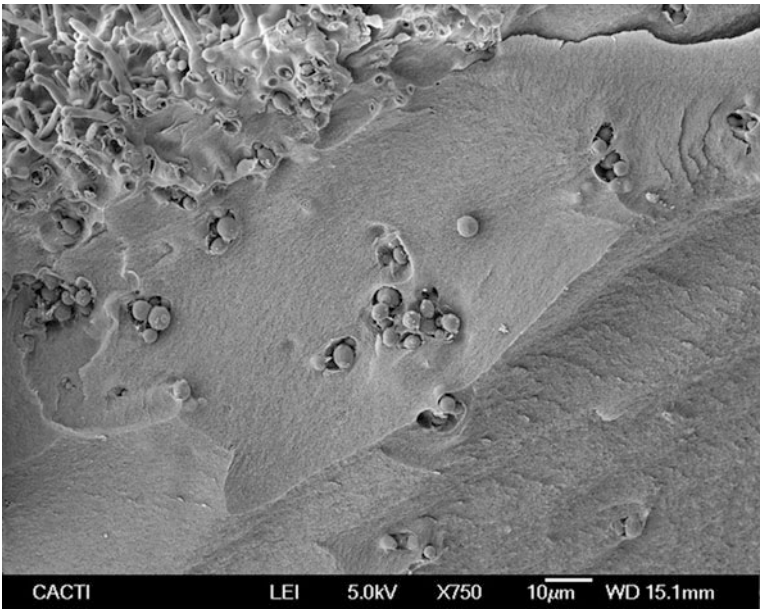

Fig. 7.1 Micrograph by scanning electron microscopy (SEM) of *Debaryomyces hansenii* cells immobilized in calcium alginate

Erlenmeyer flasks, xylitol yield increased from 0.37 g/g in the first recycling operation to 0.69 g/g after the second, and xylitol volumetric productivity from 0.31 to 2.03 g/L h after the fourth. These results improved those obtained in free cultures, where D-xylose was converted rapidly and efficiently into xylitol in the first step ($P = 106.7$ g/L of xylitol; $Q_P = 1.48$ g/L h; $Y_{P/S} = 0.84$ g/g). However, after six recycles, the bioconversion was less effective.

Cunha et al. (2006), using *C. guilliermondii* cells immobilized in 5 g of PVA-hydrogel to ferment 45 mL of sugarcane bagasse hemicellulosic hydrolyzate in 125-mL Erlenmeyer flasks, observed that the biocatalyst remained stable and exhibited a similar fermentative profile in five repeated-batch fermentations and obtained as an average $P = 35.1$ g/L, $Q_P = 0.49$ g/L h and $Y_{P/S} = 0.58$ g/g. When scaling up the process to a bench-scale stirred tank bioreactor filled with 960 mL of medium and 240 g of beads, Cunha et al. (2007) observed a small decrease in the conversion performance in the fifth cycle. The biocatalytic activity of biomass was recovered in the sixth cycle after washing the particles, and the hydrogel beads were shown to maintain their shape and size without appreciable deterioration. The best results ($P = 39.7$ g/L; $Q_P = 0.53$ g/L h; $Y_{P/S} = 0.77$ g/g) were achieved after the third cell recycling, probably because of cells adaptation to the medium.

Liaw et al. (2008) performed 52.5-day long repeated-batch fermentation in Erlenmeyer flasks, using rice straw hemicellulose hydrolyzate detoxified with activated charcoal as fermentation broth and cells of *C. subtropicalis* WF79

Table 7.1 Xylitol production in batch processes with immobilized yeasts, packed bed reactors in continuous, fluidized bed reactors and fed-batch processes

Microorganism	Carbon source	Immobilization	Reactor	Results	Reference
Batch processes with immobilized yeasts					
Candida guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Calcium alginate	Erlenmeyer flasks	$P = 11.05$ g/L $Q_P = 0.22$ g/L h $Y_{P/S} = 0.47$ g/g	Carvalho et al. (2000)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Calcium alginate	Erlenmeyer flasks	$P = 20.6$ g/L $Q_P = 0.43$ g/L h $Y_{P/S} = 0.47$ g/g	Carvalho et al. (2002[a])
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Calcium alginate	Erlenmeyer flasks	$P = 28.4$ g/L $Q_P = 0.59$ g/L h 61.5 % efficiency	Carvalho et al. (2002b)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Calcium alginate	Erlenmeyer flasks	$Q_P = 0.43$ g/L h $Y_{P/S} = 0.47$ g/g	Carvalho et al. (2002c)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Calcium alginate	Stirred tank reactor	$P = 47.5$ g/L $Q_P = 0.40$ g/L h $Y_{P/S} = 0.81$ g/g	Carvalho et al. (2005)

(continued)

Table 7.1 (continued)

Microorganism	Carbon source	Immobilization	Reactor	Results	Reference
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Calcium alginate	Stirred tank reactor	$P = 51.6$ g/L $Q_P = 0.43$ g/L h $Y_{P/S} = 0.71$ g/g	Carvalho et al. (2008)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Hydrogel based on polyvinyl alcohol (PVA)	Erlenmeyer flasks	$P = 34.2$ g/L $Q_P = 0.48$ g/L h $Y_{P/S} = 0.57$ g/g	Cunha et al. (2006)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Hydrogel based on polyvinyl alcohol (PVA)	Bench-scale bioreactor	$P = 39.7$ g/L $Q_P = 0.53$ g/L h $Y_{P/S} = 0.77$ g/g	Cunha et al. (2007)
Debaryomyces hansenii	Synthetic xylose	Calcium alginate	Erlenmeyer flasks	$P = 99.6$ g/L $Q_P = 2.08$ g/L h $Y_{P/S} = 0.79$ g/g	Domínguez (1998)
Candida subtropicalis	Rice straw hemicellulose hydrolyzates detoxified with activated charcoal	Polyacrylic hydrogel thin films	Erlenmeyer flasks	$Y_{P/S} = 0.73$ g/g	Liaw et al. (2008)
Candida boidinii	Synthetic xylose	Hydrogels based on u.v.-crosslinked poly(ethylene oxide)—matrices	Erlenmeyer flasks	$P = 4.2$ g/L	Winkelhausen et al. (2008)

(continued)

Table 7.1 (continued)

Microorganism	Carbon source	Immobilization	Reactor	Results	Reference
Packed bed reactors in continuous					
D. hansenii	*Eucalyptus globulus* wood hydrolyzate detoxified with charcoal	Calcium alginate	Upflow packed-bed bioreactors	$Q_P = 0.91$ g/L h	Domínguez et al. (1999)
C. guilliermondii	*E. globulus* wood hydrolyzate detoxified with charcoal	Calcium alginate	Upflow packed-bed bioreactors	$Q_P = 0.58$ g/L h	Domínguez et al. (1999)
Candida pelliculosa and *Methanobacterium* sp. HU	Synthetic	Polymer resins	Packed reactor	Improved stability during 2 weeks	Nishio et al. (1989)
Fluidized bed reactors					
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Porous glass spheres	Batchwise	$P = 34.2$ g/L $Q_P = 0.48$ g/L h $Y_{P/S} = 0.57$ g/g	Santos et al. (2003)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Porous glass spheres	Semicontinuous in different cycles	$P = 18.0$ g/L $Q_P = 0.32$ g/L h $Y_{P/S} = 0.44$ g/g	Santos et al. (2005a)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Porous glass spheres	Semicontinuous in different cycles	$Q_P = 0.44$ g/L h $Y_{P/S} = 0.25$ g/g	Santos et al. (2005b)

(continued)

Table 7.1 (continued)

Microorganism	Carbon source	Immobilization	Reactor	Results	Reference
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzate	Zeolite	Batchwise	$P = 38.5$ g/L $Q_P = 0.32$ g/L h $Y_{P/S} = 0.72$ g/g	Santos et al. (2005c)
Candida tropicalis	Synthetic	Porous glass	Fluidized bed reactor in continuous	$P = 90$–95 g/L $Q_P = 1.35$ g/L h	Silva and Afschar, (1994)
C. guilliermondii	Synthetic xylose	Porous glass spheres	Semicontinuous in different cycles	$Q_P = 0.52$–0.60 g/L h $Y_{P/S} = 0.79$–0.57 g/g	Silva et al. (2003)
Fed-batch processes					
Candida parapsilosis	Synthetic sugars	Free cells	2-L Setric Set 2 reactor	$Q_P = 0.46$ g/L h $Y_{P/S} = 0.72$ g/g	Furlan et al. (1997)
C. parapsilosis	Synthetic sugars	Free cells	20 L laboratory reactor	$Q_P = 0.05$ g/L h $Y_{P/S} = 0.38$ g/g	Furlan and Castro (2001)
C. guilliermondii	Synthetic sugars	Free cells	1.5 L fermenter	$P = 44.0$ g/L $Q_P = 1.06$ g/L h $Y_{P/S} = 0.76$ g/g	Godoy De Andrade Rodrigues et al. (2002)

(continued)

Table 7.1 (continued)

Microorganism	Carbon source	Immobilization	Reactor	Results	Reference
C. tropicalis	Synthetic sugars	Free cells	3.5 L fermenter	$P = 187.0$ g/L $Q_P = 0.75$ g/L h $Y_{P/S} = 3.90$ g/g	Kim et al. (2002)
C. tropicalis	Synthetic sugars	Free cells	Fermenter	$P = 237.0$ g/L $Q_P = 2.0$ g/L h $Y_{P/S} = 0.89$ g/g	Kim and Oh (2003)
C. tropicalis	Corn cob hemicellulosic hydrolyzates	Free cells	Fermenter	$P = 96.5$ g/L $Q_P = 1.01$ g/L h $Y_{P/S} = 0.83$ g/g	Li et al. (2012)
Candidu magnoliae	Synthetic sugars	Free cells	Fermentor	$P = 356.0$ g/dm^3 $Y_{P/S} = 0.75$ g/g	Nakano et al. (2000)
C. tropicalis	Synthetic sugars	Free cells	Fermentor	$P = 240.0$ g/L $Q_P = 5.58$ g/L h $Y_{P/S} = 0.80$ g/g	Oh and Kim (1997)

(continued)

Table 7.1 (continued)

Microorganism	Carbon source	Immobilization	Reactor	Results	Reference
C. tropicalis	Synthetic sugars	Free cells	5 L Fermentor	$P = 251.0$ g/L $Q_P = 4.56$ g/L h $Y_{P/S} = 0.93$ g/g	Oh and Kim (1998)
C. guilliermondii	Synthetic sugars	Free cells	5 L Fermentor	$Q_P = 0.64$ g/L h $Y_{P/S} = 0.84$ g/g	Rodrigues et al. (1999)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzates	Free cells	5 L Fermentor	$Q_P = 0.62$ g/L h $Y_{P/S} = 0.78$ g/g	Rodrigues et al. (1999)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzates	Free cells	Erlenmeyer flasks	$P = 44.0$ g/L $Q_P = 0.62$ g/L h $Y_{P/S} = 0.78$ g/g	Rodrigues et al. (1998a)
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzates	Free cells	Fermenter	$P = 26.3$ g/L $Q_P = 0.55$ g/L h $Y_{P/S} = 0.68$ g/g	Rodrigues et al. (2002)

(continued)

Table 7.1 (continued)

Microorganism	Carbon source	Immobilization	Reactor	Results	Reference
C. guilliermondii	Sugarcane bagasse hemicellulosic hydrolyzates	Ca-alginate	2 L Fermenter	$P = 28.9$ g/L $Q_P = 0.40$ g/L h $Y_{P/S} = 0.58$ g/g	Sarrouh et al. (2007)
C. boidinii	Synthetic sugars	Free cells	2 L bench top fermenter	$P = 59.3$ g/L $Q_P = 0.46$ g/L h $Y_{P/S} = 0.68$ g/g	Vandeska et al. (1996)
C. tropicalis	D-xylose and feeding D-glucose (50 g/l day)	Non-woven fabric	–	$P = 87.0$ g/L	Yahashi et al. (1996)

P maximum xylitol concentration, Q_P volumetric xylitol productivity, $Y_{P/S}$ xylitol yield

immobilized in polyacrylic hydrogel as biocatalyst. The cell immobilization procedure started with suspending yeast cells in a mixture of 2-hydroxyethyl methacrylate (HEMA, hydrophilic monomer), polyethylene glycol diacrylate (PEG-DA, crosslinking agent), and benzoin isopropyl ether (photoinitiator). The mixture was then allowed to form thin polyacrylic hydrogel films with 200 μm thickness, between two pieces of glass sheets, by UV-initiated photopolymerization. The maximum yield was 0.73 g/g, but after 40 days the fermentation activity of immobilized cells declined, and the yield was only 0.57 g/g at the end of run.

Using sugarcane bagasse hydrolyzates and *C. guilliermondii* cells entrapped in Ca-alginate beads, Carvalho et al. (2000, 2002a, b, c) studied the effects of immobilization conditions on bead chemical stability and xylitol production during repeated-batch fermentations in Erlenmeryer flasks, and successively in a 2.4 L stirred tank reactor (Carvalho et al. 2005, 2008). In bioreactor experiments, xylitol concentration increased up to 47.5 g/L within 120 h of fermentation, resulting in $Q_P = 0.40$ g/L h and $Y_{P/S} = 0.81$ g/g after one fermentation cycle (Carvalho et al. 2005). However, after five successive batches in a stirred tank reactor, the average volume of the Ca-alginate beads decreased by about 30 % after 600 h, thus demonstrating physical instability under the conditions employed in the reactor (Carvalho et al. 2008). In spite of this, almost steady xylitol production parameters were observed, with average values of xylitol concentration, overall volumetric productivity and product yield of 51.6 g/L, 0.43 g/L h and 0.71 g/g, respectively.

7.3.2.2 Continuous Fermentation

Immobilization has been employed in xylose-to-xylitol bioconversion to keep high concentration of cells as biocatalyst. Packed bed bioreactors (PBB) and fluidized bed bioreactors (FBB) are the most common bioreactors employed.

Packed Bed Bioreactors

According to Kosseva et al. (2009), PBBs have the advantage of simplicity of operation, high mass transfer rates and high reaction rates, whereas their main drawbacks are limited oxygen transfer during scale-up and periodic fluctuation in viable cell population due to nutrient depletion along the reactor length. Their long-term stability during continuous operation is one of the most important challenges.

Nishio et al. (1989) co-immobilized *Candida pelliculosa* and *Methanobacterium* sp. HU cells with glutaraldehyde and hexamethylenediamine to enhance the stability of an immobilized-cell system for the continuous production of xylitol in packed column. Using Ca-alginate beads for the same purpose, Domínguez et al. (1999) reported on the need of high sodium alginate concentrations during the immobilization step to increase the chemical stability of cell-gel beads to be employed in xylitol production from *Eucalyptus globulus* wood hydrolyzate

detoxified with charcoal. The use of immobilized cells of *D. hansenii* and *C. guilliermondii* in an upflow packed-bed bioreactor ensured volumetric productivities of 0.91 and 0.58 g/L·h, respectively.

Fluidized Bed Bioreactors

FBBs offer the advantage of good solid–fluid mixing and minimal pressure drops (Kosseva et al. 2009). The fluidization behavior is very strongly dependent on the physical properties of both liquid and solid phases (liquid viscosity, density, surface tension and solid density) (Béjar et al. 1992). Different parameters have been studied to optimize the process including the AR, the durability of the immobilization after several cycles and the influence of different hydrodynamic variables.

The influence of AR on batch xylitol production from sugarcane bagasse hemicellulose hydrolyzate in FBB was studied by Santos et al. (2003) using cells of *C. guilliermondii* immobilized onto porous glass spheres. The highest xylitol concentration (17.0 g/L) was obtained at an intermediate AR of 70 mL/min, although the specific xylitol productivity and xylitol yield were 43 and 22 % lower than the corresponding values obtained at the lowest air AR (25 mL/min), respectively.

Other authors have focused their research efforts on the number of cycles. Silva et al. (2003) employed *C. guilliermondii* FTI 20037 cells immobilized on porous glass to study the fed-batch bioconversion of xylose in a 1.4 L-fluidized bed reactor over 672 h. The fermentation was performed in seven cycles and always using fresh medium. The highest values of xylitol yield (0.79 and 0.57 g/g) and volumetric productivity (0.52 and 0.60 g/L h) were attained in the first and second cycles, respectively. Contrary to what was expected from a progressive adaptation of cells to the medium, a further increase in the number of cycles did not improve the performance of the system, due to cell wash-out that caused the cell-particles to become unsaturated, a part of xylose being consumed for the production of biomass instead of xylitol. Conversely, under similar conditions, but using sugarcane bagasse hemicellulosic hydrolyzates as carbon source, Santos et al. (2005a) obtained the lowest xylitol concentration (12 g/L) in the first cycle, and the best results ($P = 18.0$ g/L; $Q_P = 0.32$ g/L h; $Y_{P/S} = 0.44$ g/g) after five successive cycles of 72 h, because of cells adaptation to the medium. The influence of AR and carrier concentration (Cs) was evaluated by Santos et al. (2005b) during seven batch fermentation runs. The results showed that Cs had a negative influence on $Y_{P/S}$ and Q_P, whereas AR impacted positively on Q_P and negatively on $Y_{P/S}$. With AR = 0.093 min^{-1} and Cs = 62.5 g/L, $Y_{P/S}$ was low (0.25 g/g), but Q_P was the highest (0.44 g/L h), probably because cell metabolism was faster when more oxygen was available to the yeast.

Santos et al. (2005c) used *C. guilliermondii* cells immobilized on zeolite to investigate the effects of different conditions of air flowrate (0.0125–0.0375 vvm), zeolite mass (100–200 g), initial pH (4–6) and xylose concentration (40–60 g/L).

A metabolic deviation from product to biomass formation took place when raising air flowrate, while increases in pH or xylose concentration enhanced Q_P and $Y_{P/S}$ or xylitol concentration, respectively. The best results ($P = 38.5$ g/L; $Q_P = 0.32$ g/L h; $Y_{P/S} = 0.72$ g/g) were obtained using an air flowrate of 0.0125 vvm, 100 g of zeolite, pH = 6 and xylose concentration of 60 g/L.

Sarrouh and Silva (2008) evaluated the hydrodynamic characteristics (total particle density, terminal velocity, particles drag force, minimum fluidization velocity and bed porosity) and fermentation parameters (AR and fermentation time) of a bench-scale three-phase fluidized bed reactor with *C. guilliermondii* cells immobilized in calcium alginate beads for xylitol production. The reactor was shown to operate similarly to a fixed-bed bioreactor at bed porosity <0.5 and a FBB at bed porosity >0.5. The maximum flowrate needed to obtain maximum bed fluidization in the reactor was equal to the terminal velocity of the immobilized cell particles. Regarding the fermentation parameters, the best results ($P = 28.9$ g/L; $Q_P = 0.41$ g/L h; $Y_{P/S} = 0.58$ g/g) were obtained at a high AR (600 mL/min) after 70 h of fermentation, pointing out that in this system high ARs enhance oxygen transfer into the immobilized cells.

Finally, Silva and Afschar (1994) utilized a FBB for the continuous xylose-to-xylitol bioconversion by *C. tropicalis* cells immobilized on porous glass. The system exhibited a xylitol productivity of 1.35 g/L h, a value much higher than that obtained in batch culture with free cells (0.37 g/L h), thus showing the feasibility of these systems.

7.3.2.3 Fed-Batch Systems

Continuous processes can be successfully replaced by the fed-batch ones (Roberto et al. 1991) everywhere high product or substrate levels can be inhibitory to the system. In these processes, substrate concentration can be maintained at a suitable level throughout the entire course of fermentation, that is, a level sufficient to induce xylitol formation but not to inhibit yeast growth. In addition, they generally operate with high initial cell density, thereby increasing volumetric productivity (Winkelhausen and Kuzmanova 1998).

Thus, Furlan et al. (1997), using *C. parasilopsis* in synthetic medium, obtained a 40 % productivity increase compared with batch cultures, while Oh and Kim (1997), using *C. tropicalis* and maintaining high dissolved oxygen levels during the growth phase and oxygen-limited conditions during the production one, were able to increase xylitol concentration from 88 (starting with 100 g xylose/L) to 240 g/L (from 300 g xylose/L) passing from a batch to a fed-batch culture, corresponding to $Q_P = 5.58$ g/L h and $Y_{P/S} = 0.80$ g/g. Similarly, the overall performance of fed-batch cultivations with exponential feeding rate of *C. guilliermondii* on sugarcane bagasse hemicellulosic hydrolyzate was remarkably higher ($P = 44.0$ g/L; $Q_P = 0.62$ g/L h; $Y_{P/S} = 0.78$ g/g) (Rodrigues et al. 1998a) than those of batch experiments ($Q_P = 0.29$ g/L h; $Y_{P/S} = 0.50$ g/g) (Rodrigues et al. 1998b). More recently, Li et al. (2012) obtained 96.5 g xylitol/L cultivating *C. tropicalis* on corn

cob hemicellulose hydrolysate by a two-stage fed-batch fermentation process, corresponding to yield (0.83 g/g) and productivity (1.01 g/L h) about 12 and 66 % higher than those of batch fermentation, respectively, and related these improvements to a reduction of the negative effects on xylitol formation exerted by aeration and inhibitory compounds.

In fed-batch processes substrate can be added continuously or intermittently. The concentration and type of substrate as well as the optimal feeding rate were investigated by different authors. Vandeska et al. (1996), using free cells of *C. boidinii* initially in batch fermentation at high aeration levels to favor biomass production and then in three repeated fed-batch runs, observed the fastest cell growth using a mixture of glucose and xylose as a substrate. The best results were obtained performing the first fed-batch run at the highest initial xylose concentration (100 g/L) and the lowest level of aeration and using a feed rate of 0.8 g/L of xylose. Under these conditions a xylitol yield of 0.68 g/g (75 % of the theoretical yield, compared to 53 % in batch culture), a volumetric productivity of 0.46 g/L h (twice as high as the highest obtained in batch culture) and a final concentration of 59.3 g/L of xylitol were obtained. Oh and Kim (1998) using *C. tropicalis* obtained the best results ($P = 251.0$ g/L; $Q_P = 4.56$ g/L h; $Y_{P/S} = 0.93$ g/g) from 270 g xylose/L at a glucose/xylose feeding ratio of 15 % within 55 h. These results were substantially higher than those obtained in batch experiments ($P = 131.0$ g/L) starting with 150 g xylose/L after 45 h.

Although most of the efforts were directed towards the use of free cells in fed-batch processes, a few articles report on the use of cells immobilized in different supports. For example, to increase xylitol concentration up to 87 g/L by feeding 50 g D-glucose/L day, Yahashi et al. (1996) immobilized *C. tropicalis* cells in non-woven fabrics, which could be used five times in repeated fed-batch cultivations. More recently, Sarrouh et al. (2007) employed cells of *C. guilliermondii* entrapped in Ca-alginate beads to produce xylitol from concentrated hemicellulose hydrolyzate of sugarcane bagasse, in a three-phase FBB. The best results ($P = 28.9$ g/L; $Q_P = 0.40$ g/L h; $Y_{P/S} = 0.58$ g/g) were obtained at an AR as high as 600 mL/min, indicating that high ARs are required in these systems to ensure suitable oxygen transfer into the inside of the beads.

7.4 Conclusions and Future Recommendations

The microbial production of xylitol has been widely investigated in the last two decades either in synthetic media or lignocellulosic hydrolyzates, and the most suitable microorganisms and operating conditions were selected to maximize both productivity and yield. The traditional batch processes have been mostly replaced by fed-batch or continuous systems, in most of the cases using yeast cells immobilized in different matrices, where aeration plays a crucial role. Nowadays, the trends seem to be focused on the production of compounds with homogeneous composition to be obtained from cheap carbon and nutrients sources or even

wastes. In order to achieve this goal more research is needed to develop novel bioreactors for xylitol production able to keep the oxygen and energy requirements as low as possible.

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Chapter 8
Enzymatic Production of Xylitol: Current Status and Future Perspectives

Ricardo de Freitas Branco, Anuj K. Chandel and Sílvio Silvério da Silva

Abstract Enzymatic production of bio-active compounds has several advantages over chemical synthesis. Enzymatic mediated reactions are generally considered safe, highly reproducible, economical and environmentally benign. Microbial mediated xylose fermentation for xylitol production is a conventional approach with several process complexities. Enzymatic conversion of xylose into xylitol offers a promising alternative towards the commercial production of xylitol on a large scale. This chapter will discuss enzymatic production (or in vitro) of xylitol with emphasis on enzymatic catalysis using coenzymes and their enzymatic regeneration methods. Furthermore, mechanisms of the enzymatic process, operational details, advantages and disadvantages in comparison with the traditional production methods of xylitol (chemical and microbial) have been discussed at length. Special emphasis is placed on the sustainable raw material alternatives for enzymatic production of xylitol using sugarcane bagasse as the main carbohydrate source.

Keywords Enzymatic synthesis · Xylitol · Xylose reductase · Sugarcane bagasse · Multi-enzymes

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8.1 Introduction

In recent decades, the use of enzymes as industrial catalysts has increased and become almost indispensable in industries such as pharmaceutical, food and feed, textiles, detergents, biorefinery, leather processing and bioremediation (Kumari et al. 2009). Among the enzymes having profound applications in industries, cellulases, proteases, xylanases, amylases and lipases have been extensively applied and have huge demand today for the formulation of many household commodities. However, the need for more specific catalysts made researchers turn their attention to specific enzymes which could be used in food/feed, diagnostic, analytical and pharmaceutical sectors (Kumari et al. 2009; Fernandes 2010). For example, aspartate dehydrogenase, a member of the amino acid dehydrogenases group, catalyze the reversible oxidative deamination of amino acids to their corresponding 2-oxoacids, using either nicotinamide adenine dinucleotide or $NAD(P)^+$ as cofactors. Aspartate dehydrogenase has shown potential applications in the synthesis of amino acids for the production of pharmaceutical peptides (Li et al. 2012).

Fermentative production of xylitol from xylose obtained from lignocellulosic materials is the most studied and a well-established method. However, there are several mechanistic steps and process configurations involved in this process making it overall expensive and laboursome. Enzymatic production of xylitol offers several advantages over fermentative production, but less yield and requirement of a highly precise control make this method less favorable. Xylose-assimilating yeasts utilize D-xylose via two enzymatic oxidoreductive reactions with xylose reductase (XR) (EC 1.1.1.21) and xylitol dehydrogenase (XDH) (EC 1.1.1.9). D-xylose is reduced to xylitol by XR-mediated catalytic conversion. In these processes, enzymes are incorporated as chemical catalysts to improve or to substitute production steps (Bon et al. 2008; Lima et al. 2001). This chapter summarises the developments made in enzymatic conversion of xylose into xylitol utilizing sugarcane bagasse as a model substrate and presents future trends on the economization of xylitol production.

8.2 Xylose Reductase: Basic Properties and Mechanistic Action

For any enzyme catalyzed bioconversion, basic characteristics and properties of the enzymes (optimum temperature, pH, isoelectric point, kinetic, etc.) are essential to understand in detail (Fernandes 2010). Xylose reductase (EC 1.1.1.21) belongs to the superfamily of aldo-keto reductase, being a NAD(P)H dependent oxidoreductase. XR is present in some strains of yeast and fungi. Table 8.1 summarises some microorganisms capable of synthesizing the XR.

Table 8.1 Some examples of xylose reductase producing microorganisms

Microorganism	Xylose reductase specific activity (U mg^{-1} of protein)
Candida pelliculosa	1.73
C. pelliculosa var. *acetaetherius*	0.09
Candida utilis	0.26
Candida guilliermondii FTI 20037	1.10
Debaryomyces hansenii	0.16
Hansenula anômala Y1	0.09
Hormoacus platypoides AM 93	0.43
Pichia nakazawae	0.24

Adapted from Kitpreechavanich et al. (1984)

XR plays a key role in microbial metabolism, since it allows using xylose as an energy source for microorganisms. XR initiates the first step of xylose metabolism, catalyzing the reduction reaction of xylose (aldose) to xylitol (alcohol) with the aid of the NAD(P)H coenzyme as electron donor. The molecular weight of XR varies (30–70 kDa) widely depending on the producing microorganism. According to Cortez (2002), the XR present in *Candida guilliermondii* FTI 20037 is composed of one or two 30–60 kDa subunits. In another study, Kratzer et al. (2004) found that the XR from *Candida tenuis* has a molecular weight of around 36 kDa. Several studies have demonstrated that different types of XR are possible in regards to their structure, which can be monomeric or dimeric (Wilson et al. 2003) depending on the microorganism. Wilson et al. (2003) proposed a three-dimensional structure of XR from *C. tenuis* (Fig. 8.1). With regard to the iso-electric point, the XR from *Candida tropicalis* presents a pH value of around 4.00 (Yokoyama et al. 1995).

XR binds to two different substrates, i.e. coenzymes and xylose, respectively. The mechanistic action of XR is more complex than that of enzymes which use only one substrate. Rawat and Rao (1996) found that for the XR from *Neurospora crassa*, the mechanism of action is iso-ordered Bi–Bi, where XR binds necessarily first with the coenzyme and subsequently with xylose to complete the reaction. The same mechanism was demonstrated by Mayr et al. (2001) for XR from *C. tenuis*. Figure 8.2 shows the mechanism of XR where the first reaction step is not shown (with binding of the enzyme coenzyme XR), because it is a very fast step. All XR have a great affinity (few millimole) for NAD(P)H coenzyme.

The structural and action mechanism of XR became the subject of research in recent years because of the renewed interest in finding alternative energy sources (biofuels) and biotechnological production of xylitol. Industrial ethanol producing microorganisms such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* do not have the capacity to use xylose as a carbon source which is a very important aspect of 'economic' ethanol production in biorefineries (Chandel et al. 2011a, b). If these organisms have their genome complemented with XR and other necessary enzymes, production of ethanol from xylose as a carbon source could be possible

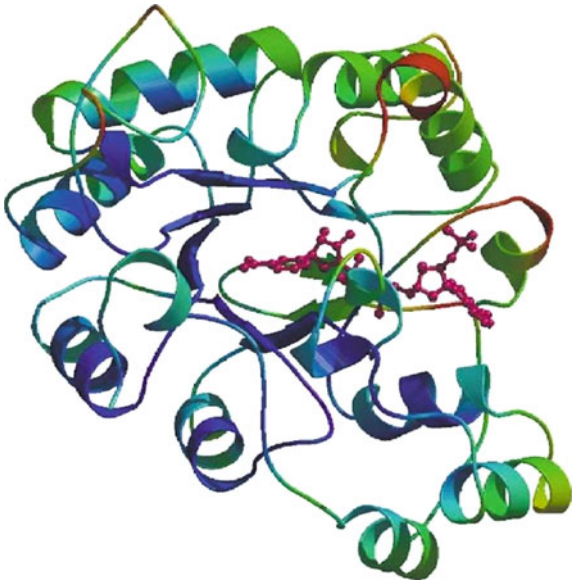

Fig. 8.1 Holoenzyme structure of the *Candida tenuis* xylose reductase (Wilson et al. 2003)

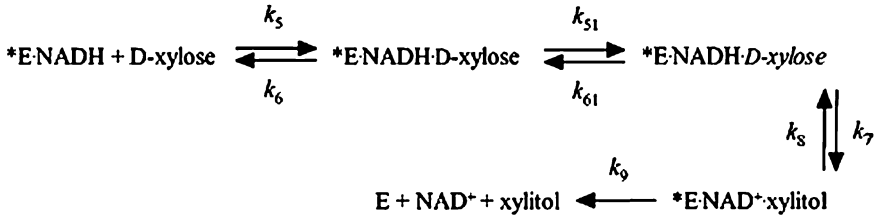

Fig. 8.2 Mechanism of action of the enzyme xylose reductase iso-ordered Bi–Bi, proposed by Mayr et al. (2001). Adapted with permission from the American Chemical Society Copyright (2001)

(Chandel and Singh 2011). In fact, several studies have been published on genetic modification to allow different microorganisms to produce XR (Baea et al. 2004; Suzuki et al. 1999; Woodyer et al. 2005).

For the enzymatic (XR) production of xylitol, XR from *C. guilliermondii* FTI 20037 has been widely reported for xylitol production (Faria et al. 2002; Santos et al. 2005; Silva et al. 2003). XR has been purified with good yields, using a simple technique of liquid–liquid extraction by reverse micelles (Cortez 2002; Cortez et al. 2004). Cells were disrupted by sonication and XR was separated by extraction into reverse micelles, using n-Benzyl-n-Dodecyl-n bis (2-hydroxyethyl) ammonium chloride (BDBAC), cetyltrimethyl ammonium bromide (CTAB) and sodium diethyl hexylsulphosuccinate (AOT) as surfactants. It was observed that the XR was recovered in yields above 90 % in the aqueous phase, and it has also been characterized for optimal temperature for enzymatic activity and stability.

Extraction of XR from this yeast following the above simple and inexpensive procedure was efficient without XDH. XDHs can hinder the process efficiency by transforming xylitol into xylulose. XR from *C. guilliermondii* FTI 20037 as main catalyst has a large affinity for the coenzyme in NADPH form as demonstrated by Silva et al. (1996).

8.3 Enzymatic Production of Xylitol: A Multi-Enzymatic Process

The first example of XR application as a main catalyst for xylitol production was reported in the mid 1980s with average results. For the enzymatic production of xylitol, Kitpreechavanich et al. (1984) reported the synthesis of this polyol from commercial xylose by XR from *Candida pelliculosa* (using intact cells and cell-free extracts) coupled to an oxide reductase system of *Methanobacterium* sp aiming at the reduction of NADP using H_2 as electron donor. The substrate was converted to xylitol with a stoichiometric equivalent of NADPH consumption (coenzyme: xylose = 1:30), where the coenzyme was regenerated and retained in a membrane reactor.

Ten years later, in another study, Nidetzky et al. (1996) studied the conversion of xylose to xylitol using a NADH-dependent XR obtained from *C. tenuis*. The reduction of xylose is coupled to an enzymatic oxidation of D-xylose or glucose dehydrogenase obtained from *Bacillus cereus*. In this study, a charged membrane reactor and an enzymatic regeneration system for the coenzyme was explored. However, results were relatively good but this technique did not get required attention due to the economics of the employed process. In 2006, a sustainable production process for enzymatic xylitol was reevaluated at the Engineering School of Lorena, University of São Paulo, Brazil. The main intention of this study was to improve the feasibility of xylitol enzymatic production.

According to Mertens et al. (2003), the regeneration of NADPH can be achieved by chemical, electrochemical and enzymatic methods (Aksu et al. 2009). The chemical and electrochemical process presents disadvantages due to the low selectivity and the occurrence of undesirable side reactions (Chenault and Whitesides 1987; Mertens et al. 2003). Indirect electrochemical processes can circumvent these problems by the use of mediators, which inhibit the process of purification and recovery of the product (Ruppert et al. 1988). Eguchi et al. (1983) reported the use of methanogenic bacteria for regenerating NADPH or hydrogen form using as a substrate. However, there are many limitations for anaerobic cultivation of bacteria. Due to the disadvantages, in recent years attention has been given to the use of enzymes in the regeneration of oxidized coenzymes. The main advantages of coenzyme regeneration using enzymes are high specificity and reduction efficiency. There are two strategies for enzymatic coenzyme regeneration (Fig. 8.3).

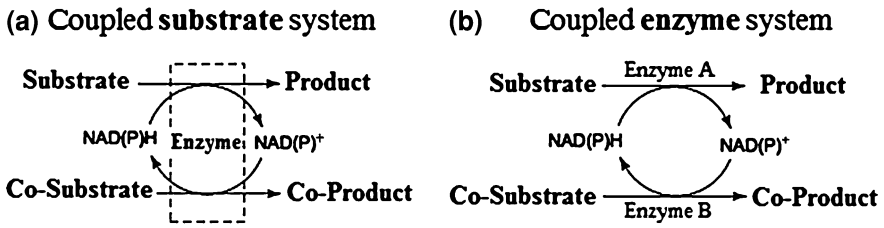

Fig. 8.3 Enzymatic systems for the coenzymes regeneration (modified from Kroutil et al. 2004). **a** Coupled substrate system. **b** Coupled enzyme system

According to Kroutil et al. (2004), the system connected to the substrate using a single enzyme, while simultaneously processing the substrate and the co-substrate (Fig. 8.3a). However, the co-substrate must be added in larger amounts than the substrate seeking to favorably shift the equilibrium of the reactions. As a consequence, these systems are commonly prevented by the co-substrate. To overcome this problem, utilization of a second biocatalyst for the coenzyme regeneration reduction reaction (Fig. 8.3b) is required. In such cases, this second enzyme (enzyme B) has to be used to restore the reduced coenzyme using a co-substrate and generating a co-product. Formate dehydrogenase (EC 1.2.1.2) from a recombinant strain of *Pseudomonas* sp. has shown promising results for the regeneration of $NAD(P)^+$ (Seelbach et al. 1996). This enzyme is commercially available and catalyzes formate oxidation to form carbon dioxide and water, using $NAD(P)^+$ as coenzyme. In fact, the formate dehydrogenase has been described as an economic alternative for the recovery of NAD(P)H in the reaction medium (Itoh et al. 1999; Kula 1994). This recombinant enzyme uses NADP (Seelbach et al. 1996). However, limitations to using this system of regeneration were found by Nidetzky et al. (1996). These authors observed that the activity of XR-NADH-dependent from *C. tenuis* was strongly inhibited in the presence of formate, which motivated the search for an alternative enzymatic regeneration system. In this case, a system was tested with glucose dehydrogenase. This regeneration system is very interesting because it co-produces gluconate, which has applications in the pharmaceutical and food industries (Crognale et al. 2008).

However, it is difficult to choose the "ideal" enzymatic regeneration system. There is no simple theoretical resolution for enzymatic regeneration due to the enzymes kinetics and interactions with other molecules. In order to elucidate this procedure, three enzymatic reactions have been summarized below:

(a) **Formate** + NADP ↔ CO_2 + NADPH
Enzyme: *Formate dehydrogenase*
(b) **Glucose** + NADP ↔ Glucono-δ-lactona + NADPH ↔ Glucono-δ-lactona + H_2O ↔ **gluconate**
Enzyme: *Glucose dehydrogenase*
(c) **Ethanol** + NADP ↔ **Acetaldehyde** + NADPH
Enzyme: *Ethanol dehydrogenase*

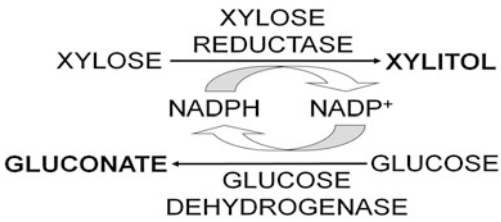

Fig. 8.4 Schematic presentation of the enzymatic reaction for xylitol production system with NADPH regenerating enzyme (Branco et al. 2011a, b)

Each system has advantages and disadvantages in relation to the other. The formate system does not generate a product, therefore facilitating purification steps and not producing a possible XR inhibitor. The glucose system uses a relatively cheap substrate (glucose) which can be obtained from lignocellulosic residues, and the system provides an interesting product, gluconate. The ethanol dehydrogenase regeneration system has the lowest price substrate (ethanol) in Brazil and produces acetaldehyde, which has huge market demand. All these dehydrogenases are similar in cost. From this point of view what will matter is the influence of this system on the xylitol production. In a "perfect" process, the in situ regeneration system would not affect the main goal; however, it is known that enzymes can be greatly influenced by different chemicals and experimental conditions. Therefore, the definitive choice can only be achieved by measuring the influence of the possible co-substrates and co-products on XR production.

The results showed that substances such as formate, glucose, gluconate, ethanol and acetaldehyde inhibited XR from *C. guilliermondii*. Acetaldehyde showed inhibition constant even at micromole scale; as a consequence the system using ethanol dehydrogenase was not found adequate. Formate and glucose dehydrogenase systems also exhibited inhibition, however the inhibition of XR was less severe. In this situation, the glucose dehydrogenase system was selected for of two reasons: the production and distribution of gluconate could cover or surpass the cost invested in the in situ coenzyme regeneration and the possibility of using glucose as a carbon source from lignocellulosic material which could lead this process to be green and sustainable.

A complete and mechanistic reaction of xylitol production using a coenzyme regeneration system with glucose dehydrogenase is shown in Fig. 8.4.

The enzymatic production of xylitol using the glucose dehydrogenase system to regenerate the NADPH is more environmentally friendly and economically attractive. The interesting concept is the use of lignocellulosic wastes as a source of xylose and glucose (substrate and co-substrate) in the main process.

8.4 Sustainable Alternative: Use of Sugarcane Bagasse

Nowadays, it is very important to understand the ecological impact and practice use of sustainable processes in biotechnological processes. In this line, harnessing of lignocellulosic materials for the sustainable production of value-added products

such as xylitol via biotechnological routes provides new hope. They are considered residues after vegetal biomass processing and disposal by industrial, agricultural and forestry activities. Lignocellulosic residues represent an abundant source of organic compounds that are potential raw material for industrial bioprocessing. In Brazil, the largest lignocellulosic residue is the sugarcane bagasse (SB) which is originated in the sugar-alcohol industry in plentiful amounts. In 2010/2011, Brazilian sugarcane harvesting was 623 million tons. Out of which, 344.5 million tons (55.3 %) were designated to produce ethanol and the rest to produce sugar (sucrose) (CONAB 2011). From 1,000 kg (1 ton) of sugarcane processed, 135 kg (dry mass) of SB is formed (Baudel et al. 2005). In general, half of the SB is burnt in boilers to generate the steam (Frollini and Pimenta 1997). Brazilian sugar-alcohol industries use approximately 90 % of SB to sell electricity for proximate cities. This means that in 2011, in Brazil alone, 84.1 millions of tons of SB were generated and, at least, 8.4 million tons were disposed. Besides energy generation, SB can be used for paper, paperboard, animal feed and fertilizer production (Pandey et al. 2000), although these products do not contribute significantly (less than 0.01 %) to the use of this residue. The excess amount of SB is considered to be an environmental problem; its accumulation for long periods can even trigger spontaneous combustion (Dawson et al. 1990).

It is known that SB is rich in polymeric compounds like cellulose, hemicellulose and lignin. According to Brienzo et al. (2009) almost one-third of SB is hemicellulose, an L-arabino-(4-O-methyl-D-glucurono)-D-xylan which is basically a xylose chain. SB also has 42.4 % of cellulose, which can almost entirely be converted to glucose (Chandel et al. 2012).

Therefore, it is important to develop green technologies for using SB to produce useful goods for mankind and, with these objectives, numerous biotechnological processes have been studied in recent decades and the research is continued with fast pace (Chandel et al. 2012). Biotechnological xylitol production from SBHH has been suggested as a promising alternative (Chandel et al. 2012).

However, there are several challenges in front of researchers for the successful and economical production of xylitol from SBHH due to inhibitors and less yields (Santos et al. 2005; Branco et al. 2007). From this point of view, enzymatic production of xylitol from SB offers new dimensions and a promising biotechnological alternative. In this context, studies were performed to investigate the influence of SBHH on the enzyme-mediated xylitol production. Table 8.2 presents some results attained from these investigations.

Promising results were attained for enzymatic xylitol production using SBHH and commercial xylose and glucose. As can be observed in Table 8.2, the process conversion efficiency is not alterated by SBHH content, and volumetric productivity is decreased with the content increase of SBHH. However, it was observed that until 40 % SBHH content was reached, volumetric productivity was not majorly influenced. These results are strong evidence for the viability to use SB as a glucose and xylose source for the enzymatic process. More details about this study can be found in Branco et al. (2011a, b).

Table 8.2 Process parameters for the enzymatic xylitol production with different sugarcane bagasse concentrations

Reaction medium SBCHH content (v v^{-1})	Conversion efficiency (%)	Q_p (g L^{-1} h^{-1})	Xylitol (mM)
Control[a]	98 ± 2.3	0.33 ± 0.025	26.0 ± 0.10
40	98 ± 2.5	0.32 ± 0.014	25.2 ± 0.012
100	98 ± 1.5	0.26 ± 0.015	20.5 ± 0.11

[a] The control experiment was carried out with commercial xylose and glucose (hydrolysate free). Adapted from Branco et al. (2011a, b)

Table 8.3 Advantages and disadvantages of the production processes for xylitol

Process type	Advantages	Disadvantages
Chemical	Optimized High productivity	High installation cost Use of natural resources
Microbial	Lower cost Sustainable	Low productivity
Enzymatic	Sustainable Maximum efficiency High productivity	Cost[a]

[a] Since it is a relatively new process there is not enough data to evaluate cost analyses

8.5 Technological Comparison of Current Processes for Xylitol Production

As mentioned before, xylitol enzymatic production results in higher yields and productivity and could be the replacement of the fermentation and chemical mediated processes. In fact, some studies aimed at the production of xylitol by the enzymatic process using xylose as a commercial substrate showed a conversion efficiency of 100 %. One limitation, that was discussed previously, refers to the addition of coenzyme NAD(P)H, a very expensive reagent.

In view of the technological processes described for xylitol production, a comparison between their advantages and disadvantages is shown in Table 8.3.

As can be seen in Table 8.3 the process for xylitol enzymatic production is the union of the other two process advantages. The advantages of the enzymatic conversion are due to: (1) better mass transfer in comparison to microbial processes, because the "barriers" are minimum (similar to a chemical reaction) consequently increasing the productivity; in microbial processes the substrate and the product have to go through many steps and cellular compartments, especially xylose membrane transportation into the cell; (2) no metabolism deviation for biomass and energy generation, therefore a stoichiometric conversion of xylose to xylitol can be achieved, different than the 0.917 g g^{-1} value (Barbosa et al. 1988) from the microbial conversion; and (3) sustainability. Similar to the microbial, the enzymatic process can be carried out using lignocellulosic residues, as shown before in this chapter. However, enzymatic production of xylitol is still in the

nascent stage, and there are therefore still several doubts about its economic viability, especially the costs associated with the enzyme and coenzyme.

Concerning results, efficiency and productivity values from the enzymatic process were 100 % and 1.58 g L^{-1} h^{-1}, respectively (Branco et al. 2011a), which is higher than found in literature for xylitol production using *Debaromyces hansenii* in synthetic medium under batch regime at 84 % and 1.49 g L^{-1} h^{-1}, respectively (Sampaio et al. 2006).

8.6 Conclusions and Future Recommendations

Xylitol enzymatic production is an alternative to the traditional ways. It is a simple coenzyme dependent process with huge potential. Coenzymes are a limiting factor in the reactions of biocatalysis. Thus, the use of isolated enzymes, especially intracellular enzymes, requires the addition of these molecules. These reactions are less attractive from an economical point of view. In order to establish an economic technological platform, it is recommended to use a regeneration system, preferably an enzymatic in situ approach. In this chapter, an in situ enzymatic regeneration system of coenzymes for xylitol production was discussed in detail. Lignocellulosic material, particularly SB, can be of great help to this enzymatic process resulting in mutual advantages for the process viability and for the environment.

Enzymatic production of xylitol is a realistic approach to increase the economic viability of this process. The following considerations are recommended for the enzymatic production of xylitol in future: (a) Use a NADH-dependent XR instead of a NADPH-dependent, because NADH is cheaper than NADPH; (b) Find or engineer a more chemically-stable XR to increase its half-life; (c) Develop in-house, and most likely in situ, NADH production using microbial biomass permeation with high content of this coenzyme; and (d) Study continuous and semi-continuous conversion processes with immobilized XR and/or using membrane reactors.

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Chapter 9
Bioenergetic Aspects of Xylitol Production from Lignocellulosic Materials

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Abstract With the aim of identifying the best experimental conditions able to optimize the industrial production of xylitol from lignocellulosic materials, this chapter provides a review about the present knowledge on the use of material and bioenergetic balances implied in xylose-to-xylitol bioconversion by yeasts. To this purpose, xylose metabolism was investigated in three different pentose-metabolizing yeasts, namely, *Pachysolen tannophilus*, *Candida guilliermondii* and *Debaryomyces hansenii*, using different lignocellulosic hydrolyzates as carbon and energy sources. The main hypotheses on which material and bioenergetic balances were based are (a) fermentative assimilation of xylose, (b) semi-aerobic xylose-to-xylitol bioconversion, (c) biomass growth from pentoses, (d) catabolic oxidation of xylose and (e) NADH regeneration by the electron transport system. Similar approaches could be proposed to investigate and model other semi-aerobic processes.

Keywords Material balances · Bioenergetics · Xylitol production · Lignocellulosics · Yeasts

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9.1 Introduction

The use of an optimized biotechnological process could be a cheaper way to produce xylitol compared to the present catalytic hydrogenation of D-xylose obtained by hydrolysis of lignocellulosic xylan (Tran et al. 2004). It would in fact take place under mild conditions and utilize directly hemicellulosic hydrolyzates (Rivas et al. 2002), even though new purification techniques have to be set up to produce food-grade xylitol (Domínguez et al. 2005; Rivas et al. 2006).

Xylose-to-xylitol bioconversion can be carried out by bacteria, filamentous fungi and yeasts (Parajó et al. 1998a), although the best known xylitol producers are undoubtedly yeasts. For this reason, most of the metabolic studies discussed in this chapter were mainly focused on pentose-metabolizing yeasts, with special emphasis on the genera *Candida* (Barbosa et al. 1988; Oh and Kim 1998; Oh et al. 1998; Sirisansaneeyakul et al. 1995) and *Debaryomyces* (Converti et al. 2002; Domínguez et al. 1997; Gírio et al. 1994; Parajó et al. 1998a, b, c). In general and with only a few differences, these pentose-metabolizing yeasts are able to grow in high saline media and to produce polyols (Gírio et al. 2000; Parajó et al. 1998a, b, c). However, as stressed by Nobre et al. (2002), to further support any program of metabolic engineering addressed to xylitol overproduction by yeasts, deeper understanding of biochemical pathways and metabolic regulation is needed. In all cases, to apply a metabolic model based on carbon material balances to xylose-to-xylitol bioconversion in hemicellulose hydrolyzate, the experimental composition of the medium in terms of both substrates and products before, during and after fermentation is needed as well as the elemental composition of biomass.

Based on the pioneering approaches of Pirt (1965) and Stouthamer and Bettenhaussen (1973), making use of global equations for substrate and ATP specific variation rates, several authors have successfully performed bioenergetic investigations on different microbial systems (van Gulik et al. 2000; Harris et al. 2006, Jørgensen et al. 1995; Mignone and Donati 2004; Tsai and Lee 1990; Zeng and Deckwer 1995). Following a similar approach, several attempts have then been made to elucidate the metabolic behaviors of different pentose-metabolizing yeasts, namely, *Debaryomyces hansenii* (Converti et al. 2001, 2002; Rivas et al. 2003, 2009; Sampaio et al. 2004, 2005, 2006, 2008), *Candida guilliermondii* (Carvalho et al. 2002, 2005; Santos et al. 2003; Sene et al. 2001) and *Pachysolen tannophilus* (Converti et al. 1999, 2001), either in synthetic media or hemicellulose hydrolyzates of different raw materials. To this purpose, the fermentative assimilation of pentoses (mainly xylose, but eventually also additional minor sugars, depending on the composition of lignocellulose hydrolysate), semi-aerobic pentose-to-pentitol bioconversion, biomass growth on sugars, partial catabolic oxidation of sugars and eventual minor carbon sources, along with NADH regeneration by the electron transport system, have been regarded as the main microbial activities and investigated using the experimental data of substrates consumption and products formation, including oxygen and carbon dioxide (Rivas et al. 2003, 2009; Sampaio et al. 2004). In this way, the main bioenergetic

parameters of the metabolism of these yeasts were estimated, among which were the *P/O* ratio (an indirect measure of oxidative phosphorylation efficiency) and the ATP requirements for growth and maintenance.

9.2 Transport and Related Energy Requirements

To utilize xylose as a carbon and energy source, the yeast must be able to transport such a pentose from the extracellular medium to the intracellular cytosol. Several species make use of a specific carrier to do this, through facilitated diffusion or proton-dependent transport.

To provide only a few examples, *Pichia stipitis* NRRL Y-7124 and CBS 7126 were shown to transport xylose and glucose by a sugar/H^+-symport. In particular, the latter strain was shown to possess two different systems for xylose transport, the one having high affinity for xylose (k_m = 0.06 mM) and being non-competitively inhibited by glucose, and the other with low affinity (k_m = 1.89 mM), which is also used for glucose (k_m = 0.26 mM) (Kilian and van Uden 1998). Similar low affinity (k_m = 67.6 mM) and high affinity (k_m = 1.9 mM) systems, both being likely sugar/H^+-symports, were detected in *Candida utilis* (Kilian et al. 1993), a yeast able to grow on xylose but not to ferment it. *Candida shehatae* CBS 2779 transports both xylose (k_m = 125 mM) and glucose (k_m = 2.0 mM) by a facilitated diffusion-system, whereas two different sugar/H^+-symports are induced under carbon shortage, the one for xylose (k_m = 1.0 mM) and the other for glucose (k_m = 0.12 mM) (Lucas and van Uden 1986). Using intact cells of *Candida mogii* ATCC 18364 marked with ^{14}C-xylose, Sirisansaneeyakul et al. (1995) observed that xylose transport in this yeast followed Michaelis–Menten type kinetics, which suggested the occurrence of carrier-mediated facilitated diffusion. Finally, Gárdonyi et al. (2003) observed biphasic transport kinetics in the yeast *Candida intermedia* PYCC 4715 that was able to grow on xylose at the same specific rate than on glucose. At low xylose levels it utilized a high-affinity (k_m = 0.2 mM), low-capacity xylose/H^+-symport that was strongly inhibited by low glucose concentrations and high L-arabinose and D-ribose concentrations, whereas at high xylose levels the transport was mediated by a low-affinity (k_m = 50 mM), high-capacity system likely based on facilitated diffusion.

9.3 Xylose Metabolism

The first step to set up a xylose-to-xylitol bioconversion model based on carbon material balances is the collection of all the information available on xylose metabolism, in order to write the network of stoichiometric equations describing the main metabolic pathways that are significant for the physiology of the selected yeast.

Briefly, in yeasts xylose is firstly reduced by a NADPH- or NADH-dependent xylose reductase (XR) (EC 1.1.1.21) that is codified by the gene *xyl1*. Xylitol is then oxidized to xylulose by a NAD^+- or $NADP^+$-dependent xylitol dehydrogenase (XDH) (EC 1.1.1.9) codified by the gene *xyl2*. This reaction is followed by the formation of xylulose-5-phosphate catalyzed by an ATP-dependent xylulose kinase (EC 2.7.1.17), which enters the pentose-phosphate pathway (PPP) (Hahn-Hägerdal et al. 1994) and is partially converted into pyruvate through a connection with the Embden-Meyerhof-Parnas (EMP) pathway, i.e. the glycolysis.

Figure 9.1 shows a general schematic of xylose metabolism in pentose-metabolizing yeasts, which leads to the formation of glycerol and ethanol in addition to xylitol. Xylitol formation is hindered by strict anaerobic conditions, owing to scarce or negligible NADH-dependent XR activity; in fact, NADH accumulated in the cytosol can be only partially regenerated, thus leading to the production of ethanol (Gírio et al. 2000; Granström et al. 2000; Nobre et al. 1999) and glycerol, a way to mitigate the redox unbalance (Granström and Leisola 2002; Granström et al. 2000). Under excess aerobiosis, oxygen can re-oxidize NADH in the respiratory chain, hence stimulating growth and equally affecting xylitol production; therefore, xylitol accumulation can only be ensured by semi-aerobic conditions (Gírio et al. 2000; Sampaio et al. 2004), under which oxygen is just sufficient to regenerate NADH, while NADPH produced by the PPP is almost entirely addressed to xylitol formation (Barbosa et al. 1988). So, the PPP is fundamental to produce NADPH, the most utilized cofactor of the first enzyme of xylose metabolism.

From these findings one can realize that, as for any enzymatic process, knowledge of the specificity for cofactors and substrates is significant for the success of xylose-to-xylitol bioconversion as well. Granström et al. (2002) purified XR from *C. guilliermondii* ATCC 20118 that was shown to accept only NADPH as cofactor and exhibited high affinity (low Michaelis constant) for L-arabinose ($k_m = 66$ mM), D-xylose ($k_m = 79$ mM) and D-ribose ($k_m = 155$ mM) but low affinity for D-lyxose ($k_m = 1427$ mM), L-xylose ($k_m = 1308$ mM) and L-ribose ($k_m = 1061$ mM).

On the other hand, Verduyn et al. (1985) demonstrated that purified XR from *P. stipitis* has dual specificity for NADPH and NADH (with that for the former cofactor higher), and is active on different haldoses such as D, L-glyceraldheyde, L-arabinose, D-xylose, D-ribose, D-galactose and D-glucose. Under anaerobic conditions, such a duality allowed for satisfactory redox balance, and the reducing power produced by xylitol oxidation could be utilized to reduce xylose. From these considerations, it is evident that strains with reduced or absent NADPH-dependent XR activity can overproduce ethanol (Verduyn et al. 1985), while those with no or with negligible NADH-dependent XR activity should be considered good xylitol producers (Gírio et al. 1989; Roseiro et al. 1991).

Based on the Michaelis constant for NADPH ($k_m = 0.036$ mM) and NADH ($k_m = 0.085$ mM), Sirisansaneeyakul et al. (1995) concluded that in *Candida mogii* ATCC 18364 xylose is preferentially reduced to xylitol by a NADPH-dependent XR, and this is re-oxidized to xylulose by a NAD^+-dependent xylitol

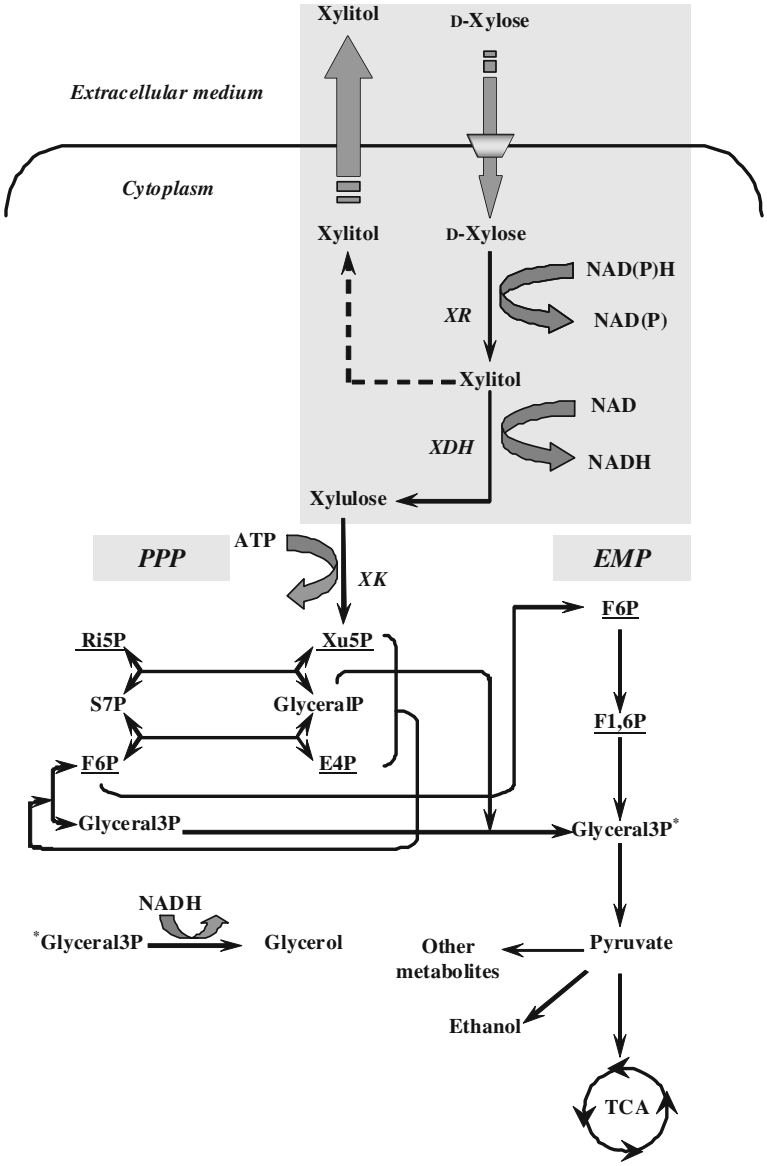

Fig. 9.1 Schematic of xylose metabolism in yeasts. *PPP* pentose-phosphate pathway, *EMP* Embden-Meyerhof-Parnas pathway, *F1,6P* fructose-1,6-diphosphate, *E4P* erythrose-4-phophate, *Ri5P* ribose-5-phosphate, *Xu5P* xylulose-5-phosphate, *S7P* sedoheptulose-7-phosphate, *F6P* fructose-6-phosphate, *Glyceral3P* glyceraldheyde-3-phosphate, *XR* xylose reductase, *XDH* xylitol dehydrogenase, *XK* xylulose kinase. Cofactors: $NADP^+$, NADPH, NAD^+, NADH and ATP (modifications made on the schematics proposed by Ostergaard et al. 2000 and Zaldivar et al. 2002)

dehydrogenase. Nolleau et al. (1995) proposed the same cofactor specificity for the enzymes of *C. guilliermondii* and *Candida parapsilosis.*

Granström and Leisola (2002) detected glucose-6-phosphate dehydrogenase and NAD^+- and $NADP^+$-dependent isocitrate dehydrogenase activities when *Candida tropicalis* VTT-C-78086 was cultivated at high xylose levels under oxygen-limited conditions, either in the presence or the absence of formate, which suggested NADPH generation mainly through the PPP. On the other hand, acetate accumulation during *C. guilliermondii* VTT-C-71006 and *C. milleri* cultivations suggested NADPH generation via acetaldheyde dehydrogenase (Granström and Leisola 2002; Granström et al. 2000). Similar $NADP^+$-dependent acetaldheyde dehydrogenase activity was reported by van Gulik and Heijnen (1995) for *S. cerevisiae*.

Other enzymes playing an important role in establishing the NADPH intracellular level are the malic enzyme (van Gulik and Heijnen 1995) and malate dehydrogenase, whose activities in *C. tropicalis* depend on NAD(P)H and NAD^+, respectively, and are subject to a "transhydrogenase cycle" that allows NADPH to be converted into NADH (Granström et al. 2002).

9.4 Xylitol Production Associated with Cell Growth

A correct material balance for a bioprocess requires knowledge of the relationship existing between cell growth and product formation, on which its stoichiometry can be based.

For this purpose, one can resort to the fermentation classification of Gaden (1959), according to which all bioprocesses can be grouped in three different categories depending on the way growth and product formation are associated with one another. The first category includes those processes, like alcohol fermentation, where the product arises directly from the energy metabolism of carbohydrates supplied, and its specific rate of formation (q_P) is directly associated with that of growth (μ) according to the equation $q_P = A\mu$. The second category includes processes, like citric or lactic acid fermentations, where products come indirectly from the carbohydrate metabolism, and their formation is associated with growth only during the idiophase because of some metabolic dysfunction or inhibition ($q_P = B + A\mu$). On the other hand, the third category gathers bioprocesses implying the biosynthesis of complex secondary metabolites (enzymes, antibiotics and vitamins) apparently unrelated to carbohydrate oxidation and dissociated with growth ($q_P = B$).

Results of some works employing different experimental strategies were already utilized successfully to establish the relationship existing between cell growth and xylitol accumulation in pentose-fermenting yeasts. To give only some examples, Saraçoğlu and Çavuşoğlu (1999) observed that *C. tropicalis* Kuen 1022 was able to uptake 85–90 % of xylose in synthetic medium and 84 % in sunflower hydrolysate after 96 h of cultivation, during which no lag phase was observed, and

cell growth and xylitol production took place simultaneously, thus highlighting a process of the first category. Analogously, Roberto et al. (1999) reported that *C. guilliermondii* FTI 20037 cultivated in rice straw hemicellulose hydrolyzate initially grew at a maximum specific growth rate (μ_{max}) of 0.15 h^{-1} consuming only glucose without any product formation, and then produced xylitol from xylose at a maximum specific rate of xylitol formation of 0.12 g g^{-1} h^{-1} when μ decreased to one-fifth of the maximum value. Based on the above Gaden classification, also such a second phase of the bioprocess was proposed to be a growth-associated production.

On the other hand, Aranda-Barradas et al. (2000), performing a kinetic study on xylitol production by *C. parapsilosis* ATCC 28474 under oxygen-limited conditions, proposed a subdivision of the process into three different phases. In the first phase of excess substrate, where the microorganism grew at μ_{max}, and the second one, during which oxygen became the limiting substrate, no xylitol accumulation was associated with xylose consumption. During the last phase the process was limited by the oxygen transfer capability of the reactor, and xylitol formation was associated with growth.

9.5 Material Balances of Xylose-to-Xylitol Bioconversion

Cell growth and product formation are the result of complex pathways composed of a large number of intracellular reactions that influence the final stoichiometry and overall kinetics of any bioprocess. For this reason, the cell elemental composition of a lot of bacteria and yeasts (Roels 1983; Shuler and Kargi 2002) was used in different attempts to make material balances. Another fundamental requisite to this purpose is the electric balance usually based on protons and electrons (Shuler and Kargi 2002).

A first attempt in the field of xylose-to-xylitol bioconversion was that proposed by Barbosa et al. (1988), where xylose metabolism in *C. guilliermondii* was modeled considering the two starting steps of xylose uptake and using stoichiometric balances based on the basic knowledge on its metabolism available at that time. To this purpose, four assumptions were made. The first of them dealt with the type of cofactor employed by the two enzymes involved in those steps, i.e. mainly NADPH and only to a minor extent NADH by XR, and NAD^+ by XDH. The second assumption was that, under aerobic (micro-aerophilic) conditions, all D-xylose is reduced to xylitol by the NADPH generated in the PPP, while xylitol is re-oxidized to xylulose using the NAD^+ regenerated in the respiratory chain as cofactor. The absence of any transhydrogenase activity responsible for the conversion of NADH into NADPH was also supposed. The fourth, and last, assumption was that, in the absence of any growth, xylitol is oxidized to xylulose only to the minimum extent necessary to furnish the NADH amount required to sustain the respiratory chain, whereas the excess is excreted outside the cell.

Under aerobiosis, xylitol would be oxidized to xylulose by the NAD^+ regenerated via respiratory chain and then catabolized only to produce glucose-6-phosphate. Subsequent oxidation of 1 mol of glucose-6-phosphate through the PPP would generate CO_2 and 12 mol of NADPH from $NADP^+$, and the cofactor (electric) balance could be only guaranteed by the consumption of NADPH associated with xylose-to-xylitol reduction catalyzed by XR. The theoretical xylitol yield on consumed xylose estimated by the above authors under these conditions and hypotheses (0.90 mol/mol) was practically the same as that estimated supposing a dual NADPH and NADH-dependent XR activity (0.905 mol/mol). On the other hand, under anaerobic conditions NADH could not be regenerated via the respiratory chain; therefore, the maximum xylitol theoretical yield lowered to 0.875 mol/mol also due to ethanol formation, according to the schematic earlier depicted in Fig. 9.1.

Based on this pioneering information, different studies based on material balances were developed using different yeasts as fermenting agents, specifically *P. tannophilus* (Converti et al. 1999, 2001), *C. guilliermondii* (Carvalho et al. 2002; Sene et al. 2001) and *D. hansenii* (Converti and Domínguez 2001; Sampaio et al. 2005, 2006, 2008).

A simplified stoichiometric network was proposed by Sampaio et al. (2004) for *D. hansenii* UFV-170 cultivation in synthetic medium (Table 9.1), which was based on the general knowledge available in the literature on xylose metabolism in pentose-fermenting yeasts (Barbosa et al. 1988) and the specificities of XR and XDH activities previously reported for this yeast (Gírio et al. 1990, 1996). Using xylose as the only carbon and energy source, it was assumed the simultaneous occurrence of (a) fermentative production of ethanol from D-xylose, (b) semi-aerobic xylose-to-xylitol bioreduction, (c) biomass growth, (d) catabolic oxidation of xylose, and (e) oxidative phosphorylation.

In particular, ethanol formation observed under strongly oxygen-limited conditions was taken into consideration by the sub-network (*a* in Table 9.1), according to which glucose-6-phosphate is produced from xylose through the PPP (Eq. a1 in Table 9.1). A fraction of this metabolite was assumed to be completely oxidized by the same pathway (Eq. a2 in Table 9.1) to provide the reducing power in the form of NADPH needed to sustain the preceding reaction, while the rest is to be fermented to ethanol and CO_2 by the EMP pathway (Eq. a3 in Table 9.1), hence leading to the stoichiometry described by Eq. (a4 in Table 9.1). According to the literature (Gírio et al. 1990; Parajó et al. 1998a), since *D. hansenii* is a yeast typically possessing exclusive NADPH-dependent XR activity, xylitol formation was assumed to be the result of the semi-aerobic metabolism of xylose in this yeast (Eq. b in Table 9.1). The rest of xylose uptaken in addition to the fractions required for the above activities was assumed to be consumed by the catabolic oxidation of glucose-6-phosphate through the EMP (Eq. d3 in Table 9.1). Besides, since the route described by Eq. (d2 in Table 9.1) is ineffective from the energetic point of view (ATP production), it was supposed that it was utilized by the cell only to produce the NADPH required for both glucose-6-phosphate (Eq. d1 in Table 9.1) and biomass (Eq. c in Table 9.1) production.

Table 9.1 Model proposed by Sampaio et al. (2004) for D-xylose metabolism in *D. hansenii* UFV-170

(a) **Fermentative Assimilation of Xylose**[a]

(a1) *Glucose 6P production by the PPP*:

$$1.2\ \text{Xylose} + 1.2\ \text{ATP} + 1.2\ \text{NADPH}_2^+ + 1.2\ \text{NAD}^+ \rightarrow \text{Glucose 6P} + 1.2\ \text{NADP}^+ + 1.2\ \text{NADH}_2^+ + 1.2\ \text{ADP} + 0.2\ \text{P}_i$$

(a2) *NADPH production by the PPP*:

$$0.1\ \text{Glucose 6P} + 1.2\ \text{NADP}^+ + 0.6\ \text{H}_2\text{O} \rightarrow 0.6\ \text{CO}_2 + 1.2\ \text{NADPH}_2^+ + 0.1\ \text{P}_i$$

(a3) *Ethanol formation in the EMP pathway*:

$$0.9\ \text{Glucose 6P} + 2.7\ \text{ADP} + 1.8\ \text{P}_i \rightarrow 1.8\ \text{EtOH} + 1.8\ \text{CO}_2 + 2.7\ \text{ATP}$$

(a4) *Total Eqs. a1–a3*:

$$1.2\ \text{Xylose} + 1.2\ \text{NAD}^+ + 0.6\ \text{H}_2\text{O} + 1.5\ \text{ADP} + 1.5\ \text{P}_i \rightarrow 1.8\ \text{EtOH} + 2.4\ \text{CO}_2 + 1.5\ \text{ATP} + 1.2\ \text{NADH}_2^+$$

(b) **Xylose-to-Xylitol Bioconversion**

$$\text{Xylose} + 0.5\ \text{H}_2\text{O} + 0.1\ \text{ATP} + 0.1\ \text{NAD}^+ \rightarrow 0.9\ \text{Xylitol} + 0.5\ \text{CO}_2 + 0.1\ \text{NADH}_2^+ + 0.1\ \text{ADP} + 0.1\ \text{P}_i$$

(c) **Biomass Growth on Xylose**[b]

$$0.219\ \text{Xylose} + 0.2\ \text{NH}_3 + 0.240\ \text{NADPH}_2^+ + Y'_{\text{ATP}}\ \text{ATP} + 0.335\ \text{NAD}^+ \rightarrow 0.095\ \text{CO}_2 + 0.405\ \text{H}_2\text{O} + 0.335\ \text{NADH}_2^+ + \text{CH}_{1.79}\text{O}_{0.50}\text{N}_{0.20} + Y'_{\text{ATP}}\ \text{ADP} + Y'_{\text{ATP}}\ \text{P}_i + 0.240\ \text{NADP}^+$$

(d) **Catabolic Oxidation of Xylose**

(d1) *Glucose 6P production by the PPP*:

$$1.2\ \text{Xylose} + 1.2\ \text{ATP} + 1.2\ \text{NADPH}_2^+ + 1.2\ \text{NAD}^+ \rightarrow \text{Glucose 6P} + 1.2\ \text{NADP}^+ + 1.2\ \text{NADH}_2^+ + 1.2\ \text{ADP} + 0.2\ \text{P}_i$$

(d2) *Glucose 6P oxidation by the PPP with NADPH production for growth*:

$$y\ \text{Glucose 6P} + 6y\ \text{H}_2\text{O} + 12y\ \text{NADP}^+ \rightarrow 6y\ \text{CO}_2 + 12y\ \text{NADPH}_2^+ + y\ \text{P}_i$$

(d3) *Glucose 6P oxidation by the EMP pathway*:

$$(1-y)\ \text{Glucose 6P} + 6(1-y)\ \text{H}_2\text{O} + 12(1-y)\ \text{NAD}^+ + 5(1-y)\ \text{ADP} + 4(1-y)\ \text{P}_i \rightarrow 6(1-y)\ \text{CO}_2 + 12(1-y)\ \text{NADH}_2^+ + 5(1-y)\ \text{ATP}$$

(d4) *Total Eqs. d1–d3*:

$$1.2\ \text{Xylose} + 6\ \text{H}_2\text{O} + 12(y-0.1)\ \text{NADP}^+ + 12(1.1-y)\ \text{NAD}^+ + 5(0.76-y)\ \text{P}_i + 5(0.76-y)\ \text{ADP} \rightarrow 6\ \text{CO}_2 + 12(y-0.1)\ \text{NADPH}_2^+ + 12(1.1-y)\ \text{NADH}_2^+ + 5(0.76-y)\ \text{ATP}$$

(e) **NADH Regeneration by the Electron Transport System**

$$2\ \text{NADH}_2^+ + \text{O}_2 + 2P/O\ \text{ADP} + 2P/O\ \text{P}_i \rightarrow 2\ \text{H}_2\text{O} + 2P/O\ \text{ATP} + 2\ \text{NAD}^+$$

[a] NADH_2^+ stands for reducing equivalents in the form either of $\text{NADH} + \text{H}^+$ or FADH_2

[b] Y'_{ATP} stands for the overall ATP requirements of biomass expressed in $\text{mmol}_{\text{ATP}}/\text{C-mol}_{\text{DM}}$

Regarding biomass synthesis, the average composition reported for dry yeast biomass ($\text{CH}_{1.79}\text{O}_{0.50}\text{N}_{0.20}$) by Roels (1983) was used in the model, due to the absence in the literature of data on the elemental composition of *D. hansenii*.

Neglecting the little difference in composition between cell mass and precursors (Roels 1983), biomass formation from xylose and ammonia-containing nitrogen sources was described by Eq. (c) in Table 9.1 (Rivas et al. 2003). To this purpose, it was assumed, on the basis of literature data for yeasts (Bruinenberg et al. 1983),

an average reducing power requirement necessary for the growth of 1 C-mol$_{DM}$ biomass of 0.24 mol NADPH, coming from xylose catabolism through the PPP (Eqs. d1 and d2 in Table 9.1).

Under pseudo-steady-state conditions, like those present inside the cell, all the energy produced by the metabolism in the form of ATP must be utilized for growth and maintenance, or even wasted by energy spilling or through futile cycles in the presence of excess substrate (Liu 1996); therefore, the coefficient Y'_{ATP} (Eq. c in Table 9.1) did refer to the sum of all these ATP requirements (expressed in mol$_{ATP}$/C-mol$_{DM}^{-1}$). Finally, all the reducing power produced by xylose metabolism in the cytosol in the form of NADH or $FADH_2$ was assumed to be utilized for oxygen reduction through the electron transport system associated with the oxidative phosphorylation (Eq. e in Table 9.1), whose average effectiveness was expressed by the *P/O* ratio.

9.6 Energetic Balances for Facultative Anaerobes

Models based on material and ATP balances can be very useful to estimate the main bioenergetic parameters, namely, (a) the effectiveness of oxidative phosphorylation (*P/O*) under different environmental conditions and (b) the overall ATP requirements for growth and maintenance ($1/Y_{ATP} + m_{ATP}/\mu$), expressed in moles of ATP per mol of biomass.

Zeng et al. (1990) proposed an interesting method to estimate the *P/O* ratio and biomass yield on ATP (Y_{ATP}) in falcultative anaerobes based on the hypothesis of negligible ATP accumulation in the cell, because of the occurrence of pseudo steady state conditions, and making use of simple equations of ATP balances expressed in terms of specific rates per cell unit mass:

$$q^c{}_{ATP} = q^f{}_{ATP} + q^r{}_{ATP} + q^o{}_{ATP} \tag{9.1}$$

where $q^f{}_{ATP}$, $q^r{}_{ATP}$ and $q^o{}_{ATP}$ are the ATP contributions related to fermentation, respiratory chain and oxidative phosphorylation, respectively, and $q^c{}_{ATP}$ the overall energetic cell requirements.

To estimate the *P/O* ratio, it was assumed that all oxygen is utilized to re-oxidize the dehydrogenase cofactors ("H_2"), either in terms of NADH or $FADH_2$, according to the equation:

$$\text{``}H_2\text{''} + (P/O)\ \text{ADP} + (P/O)\text{P}_i + \frac{1}{2}\text{O}_2 \rightarrow (\text{P/O})\ \text{ATP} + \ \text{H}_2\text{O} \tag{9.2}$$

Therefore, the term $q^o{}_{ATP}$ appearing in Eq. (9.1) was expressed as:

$$q^o{}_{ATP} = 2 \cdot (P/O) \cdot q_{O_2} \tag{9.3}$$

where q_{O_2} is the specific rate of O_2 consumption by the cells, while $q^c{}_{ATP}$ was represented as a function of Y_{ATP} and specific growth rate:

$$q^{c}{}_{\mathrm{ATP}} = \mu / Y_{\mathrm{ATP}} \tag{9.4}$$

Substitution of Eqs. (9.3) and (9.4) into Eq. (9.1) and rearranging gave:

$$q^{f}{}_{\mathrm{ATP}} + q^{r}{}_{\mathrm{ATP}} = \mu / Y_{\mathrm{ATP}} - 2 \cdot (P/O) \cdot q_{\mathrm{O_2}} \tag{9.5}$$

where the term $q^{f}{}_{\mathrm{ATP}}$ was calculated from the concentrations of fermentation products and, similarly, $q^{r}{}_{\mathrm{ATP}}$ from the rate of CO_2 formation from glycolysis and tricarboxylic acid cycle ($q^{r}{}_{\mathrm{CO_2}}$), i.e. the catabolic reaction.

A further assumption of Zeng et al. (1990) was that Y_{ATP} only depends on the specific growth rate, which is valid only in those systems where maintenance can be neglected. So, from the results of continuous semi-aerobic cultures of *Enterobacter aerogenes* DSM 30053, performed on glucose under conditions of constant μ and dilution rate (0.1 h^{-1}) and different rates of oxygen supply, the above authors were able to estimate *P/O* (1.75 mol ATP/0.5 mol O_2) and Y_{ATP} (3.5 g biomass/mol of ATP) from the slopes [−2 (*P/O*)] and intercepts on the ordinate axis (μ / Y_{ATP}) of plots of $q^{f}{}_{\mathrm{ATP}} + q^{r}{}_{\mathrm{ATP}}$ versus $q_{\mathrm{O_2}}$.

However, when maintenance cannot be neglected, an overall yield of ATP consumption (Y'_{ATP}) must be adopted, which incorporates the energy requirements not only of growth (Y_{ATP}) but also of cell maintenance (μ / m_{ATP}):

$$Y'_{\mathrm{ATP}} = Y_{\mathrm{ATP}} + \mu / m_{\mathrm{ATP}} \tag{9.6}$$

where m_{ATP} is the specific rate of ATP consumption required for this purpose.

For continuous *S. cerevisiae* CBS 426 cultivation in chemostat at different dilution rates, Larsson et al. (1995) related the overall yield of ATP for maintenance and growth, under conditions either of glucose or nitrogen limitation, to the formation of one or more products, according to the equation:

$$Y'{}_{\mathrm{ATP}} = 1/\{[Y_{\mathrm{ATP/O_2}} \cdot (\mathrm{d}O_2/\mathrm{d}t)] + [Y_{\mathrm{ATP/P}} \cdot (\mathrm{d}P/\mathrm{d}t)] - [Y_{\mathrm{ATP/P'}} \cdot (\mathrm{d}P'/\mathrm{d}t)]\}/\mu \tag{9.7}$$

where $Y_{\mathrm{ATP/O_2}}$ is the yield of ATP produced on consumed O_2 (mol ATP/mol O_2) depending on *P/O*, $\mathrm{d}O_2/\mathrm{d}t$ the specific rate of O_2 consumption (mol g^{-1} h^{-1}), $Y_{\mathrm{ATP}/P}$ the yield of ATP produced on product P formation (mol ATP/mol P), $\mathrm{d}P/\mathrm{d}t$ the specific rate of P formation (mol g^{-1} h^{-1}), $Y_{\mathrm{ATP}/P'}$ the yield of ATP consumed on product P′ formation (mol ATP/mol P′) and $\mathrm{d}P'/\mathrm{d}t$ the specific rate of P′ formation (mol g^{-1} h^{-1}).

These authors demonstrated that, under conditions of glucose limitation and *P/O* = 1.0 mol ATP/0.5 mol O_2, Y_{ATP} decreased from 20 to 13 g biomass/mol ATP with increasing *D,* which means that the energetic requirements of biomass grew. On the other hand, Y_{ATP} decreased under conditions of nitrogen limitation and excess energy at low *D* values, whereas it was not significantly influenced at high dilution rates. Moreover, at a given Y_{ATP} value (16 g biomass/mol ATP), the *P/O* ratio decreased from 1.5 to 1.0 mol ATP/0.5 mol O_2 with increasing *D* under

conditions of glucose limitation, and was only 0.1–0.2 mol ATP/0.5 mol O_2 under nitrogen-limited conditions.

These results on the whole highlight not only that environmental stress leads to higher energy requirements for biomass growth and maintenance, but also that the effectiveness of oxidative phosphorylation can be often substantially lower than the theoretical values foreseen by the chemiosmotic theory (Roels 1983).

9.7 Energetic and Material Balances of Xylose-to-Xylitol Bioconversion by Yeasts

Based on the pioneering study of Barbosa et al. (1988) on carbon material balances of xylitol accumulation in *C. guilliemondii*, Converti et al. (1999) proposed a similar model for *P. tannophilus*. More recently, applying the bioenergetic methodology of Zeng et al. (1990) as well as the findings of Larsson et al. (1995) to pentose-fermenting yeasts, integrated material and energy balances were developed for xylose-to-xylitol bioconversion, either in synthetic media or in hemicellulose hydrolyzates of different raw materials, by *C. guilliermondii* (Carvalho et al. 2005; Santos et al. 2003) and *D. hansenii* (Converti et al. 2002; Rivas et al. 2003, 2009; Sampaio et al. 2004).

9.7.1 Pachysolen Tannophilus

Converti et al. (1999) proposed a material balance to investigate the micro-aerophilic production of xylitol from acid hardwood hemicellulose hydrolyzate by *P. tannophilus* NRRL Y-2460. To this purpose, three different metabolic pathways responsible for xylose consumption were identified, specifically the reductive production of xylitol, ethanol fermentation and catabolic reaction. On the other hand, cell growth was not taken into consideration because it was always negligible under the conditions selected for that study, i.e. high starting biomass level and micro-aerobiosis. The optimum pH range for xylitol production was 6.0–7.5, outside of which the reaction rate decreased, and alternative metabolic routes that notoriously compete with xylitol formation (catabolic reaction and alcohol fermentation) became relatively more important. An outstanding fact was the growing significance of alcohol fermentation with increasing pH up to 9.0, even though the relatively low percentages of carbon source consumed by this pathway (<20 %) suggest it always kept a secondary bioenergetic alternative for the yeast. At extreme pH values (3.0 and 12) the catabolic reaction became progressively more important, consuming up to 30 % of the carbon source. It should in fact be kept in mind that, although strongly hindered by the poor oxygen availability, respiration is always possible even under micro-aerophilic conditions.

In subsequent work performed in the same medium with the same yeast under conditions optimized to exalt xylitol accumulation, Converti et al. (2001) observed that the portion of the carbon source addressed to xylitol formation reached a maximum (about 90 %) just close to the above range and confirmed that a progressive increase or decrease in pH affected xylitol production favoring catabolic reaction and alcohol fermentation. A set of batch tests was also carried out at pH 5–5.5 and with a very high starting biomass level ($19 < X_o < 29$ g_X/L) to estimate the influence of temperature on xylitol production. Between 25 and 30 °C, more than 83 % of xylose was used to produce xylitol, which means that this process can effectively be carried out over a relatively wide range of temperature; however, when temperature was lowered to 20 °C, xylitol was no longer the main product, its formation being responsible for less than 20 % of consumed xylose, while less marked decrease in xylitol production was observed at temperatures higher than 30 °C. Opposite behaviors were observed, on the other hand, for alcohol fermentation and catabolic reaction that showed minimum activities at 30 and 25 °C, respectively, and became progressively more significant outside this range.

9.7.2 Candida Guilliermondii

Sene et al. (2001) applied simple material balances to investigate batch xylitol production from concentrated sugarcane bagasse hydrolysate by *C. guilliermondii* performed by progressively adapting cells to the medium. Regardless of the adaptation level, no less than 80 % of total ethanol came within the first 12–24 h from the almost complete fermentation of glucose contained in the hydrolyzate, and the xylose percentage consumed for xylitol production reached maximum values during the exponential phase (65–67 %) after complete glucose consumption. The subsequent and progressive deceleration of ethanol production was due to the fact that during the exponential and the stationary phases this product was only linked to xylitol formation. Under such conditions, the catabolic reaction gained relative significance, consuming up to 60 % of xylose during the stationary phase. Whereas at the lowest adaptation level xylose fraction addressed to xylitol production progressively increased from the lag to the exponential phase, nearly the same percentage was consumed during them (67 %) when using well-adapted cells; moreover, irrespective of the adaptation level, such an activity progressively decreased after the exponential phase, reaching a minimum (<40 %) during the stationary one. As for ethanol, the CO_2 release remarkably accelerated at the beginning of the run because of the relatively large oxygen availability for respiration. Whereas the catabolic reaction was responsible for the largest CO_2 production throughout the whole run, glucose fermentation was significant in this sense only during the lag phase, and xylose-to-xylitol bioreduction became so only during the exponential one. Total CO_2 formation during the lag phase decreased sharply using adapted cells because of the increasing importance of xylitol formation and alcohol fermentation at the expense of respiration. The results of

this study as a whole highlighted the energetic role of secondary carbon sources contained in lignocellulosic hydrolyzates and demonstrated the suitability of using carbon material balances in metabolic studies dealing with pentose-fermenting yeasts.

To investigate the effect of cell immobilization, Santos et al. (2003) used *C. guilliermondii* cells immobilized onto porous glass spheres for batch xylitol production in fluidized bed bioreactor from sugarcane bagasse hemicellulose hydrolyzate. As expected, final levels of products from typical anaerobic and semi-aerobic activities (ethanol, xylitol, and arabitol) decreased notably with increasing air flow rate, whereas biomass production and respiration were stimulated. This observation confirmed that oxygen supply is by far the most important factor influencing xylose-to-xylitol bioconversion and that q_{O_2} regulation is fundamental to achieve satisfactory xylitol yields by yeasts. The *P/O* ratio estimated according to Zeng et al. (1990) (Sect. 9.6) using experimental q_{O_2} values (1.35 mol_{ATP}/mol_O) did not differ appreciably from that (1.40 mol_{ATP}/mol_O) obtained using q_{O_2} values calculated from the reducing power ("H_2") variation rates, and its application in a metabolic model based on the assumptions of Barbosa et al. (1988) allowed estimating an overall ATP requirement for biomass growth and maintenance of 3.4 mol_{ATP}/C-mol_{DM} along with the specific rates of ATP variations involved in the main metabolic activities.

In an alternative approach, Carvalho et al. (2005) performed a metabolic study based on carbon material and bioenergetic balances using the same raw material and *C. guilliermondii* cells entrapped in Ca-alginate. The best fermentation performance was obtained after 120 h of fermentation, at which xylitol production, xylose-to-xylitol bioconversion yield and xylitol productivity reached 47.5 g/L, 0.81 g/g and 0.40 g L^{-1} h^{-1}, respectively. Interestingly, after this period, i.e. after complete xylose depletion, xylitol yield decreased to 0.73 g/g likely due to product consumption by the yeast, a phenomenon not earlier observed with suspended cells that were somehow related to the peculiar conditions created by immobilization. About 15 % of xylose was consumed for the production of xylitol, mainly during the first 12 h, which suggests that the fast growth of immobilized cells occurred during this period could have quickly consumed oxygen and created anaerobic zones inside the Ca-alginate beads, thereby favoring the production of xylitol, arabitol, and ethanol. The highest values of the specific rates of ATP variations associated with each one of the assumed metabolic pathways were in fact observed at the start of the run, not only owing to typical aerobic activities, such as the reducing power regeneration by the electron transport system and pentose oxidation by the TCA cycle, but even to the anaerobic fermentation. To make bioenergetic balances, a negligible influence of immobilization on the oxidative phosphorylation efficiency, and the same *P/O* ratio previously estimated for cells immobilized onto porous glass (1.37 mol_{ATP}/C mol_O) were assumed (Santos et al. 2003). The overall ATP requirements estimated in this way for growth and maintenance were shown to progressively increase throughout the fermentation

from 2.1 to 6.6 mol_{ATP}/C mol_{DM}, which means that either activity became more and more expensive for the cell.

9.7.3 *Debaryomyces Hansenii*

Improving the above modeling approach, Rivas et al. (2003) applied carbon material, bioenergetic and reduction degree (Roels 1983) balances to semi-aerobic cultivations of the pentose-fermenting yeast *D. hansenii* NRRL Y-7426 in corncob hydrolyzate obtained either by prehydrolysis or by autohydrolysis-posthydrolysis, during which the oxygen level was varied between 72.8 and 80.3 % of oxygen saturation in water and the specific oxygen uptake rate between 26 and 32 $mmol_{O_2}$ C-mol_{DM}^{-1} h^{-1}. The percentage of xylose addressed to xylitol formation stayed relatively high (about 75 %) up to $q_{O_2} = 30$ $mmol_{O_2}$ C-mol_{DM}^{-1} h^{-1}, beyond which it fell together with that consumed for alcohol fermentation favoring biomass production and respiration. The selected level of micro-aerobiosis demonstrated to be optimal for xylitol production only by biomass twice-adapted to the hydrolyzate, whereas poor or excess adaptation was shown to favor alcohol fermentation or growth and respiration, respectively. These results confirmed that oxygen supply is by far the most important factor influencing xylose-to-xylitol bioconversion and that regulation of the oxygen uptake rate and selection of suited cell adaptation are fundamental requisites to achieve satisfactory xylitol yields.

Adapted yeast cells were subsequently employed in concentrated, detoxified, membrane-sterilized hydrolyzates with increasing starting xylose concentration (S_0) in the range of 37–155 g/L, using a nearly constant initial biomass level ($X_0 = 3$–4 g_{DM}/L). Owing to oxygen limitation induced by the increase in S_0, a progressive rise in the fractions of xylose addressed to xylitol and ethanol formations took place at the expense of both biomass synthesis and respiration. On the other hand, the progressive increase in the inoculum level from 3.0 to 55 g_{DM}/L reduced the fractions of xylose utilized for xylitol and biomass productions and stimulated respiration, likely because the occurrence of anaerobic conditions favored the carbon source catabolism to get enough energy for biomass sustenance in the form of ATP.

Similarly to *C. guilliermondii*, the specific rates of ATP production involved in the different metabolic activities simultaneously taking place during xylose-to-xylitol bioconversion by *D. hansenii* were calculated from data of tests performed at variable specific oxygen uptake rates, from which a *P*/*O* ratio (1.16 mol_{ATP}/mol_O) significantly lower than that of *C. guilliermondii* was estimated. The ATP necessary for the production of one C-mol biomass reached a maximum (2.6 mol_{ATP}/C-mol_{DM}) at low S_0 values and remarkably decreased with increasing S_0 as a possible result of the progressive shift from aerobic to anaerobic conditions as well as the increase in the overall fermentation time; while that of the electron transport system was always about one order of magnitude higher, thereby

confirming the significance of the oxidative phosphorylation in the ATP pool in micro-aerobic processes. On the other hand, the semi-aerobic conditions optimal for xylitol formation at intermediate starting xylose levels ($66 < S_0 < 116$ g/L) could have caused the overall specific rate of ATP production (q_{ATP}) to decrease more markedly than that of growth (μ) and the ATP necessary for biomass production to reach a minimum value around 2 $mol_{ATP}/Cmol_{DM}$. A different situation could have taken place when X_0 was progressively increased. These conditions close to strict anaerobiosis did in fact inhibit both xylitol formation and biomass growth, likely because *D. hansenii* consumed the carbon source preferentially to face the remarkable energy requirements for maintenance rather than to grow.

More recently, Rivas et al. (2009) significantly improved the aforementioned modeling approach by separately evaluating the contributions of maintenance and growth requirements to the main bioenergetic parameters. To this purpose, two sets of batch bioconversions were carried out alternatively varying the starting xylose concentration in the hydrolyzate (65.6–154.7 g/L) or the initial biomass level (3.0–54.6 g_{DM}/L), whose results were used to fit a metabolic model consisting of carbon material and ATP balances based on (a) fermentative assimilation of pentoses, (b) semi-aerobic pentose-to-pentitol bioconversion, (c) biomass growth on pentoses, (d) catabolic oxidation of pentoses and acetic acid, and (e) NADH regeneration by the electron transport system (Table 9.2).

In this way, the above authors estimated a *P/O* ratio (1.32 mol_{ATP}/mol_O) close to that previously obtained for *C. guilliermondii* (Santos et al. 2003), specific rates of ATP and xylose consumption due to maintenance of 21 $mmol_{ATP}C\text{-}mol_{DM}^{-1}h^{-1}$ and 6.5 C-$mmol_{Xyl}$ C-mol_{DM}^{-1} h^{-1}, respectively, as well as true yield of biomass on ATP and on xylose of 0.83 C-mol_{DM}/mol_{ATP} and 0.93 C-mol_{DM}/C-mol_{Xyl}, respectively. At relatively low X_0 (3.0–4.5 g_{DM}/L), the global yield of growth and maintenance on ATP did not practically depend (0.44 C-mol_{DM}/mol_{ATP}) on S_0 in the range 65–120 g/L, whereas it strongly decreased (0.19–0.37 C-mol_{DM}/mol_{ATP}) at higher S_0 as the likely result of environmental stress due to excess osmotic pressure, density and viscosity and/or of excess substrate interference on xylose transport mechanisms. The specific rate of xylose consumption progressively increased with S_0 up to a maximum of 0.254 C-mol_{Xyl} C-mol_{DM}^{-1} h^{-1} at $S_0 = 100$ g/L, where about 80 % of xylose was transformed to xylitol, and then decreased as a consequence of excess substrate inhibition. At $S_0 \approx 100$ g/L, a progressive increase in X_0 led to sharp reduction either of Y_{ATP}, likely due to a rise in maintenance requirements and progressive shortage of oxygen in the medium, or of the specific rate of ATP required for both growth and maintenance, which depended on the oxidative phosphorylation by no less than 70 %.

Separation of growth and maintenance contributions revealed that the latter did not depend on the specific growth rate, while the low values of the specific rates of ATP and xylose required only for maintenance ($m_{ATP} = 21$ $mmol_{ATP}$ C-mol_{DM}^{-1} h^{-1}; $m_{Xyl} = 6.5$ C-$mmol_{Xyl}$ C-mol_{DM}^{-1} h^{-1}) suggested that the microorganism was well adapted to corncob hydrolyzate. The energy requirements for cell maintenance dramatically increased at high values either of S_0 or X_0 because of different stress conditions.

Table 9.2 Material and bioenergetic balances proposed by Rivas et al. (2009) for D-xylose metabolism in *D. hansenii* Y-7426 in corncob hemicellulose hydrolyzate[a]

(a) **Biomass growth (a)**

$$1.095\ CH_2O + 0.200\ NH_3 + 1/Y^{b}_{ATP}\ ATP \rightarrow CH_{1.79}O_{0.50}N_{0.20} + 0.095\ 'H_2'^{c} + 0.095\ CO_2 + 0.405\ H_2O \quad (a)$$

(b) **Formation of xylitol (XYT) and arabitol (ARL) (p)**[d]

$$CH_2O + 0.087\ H_2O \rightarrow 0.875\ CH_{2.4}O + 0.025\ CH_3O_{0.5} + 0.100\ CO_2 \quad (b)$$

(c) **Fermentations of glucose (fG) and xylose (fX) to ethanol (EtOH)**

$$CH_2O \rightarrow 0.667\ CH_3O_{0.5} + 0.333\ CO_2 + 0.333\ ATP \quad (c)$$

(d) **Catabolic reactions of xylose (rX) and acetate (rA)**

$$CH_2O + H_2O \rightarrow CO_2 + 2\ 'H_2'^{c} + c^{e}\ ATP \quad (d)$$

(e) **Oxidative phosphorylation (O)**

$$'H_2'^{c} + \tfrac{1}{2}\ O_2 \rightarrow H_2O + P/O\ ATP \quad (e)$$

(f) **Bioenergetic balances**

$$q^{a}_{ATP} = q^{fG}_{ATP} + q^{fX}_{ATP} + q^{rX}_{ATP} + q^{o}_{ATP} \quad (f1)$$

$$q^{o}_{ATP} = 2P/Oq_{O_2} = P/O\left(q^{a}_{H_2} + q^{rX}_{H_2} + q^{rA}_{H_2}\right) \quad (f2)$$

$$q^{a}_{ATP} = \mu/Y^{max}_{ATP} + m_{ATP} \quad (f3)$$

$$q^{fG}_{ATP} = 0.333\ q_G \quad (f4)$$

$$q^{fX}_{ATP} = 0.500q_{EtOH} - 0.014(q_{XYT} + q_{ARL}) - 0.333q_G \quad (f5)$$

$$q^{rX}_{ATP} = 0.633q_X - 0.693\mu - 0.697q_{XYT} - 0.950q_{EtOH} + 0.027q_{ARL} + 0.633q_G \quad (f6)$$

$$q^{a}_{H_2} = 0.095\mu \quad (f7)$$

$$q^{rX}_{H_2} = 2.000q_X - 2.190\mu - 2.200q_{XYT} - 3.000q_{EtOH} + 0.086q_{ART} + 2.000q_G \quad (f8)$$

$$q^{rA}_{H_2} = 2q_A \quad (f9)$$

[a] Stoichiometric coefficients are expressed in mol or C-mol (Eqs. a–e), while the specific rates are in mol or C-mol C-mol$^{-1}_{DM}$ h^{-1} (Eqs. f1–f9)

[b] $1/Y_{ATP}$ is the overall ATP requirement for biomass growth and maintenance

[c] 'H_2' stands in this equation for reducing equivalents in the form of NADH or any other equivalent form of metabolic reducer

[d] NADP(H) produced in the pentose-phosphate pathway is completely consumed in this bioreduction

[e] $c = 0.633$ for xylose and 0.000 for acetate

At $S_0 > 130$ g/L, the ATP consumption to sustain such an activity did in fact become 7–8-fold that observed at low S_0, becoming responsible for no less than 80 % of the overall ATP consumption, while the very high percentage of ATP addressed to maintenance (>96 %) at very high X_0 values was ascribed to substrate limitation of growth. Finally, more than one half of the ATP employed for both growth and maintenance was shown to be associated with the reducing power generated from acetate consumption by the TCA cycle, which confirms the dominant role of acetic acid catabolism in sustaining ATP production in xylitol bioconversion using hemicellulosic hydrolyzates.

9.8 Conclusions and Future Recommendations

In order to make the biotechnological production of xylitol effective and economically competitive with the traditional chemical synthesis, deeper knowledge of xylose metabolism in the most effective pentose-fermenting yeasts is a challenge. To this purpose, a new modeling approach based on material and bioenergetic balances and derived from facultative anaerobes was successfully applied to the known reactions involved in the primary metabolism of pentose-fermenting yeasts. This chapter presents and reviews the efforts made mostly by our research-group in the last decade on xylose-to-xylitol bioconversion in different hemicellulose hydrolyzates by the yeasts *Pachysolen tannophilus*, *Candida guilliermondii* and *Debaryomyces hansenii*. Comparison of literature results confirms the significance of the oxidative phosphorylation in the ATP pool of these yeasts even in microaerobic processes and suggests that regulation of the oxygen uptake rate and selection of suited cell adaptation are fundamental requisites to achieve satisfactory xylitol yields. The energy requirements for cell maintenance dramatically increased at high values either of S_0 or X_0 because of different stress conditions. In general, the semi-aerobic conditions optimal for xylitol formation took place at intermediate starting xylose levels as the likely result of a decrease in the overall specific rate of ATP requirements to a minimum threshold.

Further developments in this field are expected from the application of similar approaches to new, more effective and quicker xylitol producers to be obtained by simple screening/isolation, mutagenesis or genetic engineering. Especially in the last case, in which the modifications to be introduced into the host are known in advance, it should be relatively easy to mathematically model new overall networks incorporating both the new enzymatic activities leading to xylitol overproduction and those already known for the wild type. Modeling techniques like those depicted in this chapter have the potential to acquire deeper knowledge on the metabolism and bioenergetics of the microbial agent responsible for xylitol accumulation; therefore, they may play, in the future, a paramount role in seeking solutions to make the biotechnological production of xylitol competitive with the traditional chemical methods.

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Part III
Recovery of Xylitol and Analytical Methods for Xylitol

Chapter 10
An Assessment on Xylitol Recovery Methods

Bahar Aliakbarian, Danilo de Faveri, Patrizia Perego and Attilio Converti

Abstract The interest in dietary sugars and polyols has increased considerably in recent years, as they are candidates for many commercial applications in different sectors like food and pharmaceutical industries. This chapter aimed at providing an overview on the xylitol recovery using different approaches. After a brief description of the applications of such an important polyol component in the food and medicine industries along with its physicochemical properties, the traditional ways of providing xylitol, specifically the chemical synthesis, are described and compared with the new biotechnological options. Emphasis has been addressed on the present literature on xylitol recovery assessment, specifically focusing on xylitol recovery by crystallization. The effects of the most influencing factors such as pretreatment, initial xylitol super saturation value, crystallization temperature as well as solvent on xylitol recovery were discussed. The chapter points out that even if in the last few years xylitol crystallization has drawn more attention, important research efforts are still required to develop downstream technologies able to economically recover this compound in a very pure form, suitable for commercial purposes.

Keywords Xylitol recovery · Crystallization · Membrane separation · Chromatographic separation

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Table 10.1 Natural occurrence of xylitol in fruits, vegetables and related products (Jaffe 1978)

Product	Xylitol (mg/100 g dry substance)
Banana	21
Raspberry	268
Strawberry	362
Yellow plum	935
Carrot	86.5
Endive	258
Onion	89
Lettuce	131
Cauliflower	300
Pumpkin	96.5
Spinach	107
Kohlrabi	94
Eggplant	180
Leek	53
Fennel	92
White mushroom	128
Brewer's yeast	4.5
Chestnut	14
Carrot Juice	12
Lamb's lettuce	273

10.1 Introduction

Xylitol is an important sugar substitute with certain interesting physical and chemical properties that make it a high value compound for pharmaceutical, odontological and food industries (Misra et al. 2011). It has similar sweetness as sucrose, is non-cariogenic, tolerated by diabetics, recommended for obese people and, because of its negative heat of dissolution, used as a part of pharmaceutical products coating (Parajó et al. 1998a, b; Ylikahri 1979). From the standpoint of special dietary applications, xylitol is one the most promising sweeteners, especially in the areas of non-cariogenics and carbohydrate metabolism disturbances.

Xylitol is a normal metabolic intermediate in mammalian carbohydrate metabolism, including that of man (Hyuönen and Koivistoinen 1982). It can be found naturally in lichens, seaweed, yeasts and many fruits and vegetables (Table 10.1). Commercially produced xylitol is a product similar in structure and properties to that of the natural substance.

10.2 Physico-Chemical Properties

Xylitol can be chemically characterized as a five-carbon sugar alcohol (1,2,3,4,5-pentahydroxy pentane) with molecular formula of $CH_2OH(CHOH)_3CH_2OH$. For many years, xylitol has been considered an unwanted byproduct of the

Table 10.2 Physico-chemical properties of xylitol and other sweeteners (Nestl et al. 1989)

Property	Sweetener					
	Sucrose	Fructose	Xylitol	Mannitol	Isomannitol	Sorbitol
Sweetness	100	–	100	50	–	60
Solubility in water at 20 °C (%)	67	70	66	62	25	70–74
Enthalpy of dissolution (Jg^{-1})	−18.16	−37.69	−153.07	−70.12	−39.40	−110.99
Viscosity in water at 20 °C	(+++)	(+)	(+)	(++++)	(+++)	(++)
Fusion point (20 °C)	160–185	103	94	130–135	145–150	96–97
Boiling point (°C)	–	–	216	295	–	–
Maillard reaction	(+)	(++++)	(−)	(−)	(−)	(−)
Energy (kcal/g)	3.68	3.68	2.48	3.68	3.70	3.69

Symbols between brackets stand for the level of the physico-chemical properties: not present (−), moderate (+), high (++), very high (+++), maximum (++++)

fermentation of D-xylose to ethanol. Reasonable yields and high productivity of this sugar alcohol have raised researchers interest in uncovering its biochemical formation mechanisms and physico-chemical properties.

The physico-chemical properties of xylitol are listed in Table 10.2 and compared to those of some frequently used sweeteners in food and pharmaceutical industries.

10.3 Applications of Xylitol

Because xylitol is as sweet as regular table sugar and it does not require insulin to be absorbed, it became a food supplement sweetener in the diabetics diet (Makinen 1994). Compared with glucose in healthy subjects, xylitol causes a much smaller increase in serum insulin and blood glucose levels with no "rebound" hypoglycemia (glycemic index: xylitol = 7, glucose = 100) (Natah et al. 1997).

Another important application of xylitol is in parenteral nutrition (infusion therapy) (Georgieff et al. 1985). Especially German physicians have used xylitol in substantial quantities for intravenous feeding of patients with impaired glucose tolerance. When used in this way xylitol was found to have a strong anticatabolic muscle-sparing effect.

Xylitol can help in preventing ear infections, thereby reducing the need for antibiotics. Usage of xylitol chewing gum or syrup by young day-care center subjects was associated with reduced rate of acute otitis media (middle ear infections) and a lowered nasopharyngeal carriage rate of pneumococci (Kontiokari et al. 1995; Uhari et al. 1996).

Even though most dietary sugars and polyols are hexoses or hexitols, structurally based on a 6 carbon unit, xylitol is a 5 carbon acyclic polyol or pentitol that exhibits various clinical advantages. The xylitol structure is stoichiometrically

unfavorable for extensive acid (especially lactic acid) fermentation, which renders it hydrophilic and capable of forming weak interactions with calcium in solution. These complexes (also formed by sorbitol and mannitol) are believed to stabilize the calcium phosphate systems present in saliva, but are not strong enough to dissolve solid calcium salts. Animal experiments showed that dietary xylitol improves calcium absorption and prevents osteoporosis (Mattila et al. 1996; Svanberg and Knuuttila 1994; Uhari et al. 1998).

10.4 Xylitol Recovery

As shown in Table 10.1, xylitol can be found naturally in some fruits and vegetables, but, due to its low concentration, extraction from these sources is not a cost-effective process (Saha and Bothast 1997). Commercially it can be produced by a chemical process consisting of catalytic hydrogenation of xylose present in lignocellulosic hydrolyzates, but this recovery process is also not cost-effective; therefore, the final product is more expensive than other polyols (de Faveri et al. 2004; Winkelhausen and Kuzmanova 1998). Alternatively, it can be produced by biotechnological methods, based on the use of microorganisms, in particular yeasts and/or enzymes (Parajó et al. 1998a, b). Such bioprocesses consist of fermentation of agro-industrial residues, which could potentially compete with the traditional chemical method. *Pachysolen tannophilus* (Converti et al. 2001), *Candida guilliermondii* (Converti et al. 1999, 2000; Felipe et al. 1997; Roberto et al. 1996) and *Debaryomyces hansenii* (Cruz et al. 2000; Gírio et al. 1994, 2000; Parajó et al. 1997) were widely employed as xylitol producers in previous studies.

10.4.1 Xylitol Recovery from Chemical Production

The conventional chemical process of xylitol production includes four main steps: acid hydrolysis of plant material, purification of the hydrolyzate to obtain either a pure xylose solution or a pure crystalline xylose, hydrogenation of xylose to xylitol, and xylitol crystallization (Winkelhausen and Kuzmanova 1998).

Because crystalline xylitol from saturated aqueous solution is moisture sensitive, gum processing is difficult to manage. Duross (1992) used melt-crystallized xylitol to produce crystals, which were moisture resistant and more easily formulated into chewing gum. A 70 % xylitol solution was made, heated to 170 °C and then cooled to 90 °C under agitation in a water bath (80 °C). The solution was seeded with 1 g of xylitol crystals, and the agitation was continued until a noticeable increase in viscosity due to crystal formation (50 %) was observed. The solution was poured onto a tray and covered with aluminum foil to crystallize. The result was an agglomerated crystal structure with 99.5 wt% xylitol.

Heikkila et al. (1999) patented the production of xylitol from xylose and xylonic acid present in the cooking liquor from pulping as an alternative method to reduce the environmental impact of pulping residues. Hydrogenation of xylonic acid crystals was performed using ruthenium at 110 °C and 1300 kPa for 3 h; this solution was filtered and then seeded with 0.05 g of xylitol crystals. A crystallization yield of 0.297 g/g with 68 % purity was achieved after centrifugation of the solution at 4500 rpm for 5 min.

10.4.2 Xylitol Recovery from Fermented Media

As noticed in the previous paragraph, the critical step in the conventional xylitol production is the purification process from the acid hydrolyzate. Crystallization and purification of xylitol prove to be complicate processes since acid hydrolysis releases considerable amounts of various sugars, depending on the feedstock (Winkelhausen and Kuzmanova 1998), such as D-galactose, D-mannose and L-arabinose in addition to L-xylose. The yield of xylitol from xylan fractions is consequently low (8–60 % depending on the raw material employed) (Nigam and Singh 1995) and is considered the main existing drawback of conventional xylitol production. Alternative methods such as microbial processes could resolve not only the aforementioned drawback but also waste-treatment concerns. The biotechnological production of xylitol makes use of renewable resources such as agro-industrial residues, which represent an inexpensive source of raw materials, and helps to minimize environmental and energetic problems (Roberto et al. 1996). Until now, literature on polyols was focused on pentose bioreduction in hemicellulosic hydrolyzates along with related bioconversion pathways, whereas very little information is available about xylitol recovery (de Faveri et al. 2004; Martínez et al. 2007; Misra et al. 2011). Xylitol characteristics and impurities found in fermented broths, such as molecule size, sugars and sugar alcohols, inorganic salts and polypeptides are critical factors to select a recovery method (Counsell 1978; Jaffe 1978; Kiysawa 1991; Sommer 1996).

In the following sections, different methods reported in the literature for xylitol recovery from fermentation media are summarized and compared.

10.4.2.1 Chromatographic Methods

Melaja and Hamalainen (1977) hydrogenated birch wood hemicellulose hydrolyzate in the presence of Raney nickel catalyst in autoclave at 135 °C and 40 atm of hydrogen pressure for 2.5 h, thus obtaining a xylose-rich solution whose composition is listed in Table 10.3. Xylose and other carbohydrates were hydrogenated to their respective polyols, and approximately 60 % of the original xylan material was recovered as xylitol.

Table 10.3 Birch wood hemicellulose hydrolyzate composition for catalytic xylitol production (Melaja and Hamalainen 1977)

Sugar	Dry solid basis (%)
Xylose	73
Arabinose	6.1
Mannose	9.0
Galactose	5.1
Glucose	6.8

Xylitol was crystallized from the hydrogenated solution, and the uncrystallized xylitol fraction was separated by liquid chromatography. The chromatographic separation was performed in a 1-m high column with 94-cm diameter containing cross-linked sulfonated polystyrene divinylbenzene cation exchange resin in calcium or strontium form. Eluents collected from the chromatographic column contained 0.1–1.95 g of arabitol, 0.45–1.70 g of mannitol, 0.1–1.0 g of galactitol, 0.2–0.85 g of sorbitol and 0.85–14.3 g of xylitol. The fractions with very high xylitol concentrations were crystallized, and xylitol was recovered. Crystallization was able to ensure a maximum xylitol recovery of 60 wt% of mother liquor.

On the other hand, Munir and Schiweck (1981) proposed the use of a strongly acidic, weakly cross-linked divinylbenzene cation exchange resin in calcium form to separate xylitol from centrifuged mother liquor. According to their patent, xylitol readily crystallized and was easily separated in a wire basket, and more than 34 % of xylitol present in the original mother liquor was recovered.

More than one decade later, Gurgel et al. (1995) used both anion (Amberlite 94S) and cation exchange (Amberlite 200C) resins to purify xylitol from fermented sugar cane bagasse hydrolyzate, which resulted in 40–55 % loss of product owing to xylitol adhesion to the resin surface. The fermented broth was then treated with 200 g/L activated carbon at 80 °C, pH 6 for 60 min to remove color and proteins, but this treatment implied the undesired adsorption of about 20 % of xylitol. Subsequent filtration and concentration resulted in a colored and viscous solution, in which xylitol crystallization was very difficult and took almost six weeks at –15 °C.

Vourinen (1996) patented the production of xylitol from D-glucose, D-fructose, and D-galactose in three steps comprising oxidation, treatment and xylitol separation. D-Glucose (1050 g) was oxidatively cleaved in an autoclave with an aqueous solution containing sodium hydroxide (18 g), water (264 g), methanol (100 g) and sodium arabinonate (16 g). The reactor was pressurized with oxygen (at 85 °C) to form sodium arabinonate crystals, which were recovered by centrifugation, dissolved in water and acidified to arabinonic acid on an acid cation exchange resin. The acidic product was vacuum concentrated and crystallized to successively produce D-arabino-1,4-lactone crystals. These crystals were then converted to arabitol in the presence of ruthenium-on-carbon catalyst with a molar yield of 90 %. After crystallization, a second reaction allowed the arabitol crystals to be converted to xylitol (90 % molar yield) in the presence of ruthenium-on-carbon catalyst. However, the procedure has been found to be too costly for large-scale production.

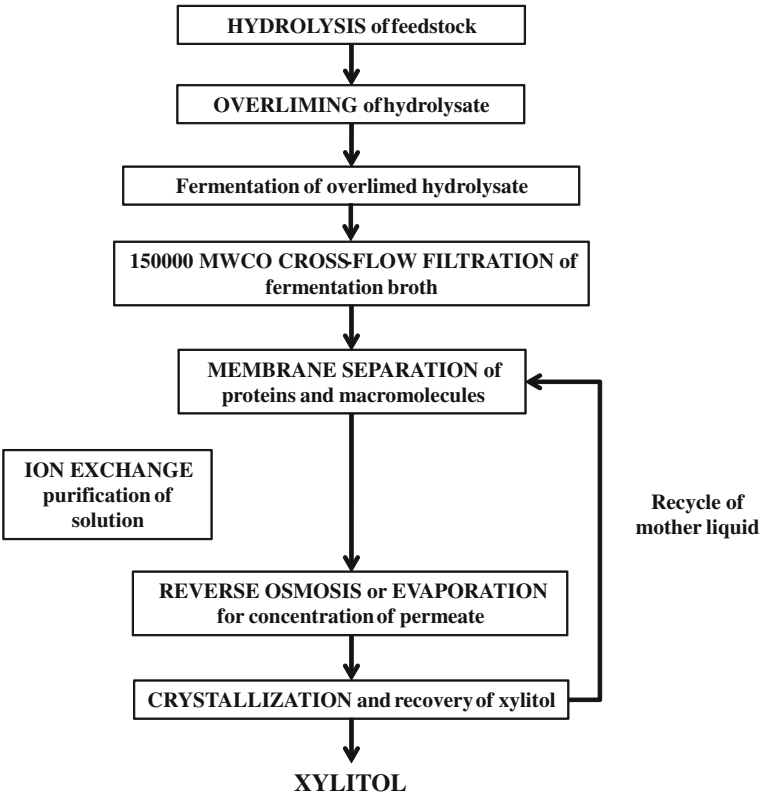

Fig. 10.1 Microbial xylitol production and recovery using the membrane method (Affleck 2000)

10.4.2.2 Membrane Methods

Membrane separation was proposed by Affleck (2000) as an alternative method for the recovery of xylitol from fermented broth, because it has the potential for energy savings and higher purity. Figure 10.1 illustrates the xylitol microbial production and membrane separation proposed by him.

Such a process included a pretreatment such as acid extraction and enzyme hydrolysis of corn fiber prior to fermentation. The liquor resulting from acidic treatment and hydrolysis was neutralized with $Ca(OH)_2$ or other calcium chemicals and then fermented by *Candida tropicalis* under high air flow rate (1.5 vvm). The produced xylitol was separated from the impurities contained in the fermented broth with a yield of 0.6 g of xylitol per g of xylose. A 10,000 nominal molecular weight cutoff (MWCO) polysulfone membrane was found to be the most effective tool for the separation and recovery of xylitol. Such a membrane did in fact allow 82.2–90.3 % of xylitol contained in the fermentation broth to pass through, while retaining 49.2–53.6 % of the Lowry's method positive material, i.e. oligopeptides and peptides. Permeate from the membrane was collected and crystallized, while

crystals, analyzed by HPLC for xylitol and impurities, were shown to have purity up to 90.3 %. The activated carbon treatment was able to remove as much as 79.5 % of the UV absorbing material responsible for the fermentation broth color, but adsorbed about 25–50 % of xylitol present in the solution.

A common problem for all of xylitol crystals recovered by membrane separation was the inability of this treatment to remove impurities, which mainly consisted of phosphates and other salts such as $MgSO_4$. The low yields were due to the presence of colored, viscous mother liquor, caused by Lowry positive material, which inhibited crystallization and made crystal recovery difficult. Whereas this polysulfone membrane was not very effective in removing impurities, it could be used as a pretreatment for further purification steps (Affleck 2000).

10.4.2.3 Crystallization

Crystallization is a separation technique to produce particulate material, especially in the chemical industry. Crystallization is often critical because it largely determines product purity and handling characteristics such as caking, wetting or losses due to dusting (Giulietti et al. 2001). Crystallization is employed in many cases as an energetically advantageous way to separate an individual compound from a mixture of substances represented by raw materials or reaction by-products (Giulietti et al. 2001; Misra et al. 2011). It may occur as the formation of solid particles from a vapor, as solidification from a liquid melt or as the formation of dispersed solids from a solution. The elementary steps involved in this process are creation of supersaturation, nucleation, crystal growth, aggregation and other secondary processes. When performed at low temperature, it allows minimizing the thermal degradation of heat-sensitive compounds; besides, its unit operating cost is particularly low if compared with other recovery techniques, because of the use of very high product concentrations (Sampaio et al. 2006).

Continuous operation is usually applied for large capacities and offers good control of average product size. Continuous processes are easier to operate than batch ones, requiring less manpower and less physical space for the same production capacity (Giulietti et al. 2001), but are not recommended for products with a strong scaling tendency.

On the other hand, batch operation is usually preferred for small-scale production, but there are exceptions. Batch operation normally involves simpler equipment, and the same crystallizer can be used for more than one product. Moreover, batch is the recommended mode of operation for crystallization of substances with low growth rates. Good practices for batch operation involve a sound selection of cooling profiles and seeding procedures since they influence product quality (Derenzo et al. 1996; Giulietti et al. 1995, 1996, 1999, 2001).

As described by Giulietti et al. (2001), the high complexity of the process is due to a number of factors, specifically (a) simultaneous heat and mass transfer with a strong dependence on fluid and particle mechanisms; (b) multiphase and multicomponent system; (c) concentration, particle size and size distribution that could

vary with time; (d) lack of data; (e) low reproducibility of the experiments to determine both nucleation and growth rates; (f) secondary effects like agglomeration and those exerted by impurities that can alter the morphology and the quality of the crystalline product.

In order to design an industrial crystallizer, selections of the solvent and crystallization method are two important steps. The morphology of crystals depends on the solvent. This choice can be based on experimental tests and also on molecular modeling techniques (Giulietti et al. 2001; Misra et al. 2011; Myerson 1999; Nývlt and Ulrich 1995). The crystallization method (cooling, evaporation, flash, precipitation or second solvent addition) is in general chosen on the basis of the physical and thermodynamic properties of the solute and solvent as well as the required purity of the final crystalline product. The second criterion for the choice of the crystallization mode is based on the solubility curves and in particular on the absolute supersaturation (c*) and temperature (T) (Giulietti et al. 2001; Martínez et al. 2007; Misra et al. 2011), as specified below:

i. for very soluble substances ($c^* > 0.2$ g/g), by cooling the solution;
ii. for soluble substances ($c^* < 0.2$ g/g), by cooling or evaporating the solution or by combining them by flash evaporation;
iii. for substances with a small dc^*/dT (<0.005 g/g °C), by evaporating the solution;
iv. for slightly soluble substances ($c^* < 0.01$ g/g), supersaturation can be created by the chemical reaction of two or more reactants.

In accordance with literature reports, it is well known that xylitol crystallization, in the past two decades, has drawn more attention and is believed to be the final step for obtaining highly purified products (de Faveri et al. 2002; Guerrieri 1998; Misra et al. 2011; Vyglazov 2004).

According to Glasgow (1983), to produce pure crystalline solids in an efficient manner, a supersaturated solution must be produced, and the appearance of the crystal nuclei as well as the crystal growth must be controlled. With regard to products obtained by fermentation, crystallization of the product of interest only can be performed after purification of the fermented broth (Belter et al. 1988). In the case of xylitol bioproduction, several impurities coming from the hydrolysis of the lignocellulosic matrix (sugars, metallic ions, phenolic compounds, furfural, hydroxymethylfurfural, etc.) or nutrients added for medium preparation (amino acids, peptides, proteins and inorganic salts) are recognized as prejudicial to the formation of xylitol crystals (Affleck 2000; Cruz et al. 2000; Domínguez et al. 1996; Gurgel et al. 1998; Parajó et al. 1998a, b; Vyglazov et al. 1990). Moreover, due to the fermentation broth complexity, the purity grade of xylitol obtained by direct crystallization is not acceptable. For these reasons, purification of the fermented broth prior to crystallization is required to produce pure xylitol crystals (Mussatto et al. 2006).

To simulate the actual composition of hemicellulose hydrolyzates, de Faveri et al. (2002) tested xylitol separation from solutions with relativity high concentrations of xylose and xylitol in a bench-scale system composed of (a) a rotavapor

used as low pressure–concentration unit, provided with temperature and pressure control system and a vacuum pump, (b) a crystallization unit working at atmospheric pressure, (c) a centrifuge equipped with process parameters control system, and (d) a vacuum filtration system (Fig. 10.2). Crystallization assays were done using either xylitol–xylose synthetic solutions or fermented and centrifuged hardwood hemicellulose hydrolyzate solutions with variable concentrations. *D. hansenii* (NRRL Y 7426) was used for preliminary fermentations of hemicellulose hydrolyzate, which were performed in a 3 L-working volume fermenter.

Xylitol was recovered by a crystallization protocol consisting of dilute solution evaporation up to super saturation, supersaturated solutions cooling, separation of crystals by centrifugation, and final filtration. The best results in terms either of crystallization yield (56 %) or purity degree (100 %) were obtained with quite concentrated solutions (730 g/L) at relatively high temperature (−5 °C).

Some years later, de Faveri et al. (2004) used a 3^2-full-factorial design combined with response surface methodology to determine the influence of super saturation level (582–730 g/L) and cooling temperature (−10, −5 and 0 °C) on xylitol crystallization as well as to identify the optimum crystallization conditions. The optimal operating conditions in the same bench-scale system described above corresponded to a 728 g/L xylitol super saturation value and a cooling temperature of −6.0 °C, which led to 97 % purity degree and 54 % xylitol crystallization yield.

Vyglazov (2004) investigated xylitol crystallization from aqueous-ethanolic solutions under isothermal conditions at 5, 25 and 40 °C as a function of the super saturation degree of the initial solution, solvent composition and temperature, and determined the main kinetic characteristics of the process, namely, the maximal super saturation of solutions in the form of the first and second metastability boundaries, induction period, nucleation reaction order, and rate constant of xylitol crystal growth. Food xylitol used was preliminarily recrystallized and dried to a moisture content of 0.03 %. The xylitol purity and melting point of crystals were 99.9 % and 93.5–94.0 °C, respectively. The solvent was prepared by mixing ethanol (rectificate) with distilled water at 20 °C. Xylitol crystallization was performed in a three-necked flask equipped with a stirrer and thermometer. Kinetic analysis of curves showed that the increase in ethanol concentration in the intercrystallite liquor of xylitol suspension accelerated xylitol separation into the solid phase (Table 10.4). For example, at 25 °C, xylitol crystallization was 11 times faster in 90 % than in 60 % ethanol. It was also found that, as the ethanol concentration in the mixed solvent was increased (e.g., from 80 to 90 vol%), the xylitol crystallization rate increased by a factor of approximately 3.

Sampaio et al. (2006) focused on the study of the kinetics of xylitol crystallization from solutions fermented by the new yeast strain *D. hansenii* UFV-170 as a function of the initial solution supersaturation (from 675 to 911 g/L) and cooling temperature (from −10 to 15 °C). The results of different treatments with activated charcoal showed that the use of 20 g/L charcoal at 25 °C for 1 h was an efficient method to clarify fermented media. Such a treatment was in fact considered to ensure the best compromise between the opposite economic requirements of obtaining the clearest and purest solution as possible with the lowest amount of

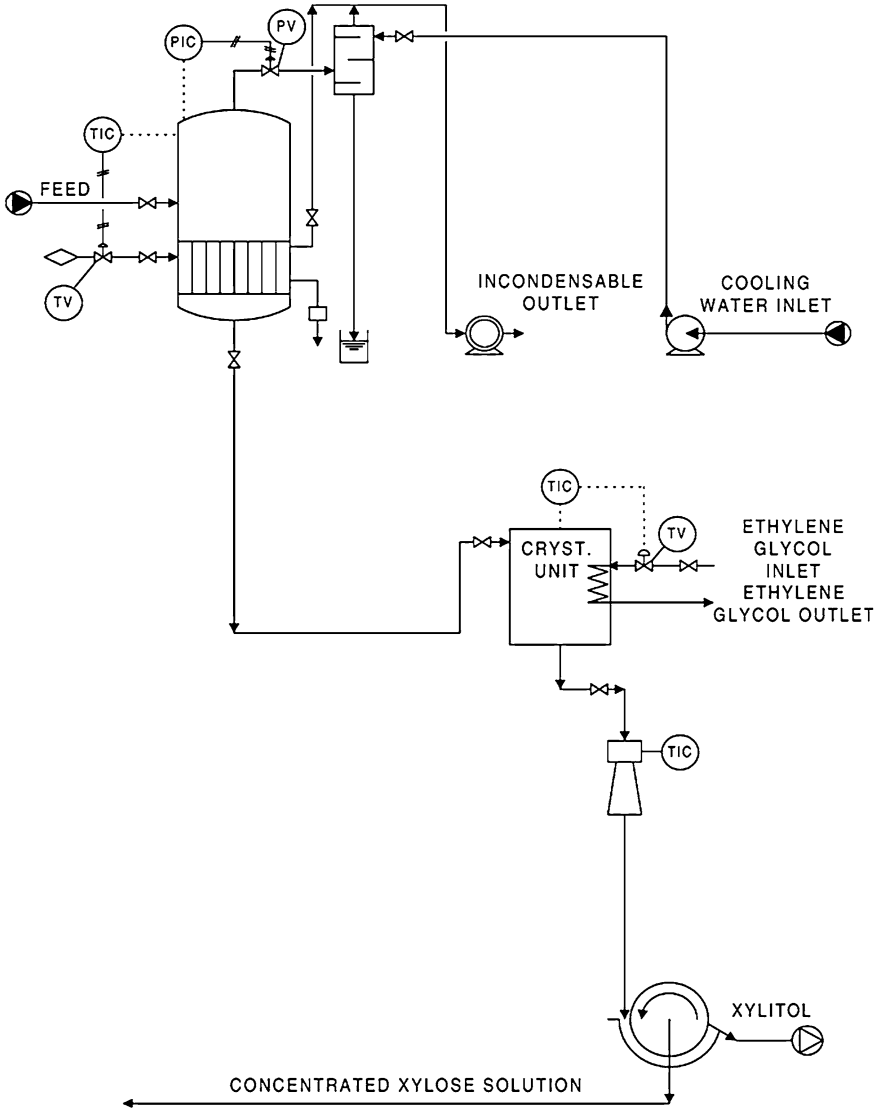

Fig. 10.2 Flow-sheet of the bench-scale system used for xylitol crystallization tests performed by de Faveri et al. (2002)

adsorbent, being able to remove nearly 79 and 94 % of the contaminants detected at optical density of 540 (clarification effect) and 280 nm (amino acids), respectively, and 69 % of total proteins, while xylitol recovery was almost complete. The crystallization technique showed good performance and demonstrated that:

Table 10.4 Duration of xylitol crystallization from aqueous ethanolic solutions (Vyglazov 2004)

Ethanol concentration in water–ethanol mixture (vol %)	*t*, min, at indicated temperature, °C		
	5	25	40
60	285	345	930
70	110	122	380
80	70	80	117
90	25	32	85

i. an increase in xylitol concentration (Fig. 10.3) remarkably accelerated xylitol separation into the solid phase (by a factor of 14–15);
ii. the lower the temperature, the more effective the xylitol crystallization in terms of crystallization yield, while the crystals purity degree showed a completely opposite behavior, and
iii. the simultaneous presence of residual xylose reduced the xylitol content of crystals (from 97.7 to 85.3 %), but ensured a 1.6-fold increase in the crystallization yield (from 27 to 42 %).

Finally, the kinetic study of xylitol crystallization revealed the positive effect of the presence of residual xylose, which ensured a 1.6-fold increase in the crystallization yield and enhanced the rate constant of crystal growth, thus permitting operation at a higher crystallization temperature and lower xylitol concentrations.

Mussatto et al. (2006) recovered xylitol from sugarcane bagasse hemicellulosic hydrolyzate fermented by the yeast *C. guilliermondii* by adsorption and crystallization procedures. Silica gel adsorption was employed to purify the broth containing xylitol. In this step, different solvent mixtures of ethyl acetate, ethanol and acetone were used as eluents, and different proportions of fermented broth volume per gram of silica gel were used to pack the column employed as stationary phase bed. The purified broth was finally submitted to different crystallization procedures as follows: (a) column 1: the fractions were separately maintained at 4 °C for 24 h; (b) column 2: the fractions were separately maintained at room temperature for 12 h and subsequently at 4 °C for 72 h; (c) column 3: the fractions were separately maintained at 4 °C for 24 h, combined and 6.5-fold concentrated in a rotavapor at low temperature (50 °C), supplemented with 45 mg of finely ground standard xylitol and thus maintained at 4 °C for 24 h; (d) column 4: the fractions were combined, 6.5-fold concentrated in a rotavapor at low temperature (50 °C), maintained at 4 °C for 24 h, and finally supplemented with 400 mg of finely ground standard xylitol to force the precipitation. The best results (60 % crystallization yield and 33 % total recovery of xylitol from fermented broth) were obtained when the column 4 was used, and the broth was purified with a mixture of ethyl acetate and ethanol, 6.5-fold concentrated and supplemented with commercial xylitol.

Recently, Misra et al. (2011) reported, for the first time, an economically viable purification of xylitol produced through fermentation of synthetic xylose and corn cob hemicellulosic hydrolyzate by an adapted strain of *C. tropicalis*. The

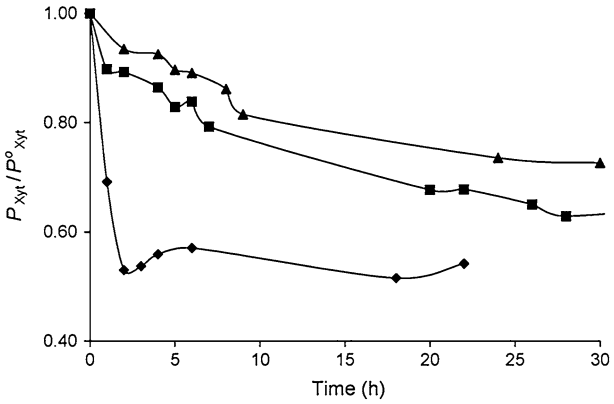

Fig. 10.3 Time behavior of the actual (P_{Xyt}) to starting (P^{o}_{Xyt}) xylitol concentrations ratio during crystallization tests at different xylitol concentrations: (◇) 911 g/L; (■) 782 g/L; (▲) 675 g/L (Sampaio et al. 2006)

Table 10.5 Comparison of different processes for xylitol purification as described by Misra et al. (2011)

Recovery process	Description	Xylitol recovery
Liquid–liquid extraction	Broth to ethylacetate ratio: 1:5 (v/v) Extraction at 30 °C	32.52 % in organic phase; 65.26 % in aqueous phase
Precipitation	Solvent: acetone Stand for 60 min at 4 °C followed by centrifugation	30.60 % in organic phase; 67.44 % in aqueous phase
Vacuum concentration followed by crystallization	Two phase crystallization temperature (−20. 8 °C), 4 cycles	76.20 % in 50 mL synthetic xylose fermentation broth 68.06 % in 5 L synthetic xylose fermentation broth

fermented media were treated with activated charcoal and vacuum concentrated, while final crystallization was employed to recover xylitol. Optimum conditions of treatment with 15.0 g/L charcoal at 30 °C for 1 h with 10 times supersaturation of initial concentration and crystallization temperature of −20 °C for initiation and then of 8 °C ensured a yield of 44.0 %. After four cycles of crystallization, 76.2 % and 68.1 % xylitol crystallization yields were obtained in 50 mL and scaled-up volume of 5.0 L of the synthetic xylose fermentation broth, respectively. The authors also compared the purification process using other strategies. A summary of results obtained in this research is shown in Table 10.5.

10.5 Conclusions and Future Recommendations

Xylitol recovery using different approaches such as chromatographic methods, membrane separation and crystallization were discussed in this chapter, and the effect of operating parameters was evaluated. However, to make large-scale industrial production of this sugar alcohol viable, it is crucial to increase xylitol production rate by optimization strategies. In addition to this, crystallization as an energetically advantageous and environmentally friendly method has drawn more attention as the final step to obtain highly purified xylitol. To such a purpose experimental designs must be taken into account, and an important research effort is still required to develop alternative downstream technologies able to economically recover this compound in a very pure form, suitable for commercial purposes.

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Chapter 11
Current Analytical Methods for Qualitative and Quantitative Measurement of D-Xylitol

Zhongqi Jiang, Samuel Amartey, Zi-Hua Jiang and Wensheng Qin

Abstract D-xylitol is a naturally-occurring five-carbon sugar alcohol. It can also be derived from the chemical reduction of D-xylose. It is widely used in recent years and will continue to be used as a food additive and sweetening agent in the food industry. The qualitative detection and quantification of D-xylitol in the presence of other sugars and sugar alcohols in fruits, vegetables and other natural sources is essential for industry production. A number of analytical methods have been developed over the years for qualitative detection and quantitative measurement of D-xylitol. Since most samples to be analyzed contain a mixture of compounds, highly efficient and sensitive analytical methods for D-xylitol in the mixture are required. Current analytical methods are usually comprised of two components: (1) an efficient separation unit, and (2) a structure identification unit. In this chapter, we provide an overview on these analytical methods used for the qualitative and quantitative determination of D-xylitol in samples from various sources. Chromatography-based techniques including GC, HPLC and CE methods with different detection options, such as UV, RI, ELS, etc., have been widely used. More advanced analytical instruments derived from hyphenation of

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chromatography with structure determination tools such as MS and NMR are becoming more and more accessible. The GC–MS, LC–MS and LC–MS/MS have now become routine methods for D-xylitol measurement. The coupling of spectroscopic methods such as NMR and MS to the chromatography methods can also provide structural information of the compounds being analyzed. Other methods such as the immunoassay and enzymatic assay methods are also discussed.

Keywords: D-xylitol · Analytical method · Detection method · Chromatographic separation · Spectroscopy · Biosensor

Abbreviations

H NMR	Proton nuclear magnetic resonance
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
ELISA	Enzyme-linked immunosorbent assay
ELS	Evaporative light-scattering
ESI-MS	Electrospray ionization mass spectrometry
FIA	Flow injection analyisis
FID	Flame Ionization Detector
FT-ICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
HPAEC	High-pH anion exchange chromatography
HPLC	High-performance liquid chromatography
IC-ELISA	Indirect competitive enzyme-linked immunosorbant assay
IDC	1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate
Ig E	Immunoglobulin E
Ig G	Immunoglobulin G
ISTD	Internal standard
ITP	Capillary isotachophoresis
LC-MS	Liquid chromatography-mass spectrometry
LC-NMR	Liquid chromatography-nuclear magnetic resonance
MS	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
OPLC	Optimum performance laminar chromatography
PAD	Pulsed amperometric detector
RI	Refractive index
RSD	Relative standard deviation
SIM	Selective-ion-monitoring
TLC	Thin layer chromatography

TMS	Trimethylsilylation
XDH	D-xylitol dehydrogenase
XYO	D-xylitol oxidase

11.1 Introduction

D-xylitol is a naturally-occurring five-carbon sugar alcohol derived from the reduction of D-xylose. It is also known as 1, 2, 3, 4, 5-pentahydroxypentane, and its IUPAC name is designated as (*2R, 3r, 4S*)-pentane-1, 2, 3, 4, 5-pentol. It has been used as a food additive and sweetening agent in the food industry since the 1960s (Mickenautsch and Yengopal 2012). D-xylitol was first discovered and reported in 1891 by the German Nobel Prize winning chemist Emil Fischer (Granström et al. 2007). It is present in a wide variety of fruits and vegetables, as well as in corn husks and mushrooms (Jaffe 1978; Mitchell 2006). Currently D-xylitol is produced commercially by the chemical reduction of D-xylose which is derived mainly from birch bark, corn husks and stone fruit (James 2009). It has attracted huge interest worldwide because of its usefulness in treating a number of health-related conditions. D-xylitol has almost the same sweetness as sucrose but has lower energy value than sucrose (2.4 cal/g vs. 4.0 cal/g). It has been shown to resist fermentation by microorganisms into acids (Russo 1976), thus it has been used as a sugar substitute in dietary foods, especially for insulin-deficiency patients. In addition, D-xylitol is believed to have 'active' anticariogenic properties and has been widely used in the odontological industry. Furthermore, studies have shown that D-xylitol can prevent acute ear infection (otitis media) in small children (Mäkinen 1992; Uhari et al. 1996).

D-xylitol is currently sold at the price of $4–5/kg and its global market is estimated to be $340 million per year, which will definitely increase in the future (Chen et al. 2010). It is un-economical to extract large amounts of D-xylitol from vegetables and fruits due to its low content. In industrial scale production, D-xylitol is manufactured through catalytic chemical reduction of pure D-xylose which is obtained from hemicellulose (Chen et al. 2010).

The importance of simple, fast, low cost, but sensitive and accurate methods of separating and measuring D-xylitol in various sources cannot be over-emphasized. The qualitative detection and quantification of D-xylitol in the presence of other sugars and sugar alcohols in fruits, vegetables and other natural sources are essential because such information reveals very important characteristics of these natural foods, such as the flavor, maturity, quality, authenticity, and storage conditions (Martínez Montero et al. 2004). The product analysis and quality control of processed food, beverage, and health care products also require sensitive determination methods of D-xylitol. In more recent years, increasing efforts are

directed toward the discovery and development of new bioprocesses for the production of D-xylitol as a natural occurring sugar substitute. Such investigations also depend on efficient detection methods for D-xylitol.

A good number of analytical methods have been developed for the qualitative and quantitative measurement of D-xylitol. Since most samples to be analyzed contain a mixture of compounds, efficient analytical methods for D-xylitol are usually comprised of two components: (1) an efficient separation unit, and (2) a structure identification unit. Earlier methods mainly rely on the retention time of analytes in chromatography for the identification of D-xylitol, while more recently developed methods incorporate advanced spectroscopic methods such as mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy for structure determination of D-xylitol. In the following sections, we provide an overview on current analytical methods for the qualitative and quantitative determination of D-xylitol-containing samples from various sources.

11.2 Detection Methods of D-Xylitol

Based on their separation principles, the current methods for D-xylitol detection may be classified as gas chromatography (GC)-based methods, high performance liquid chromatography (HPLC)-based methods, and capillary electrophoresis (CE)-based methods. The relatively newly developed biotechnology-based methods can analyze compound mixtures without prior separation because D-xylitol is recognized specifically by biomolecules through non-covalent molecular interactions.

11.3 GC-Based Methods

11.3.1 Uncoupled GC

GC is a very useful technique for the analysis of volatile compounds. For the determination of sugars and sugar alcohols, the gas chromatographic method has been widely used due to its good selectivity and small amount of sample needed. It is also rapid and very sensitive. However, the main drawback is that sugars and sugar alcohols including D-xylitol are not volatile and must therefore be converted to volatile derivatives prior to measurement. The common derivatization methods include trimethylsilylation (TMS) (Sweeley et al. 1963; Namgung et al. 2010) and acetylation of all hydroxyl groups present in a molecule (Mäkinen and Söderllng 1980; Lee and Chung 2006). The flame ionization detector (FID) is a type of gas detector used in GC for detecting organic compounds such as proteins, nucleotides, and pharmaceuticals. FID is best for detecting hydrocarbons and other easily flammable components. They are very sensitive to FID and their response tends to

be linear across a wide range of concentrations (Namgung et al. 2010). GC with FID is one of the early methods developed for the detection of D-xylitol (Mäkinen and Söderllng 1980), and it is still the method of choice for the determination of D-xylitol in a complex matrix (Sreenath and Venkatesh 2010; Namgung et al. 2010).

In evaluating the quality of Doenjang, a traditional fermented food widely consumed in Korea, which varies considerably by its basic ingredients, species of microflora, and the fermentation process, the classification of metabolites present in Doenjang samples was performed by using GC-FID. A significant amount of D-xylitol was detected among other metabolites such as amino acids, organic acids, sugars and sugar derivatives, and fatty acids (Namgung et al. 2010).

11.3.1.1 GC–MS

Gas chromatography-mass spectrometry (GC–MS) is one of the so-called hyphenated analytical method in which the gas chromatography separates the components of the mixture and the mass spectroscopy characterizes each of the components. The coupling of GC with MS has greatly enhanced the capacity of this analytical method. GC–MS has high reproducibility, high resolution and few matrix effects (Kopka 2006). It has become one commonly used method for D-xylitol detection (Namgung et al. 2010; Lee and Chung 2006; Clayton et al. 2008). Figure 11.1 shows a typical ion chromatogram and mass spectra of polyols, which were separated as peracetylated derivatives and detected in selective-ion-monitoring (SIM) mode (Lee and Chung 2006). The method was successfully used to simultaneously measure urinary polyols and quantify as little as 0.5–1.0 ng/μL of these polyols.

GC–MS has been used extensively in the medical, pharmacological, environmental, and law enforcement fields. Roboz et al. (1984) used gas chromatography-chemical ionization mass spectrometry with selected ion monitoring of the M-59 ions to determine polyols (as their peracetyl derivatives) in serum. They used 2-deoxygalactitol as an internal standard. A rapid capillary gas chromatographic method was also described by Haga and Nakajima (1989) for the profile analysis of urinary polyols as their trifluoroacetyl derivatives. Ten urinary polyols including D-xylitol and myo-inositol were measured for the first time and verified by GC–MS method. Polyol species in cerebrospinal fluid and plasma-ribitol, arabitol, D-xylitol, 1, 5-anhydrosorbitol, myo-inositol, mannitol, sorbitol, and galactitol were quantified by a capillary gas chromatography/ion trap mass spectrometric method. Microliter volumes of cerebrospinal fluid or plasma were mixed with internal standard (deuterium labeled myo-inositol), deproteinized, and evaporated to dryness. The samples were converted to acetylated derivatives resolved on a capillary column bonded with 50 % phenyl-50 % methyl polysiloxane. Chemical ionization mass spectra for the acetate derivatives of polyols were generated in an ion trap using acetonitrile as reagent gas (Shetty et al. 1995).

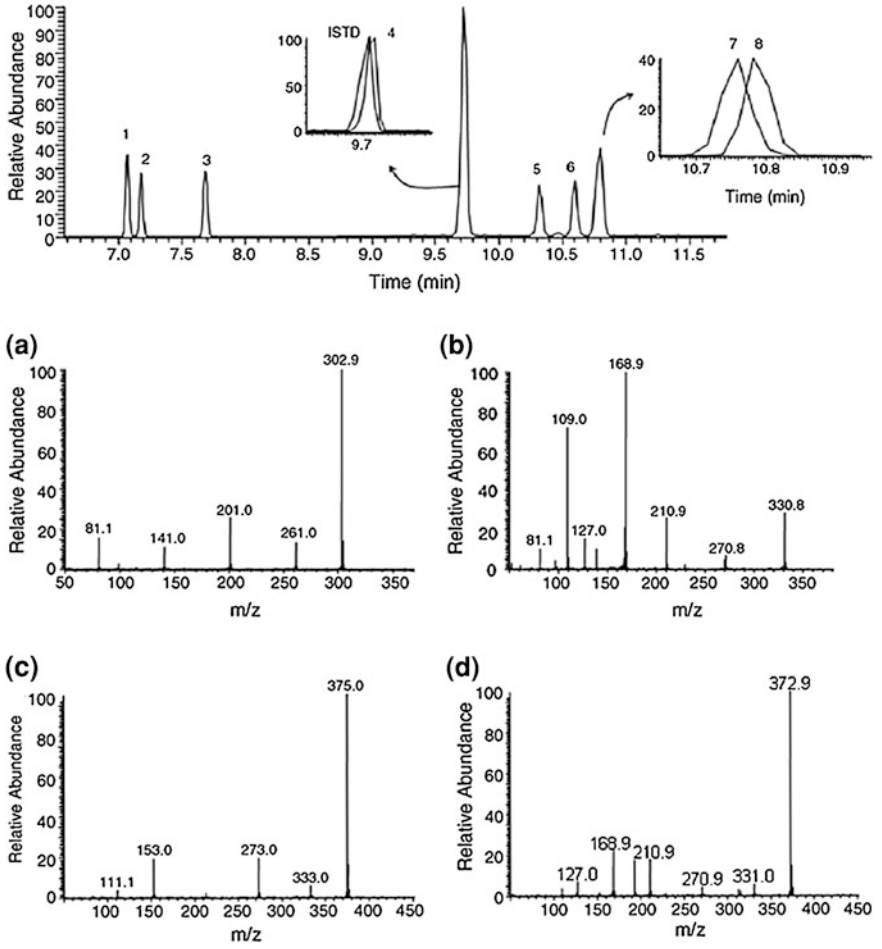

Fig. 11.1 GC-MS analysis of standard polyols as acetylated derivatives separated on an SPB-1701 column (15 m × 0.25 mm i.d., 0.25-μm film thickness). Representative total ion chromatogram (upper layer) and mass spectra (**a**–**d**) obtained in selective-ion-monitoring (SIM) mode. The magnified figures were ion chromatogram at *m/z* 169 and 171 for peak 4 and the internal standard (ISTD), at *m/z* 375 for peak 7, and at *m/z* 373 for peak 8. Peak 1: adonitol; peak 2: arabitol; peak 3: xylitol; peak 4: glucose; peak 5: mannitol; peak 6: dulcitol; peak 7: sorbitol; peak 8: myo-inositol; ISTD: 6, 6-D_2-glucose. Mass spectrum: (**a**) adonitol, arabitol and xylitol; (**b**) glucose; (**c**) mannitol, dulcitol and sorbitol; (**d**) myo-inositol. Adapted from reference Lee and Chung (2006), copyright 2006 Elsevier

The coupling of MS to GC has also increased the accuracy of the GC method in terms of structure determination. Li et al. (2011) reported a GC–MS method for profiling tobacco leaves rich in D-xylitol. From water–methanol-acetonitrile (v/v/v, 3:1:1) extract, nine saccharides, nine alcohols, nine amino acids, 16 organic acids, and phosphoric acid were identified based on standard compounds. Rainey

et al. (2011) also described the chemical characterization of D-xylitol from ground tobacco product. The tobacco product was first derivatized with trimethylsilylation and then analyzed by GC–MS. D-xylitol was detected in mellow Camel sticks.

11.3.2 HPLC-Based Methods

HPLC is a method of separating, identifying, and quantifying compounds in a mixture. Using HPLC does not require the derivatization of the compounds to form volatile derivatives. As early as the 1980s, there were reports on the determination of D-xylitol by HPLC (Galensa 1983; Kertesz et al. 1983; Lohmander 1986). Development in recent years has improved the separation efficiency, sensitivity, and detection accuracy. The HPLC method is simple, fast, and accurate, and is currently the main method for qualitative and quantitative determination of D-xylitol.

11.3.2.1 Uncoupled HPLC

HPLC columns and mobile phases

The separation of D-xylitol from sugars and other sugar alcohols can be quite challenging due to their structural similarity. Different types of columns have been used for that purpose with good separation result. These include amino-based carbohydrate column (Wan and Yu 2006; Bhandari et al. 2008), HPX-87H organic acid column (Park et al. 2005), TSK amide 80 column (Katayama et al. 2006), phenyl column (Wan and Yu 2007), ODS column (Nojiri et al. 1999), and ion-exclusion column (Cheng et al. 2010; Ohsawa et al. 1986). For optimum analytical results, sample pretreatment is usually required prior to applying onto the column. While simple dilution and filtration suffice for liquid samples, solid samples may undergo solid phase extraction (SPE) with different solvents to eliminate a great variety of interfering substances present in the sample matrix (Martínez Montero et al. 2004). The mobile phases most frequently utilized in HPLC separations are mixtures of water/acetonitrile, solutions of NaOH, pure water, sulphuric acid solutions or gradient elution systems (Martínez Montero et al. 2004). The presence of Ba^{2+} ions in an alkaline mobile phase improves selectivity and the reproducibility and reduces the analysis time, because Ba^{2+} ions allow the precipitation of carbonate which would otherwise cause interference (Cataldi et al. 1999).

An improved chromatographic technique known as high-pH anion exchange chromatography (HPAEC) was also developed to separate carbohydrates. It takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at high pH. Coupled with a pulsed amperometric detector (PAD), it allows direct quantification of non-derivatized carbohydrates at low picomole levels with minimal sample preparation and clean-up. This has allowed the detection of carbohydrates in a variety of complex matrices, for instance, foods,

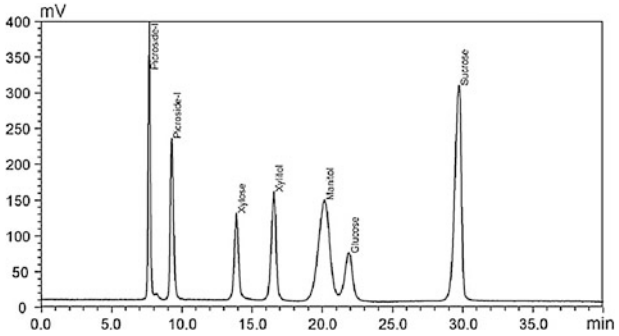

Fig. 11.2 HPLC-ELSD chromatogram of sugars and sugar derivatives: picroside-II, picroside-I, xylose, xylitol, mannitol, glucose and sucrose. Adapted from reference Bhandari et al. (2008), copyright 2008 Elsevier

beverages, dairy products, etc. Both HPAEC and PAD need high pH mobile phases to allow the chromatographic separation of the anionic sugars through ion exchange mechanisms (Martínez Montero et al. 2004).

HPLC detection methods

A variety of detection methods, including UV detection, electrochemical detection, infrared detection, refractive index (RI) detection, and evaporative light-scattering (ELS) detection are available for HPLC methods. The ideal detector must have an adequate sensitivity, good stability and reproducibility, linear response and must be non-destructive. Since D-xylitol lacks chromophoric and fluorophoric moieties, less sensitive detection methods such as RI (Park et al. 2005; Cheng et al. 2010; Ling et al. 2011; Salgado et al. 2010; Altamirano et al. 2000) and ELS (Bhandari et al. 2008) are more commonly used for its determination. Detection limits and sensitivity depend on the type of detector hyphenated to the HPLC. The RI and ELS detection methods typically provide detection limits in the range of 0.05–1.2 μg/injection for D-xylitol (Wan and Yu 2006). Pulsed amperometric detection has also be used for the detection of D-xylitol when coupled with ion chromatography, e.g., using Dionex column and a strongly basic mobile phase (pH > 12).

Bhandari et al. (2008) established a sensitive, selective and reliable HPLC method based on ultrasonic extraction and ELS detection for the simultaneous determination of sugars and sugar alcohols including D-xylitol (Fig. 11.2). The method showed good reproducibility for the quantification of seven analytes in *Picrorhiza* species. The method is simple, accurate and specific and can be used in laboratories that lack sophisticated analytical instruments, such as LC–MS or GC–MS.

Derivatization for more sensitive HPLC detection

Since RI and ELS detection methods are of relatively low sensitivity, pre-column derivatization of D-xylitol for a more sensitive HPLC detection has been

investigated. Derivatization enhances selectivity and sensitivity, but has some disadvantages as well, e.g., it is time consuming and the presence of reagents in analytical samples can interfere with the analysis. Several derivatization methods have been reported in the literature suitable for more sensitive detections based on UV absorbance, fluorescence, and specific optical rotation (Galensa 1983; Katayama et al. 2006; Yamamoto et al. 1998). A simple and sensitive derivatization method with benzoic acid was reported by Katayama et al. (2006) for the determination of diabetic markers in serum, including D-glucose, 1, 5-anhydro-*D*-glucitol, D-xylitol, and other related sugar alcohols. The samples were first treated with benzoic acid in the presence of 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate (IDC) and 4-piperidinopyridine at 80 °C for 60 min. The benzoylated derivatives were separated on a TSK amide 80 column and detected with a fluorescence detector at λ_{ex} 275 and λ_{em} 315 nm. D-xylitol was detected as its mono-benzoyl ester with the detection limit of 10 ng/mL.

Similarly, nitrobenzoylation has been reported as a highly sensitive method for the determination of D-xylitol among other sugar alcohols using UV detection at 260 nm (Wan and Yu 2007; Martínez Montero et al. 2004; Nojiri et al. 1999 and 2000). P-nitrobenzoyl chloride is a rapid and quantitative derivatizing agent for amines and hydroxylated compounds, providing strong UV-absorbing amine and sugar alcohol derivatives allowing for more sensitive detection (Martínez Montero et al. 2004). Nojiri et al. (2000) determined D-xylitol and five other sugar alcohols after derivatization with p-nitrobenzoyl chloride and analyzed on a phenyl column with UV detection. The detection limit of this method was 0.1 % for the above sugar alcohols contained in the samples. The sensitivity of the p-nitrobenzoylation method was 10–1000 times higher than that of HPLC with RI detection or GC with flame ionization detection (Nojiri et al. 1999).

Another derivatization method for UV detection employs phenylisocyanate as a reagent forming phenylurethanes (Indyk and Woollard 1994). Isocyanates have high reactivity with compounds possessing active hydrogen atom(s) and provide highly stable derivatives with carbohydrates and sugar alcohols, allowing detection down to nanogram levels. Interestingly, Yamamoto et al. (1998) reported a method of forming metal complex for the detection of sugars and sugar alcohols. The multi-hydroxylated alditols readily form anionic complexes with molybdate which have remarkably large specific optical rotations (in contrast to the initial alditols showing almost no optical rotation), thus providing a highly selective detection system for these sugar alcohols.

Column-switching HPLC

Recent development in column-switching techniques for chromatography allows the coupling of different separation modes to resolve a wide range of compounds in complex samples (Cheng et al. 2010; Fukushima et al. 2007). Cheng et al. (2010) reported a column-switching HPLC technique by coupling H^+ and Pb^{2+} ion-exclusion columns to study the enzyme hydrolysis of waste cellulosic biomass. The column-switching HPLC with RI detection was connected on-line to an immobilized enzyme reactor for successive on-line desalting and simultaneous

analysis of carbohydrates and D-xylitol in a hydrolysate of waste paper and waste tree branch by incorporating the heart-cut and the elution-time-difference techniques.

11.3.2.2 LC–MS and LC–MS/MS

Electrospray ionization mass spectrometry (ESI–MS) is one of the most important advancements in MS technology. ESI is a desorption ionization method that can be performed on solid or liquid samples, thus allowing the analysis of nonvolatile or thermally unstable samples such as carbohydrates, peptides, proteins, and some inorganic molecules. The ions observed by ESI–MS are usually quasimolecular ions created by the addition of a proton ($\rightarrow [M + H]^+$) or another cation such as sodium ion ($\rightarrow [M + Na]^+$) under positive mode, or the removal of a proton ($\rightarrow [M - H]^-$) under negative mode. Multiply charged ions such as $[M + H + Na]^{2+}$ and $[M - 2H]^{2-}$ are often observed as the result of adding/removing multiple protons and/or cations under positive and negative ionization mode, respectively. The method has high sensitivity and can be used for accurate quantitative and qualitative measurements.

Although the advent of ESI–MS has significantly increased the speed of MS analysis of complex mixtures, mass spectrometry alone is seldom used to analyze D-xylitol-containing samples. Watkins et al. (2005) reported an ESI–MS method equipped with Fourier transform ion cyclotron resonance (FT-ICR) in order to gain detailed structure information of polyols and polyol mixtures including D-xylitol. The analytes were first ionized by positive mode ESI and then allowed to react with the neutral reagent diethylmethoxyborane. Consecutive ion–molecule reactions between the hydroxyl groups of polyols and the borane reagent resulted in products which were separated by 68 mass units, along with 30 mass shifts as a result of intra-molecular derivatization of the primarily formed products. The data provided structural information about the number of hydroxyl groups and their relative positions in the polyols.

In general, MS analysis provides better results for clean samples than mixtures or dirty samples. Thus, the incorporation of a purification/separation component such as HPLC greatly enhances the analytical capacity of MS. Liquid chromatography hyphenated to mass spectrometry (LC–MS) has emerged as a popular and powerful tool for analyzing samples containing multiple compounds. Compared to GC–MS, sample pretreatment is usually simplified with LC–MS because sample derivatization is usually not required.

Wan and Yu (2006) reported the determination of D-xylitol in atmospheric aerosols using LC–MS with positive ESI. Samples were extracted from aerosol filters in methanol and after the evaporation of the solvent reconstituted with 5 mM ammonium acetate in water prior to the LC–MS analysis. D-xylitol was efficiently separated on polymer-based amino columns from eight other sugars and sugar alcohols (glycerol, erythritol, D-mannitol, D-xylose, D-glucose, levoglucosan, sucrose and a trisaccharide melezitose). Isocratic elution was applied with a

mobile phase consisting of 20 % of 10 mM NH_4Ac aqueous solution, 8 % of methanol, and 72 % of water. The addition of an ammonium ion resulted in $[M + NH_4]^+$ which was found to be abundant and used for monitoring and quantification of D-xylitol. The analytes were measured at the levels of 100, 500 and 1000 μg/L and their recovery rates were in the range of 78–102, 94–112, and 92–110 %, respectively. The limit of detection for D-xylitol was 4.7 pmol/injection.

In a similar study by Wan and Yu (2007), LC–MS with negative ESI was used to analyze sugars and sugar alcohols in atmospheric aerosols. Sugars and sugar alcohols do not deprotonate easily to form $[M - H]^-$ ions because they lack highly acidic groups, therefore their ionization is not effective under negative mode ESI without derivatization. However, the post-column addition of chloroform into acetonitrile was found to greatly enhance the ionization of these compounds by forming chloride adduct ion under negative mode ESI. The detection limit of D-xylitol was 0.016 μM based on the quantification of $[M + {}^{35}Cl]^-$ ion.

Tandem mass spectrometry, also known as MS/MS or MS^2, involves multiple steps of mass spectrometry with some form of fragmentation occurring in between the stages. Specific precursor ion(s) generated in the first mass analyzer may be selected for fragmentation, and the product ions are scanned in the second mass analyzer. The first step MS may be considered as a separation unit to provide individual molecular ions (and their daughter ions) of the compounds present in the sample being analyzed. As such, MS/MS not only provides more detailed structural information of the analyzed molecule, but also allows the analyis of samples containing a mixture of compounds. Combined with liquid chromatogrphy, LC–MS/MS has become a routine technique for analyzing samples of complex mixtures.

Wamelink et al. (2005) reported an LC–MS/MS method used to analyze the content of sugar alcohols in urine samples. The urine samples were supplemented with isotope-labeled internal standards ($[^{13}C_4]$ erythritol, $[^{13}C_2]$ arabitol and $[^{2}H_3]$ sorbitol) and desalted by a mixed-bed ion-exchange resin. Separation was achieved by using an Aminex HPX-87C column. Multiple reactions monitoring polyol detection were achieved by tandem mass spectrometry with an ESI source operating in the negative mode. Age-related reference ranges of D-xylitol and other polyols (erythritol, treitol, arabitol, ribitol, galactitol, mannitol, sorbitol, sedoheptitol and perseitol) in urine were established. The method was also applied to the quantification of the abnormal polyol concentrations observed in patients with transaldolase deficiency, ribose-5-phosphate isomerase deficiency and classical galactosaemia.

11.3.2.3 LC–NMR

Proton nuclear magnetic resonance (^1H NMR) spectroscopy is a routine tool for fast and comprehensive characterization of organic compounds. However, compound mixtures often result in extensive signal overlapping and spectral

complexity which can be a serious hindrance in analyzing mixtures. The development of the LC–NMR technique, which couples an HPLC step immediately prior to NMR measurement, allows compound separation and analysis to be carried out quickly (Griffiths 1995).

Graça et al. (2008) reported an LC-NMR method for the metabolic profiling of human amniotic fluid and identified more than 60 compounds including D-xylitol in the fluid. Both reversed-phase and ion-exchange liquid chromatography were carried out in order to separate a range of metabolites including amino acids, sugars, xanthenes, organic acids and their derivatives. The NMR spectrometer was equipped with a 3-mm probe head (60 μL active volume) and coupled to an ION300 ion exchange column with a mobile phase composed of 2.5 mM H_2SO_4 in 100 % D_2O. Subsequent NMR and MS analysis enabled the rapid identification of over 60 compounds, five of which were detected for the first time from the human amniotic fluid. Since NMR spectroscopy provides structural information about the atom connectivity and the stereochemistry of molecules, the LC-NMR method has a higher degree of accuracy in the determination of D-xylitol compared to other methods.

11.3.2.4 Optimum Performance Laminar Chromatography

Thin layer chromatography (TLC) is a simple chromatography technique used to separate mixtures for both analytical and preparative purposes. Due to its simplicity and low cost, it is widely used for rapid sample/product profiling. Altamirano et al. (2000) used TLC to detect D-xylitol while trying to rapidly identify the best D-xylitol producing yeast strains isolated from different natural sources. Silica gel TLC analysis was carried out with ethyl acetate: 2-propanol: water (130:57:23) as the developing solvent and bromocresol green-boric acid as the staining agent. The visualization of a yellow spot on a blue background of the TLC plate indicated the presence of D-xylitol, and the relative intensity of the yellow spot correlated with the amount of D-xylitol present in the analyzed samples.

OPLC, also known as over-pressured layered chromatography or forced-flow TLC, is a pumped-flow chromatography system that combines the user friendly interface of HPLC with the capacity of flash chromatography and the inexpensive and multidimensional aspects of TLC (Tyihak et al. 2001). OPLC may be considered as a TLC technique of which the mobile phase is moved under pressure, or alternatively, an HPLC technique that uses a square and planar two-dimensional column format. OPLC allows side-by-side analysis of several samples in a single run, provides added loading capacity for a single sample, and improves separation efficiency. Several types of detection methods have been used in conjunction with OPLC including radiation detection, fluorescence detection, UV detection, and Raman spectroscopy detection (Poole and Poole 1995).

Tamburini et al. (2006) reported an OPLC method for the separation and quantitative determination of three alditols (D-xylitol, L-arabitol, and D-glucitol)

and four aldoses (D-xylose, L-arabinose, D-glucose, and L-rhamnose). The four sugars were present in hemicellulose hydrolyzates used as substrates for the production of D-xylitol from D-xylose by yeast. All four aldoses and three alditols were detected in the final fermented broth. The separation was performed on aluminum foil-backed silica gel OPLC–HPTLC plates with acetonitrile–acetic acid–water (63:33:5, v/v/v) as a mobile phase. The developed plates were derivatized with lead (IV) acetate dichlorofluorescein reagent (Jork et al. 1990) and the fluorescence intensity measured at $\lambda = 313$ nm. The upper limits of linearity were determined to be in the range of 140–600 ng and the detection limits were 15–50 ng per spot.

11.3.2.5 Capillary Electrophoresis

Analytical separation of aldoses and alditols in complex mixtures has been achieved by several other methods including capillary electrophoresis (CE) (Corradini et al. 1998; Guttman 1997; Martínez Montero et al. 2004). CE is a powerful separation technique for the separation of a variety of analytes in relatively complex matrices. The application of CE for the analysis of sugar polyols has been reviewed previously (Martínez Montero et al. 2004). Sugar polyols lack both a charge and a strong UV chromophore, and thus their detection by UV absorbance can be carried out after derivatization (Guttman 1997). Alternatively, underivatized sugar polyol may be detected by indirect UV detection by using high-alkaline pH medium to ionize the polyol in a buffer solution.

A CE method with indirect UV detection was reported for the simultaneous analysis of underivatized acidic, neutral and amino sugars, and sugar alcohols including D-xylitol (Soga and Heiger 1998). Separations were carried out on fused silica capillaries and a combination of 2, 6-pyridinedicarboxylic acid (20 mM) and cetyltrimethylammonium bromide (0.5 mM) was used as the electrolyte. Optimum separation of carbohydrates and sugar alcohols was achieved at pH 12.1 and the minimum detection level for carbohydrates was in the range of 23–71 μM. Under these conditions, D-xylitol was measured along with 18 monosaccharides and other sugar polyols. The applicability of this method was demonstrated in the determination of the monosaccharide composition in the acid hydrolysate of a model glycoprotein fetuin (Soga and Heiger 1998). The advantages of the CE method include direct analysis of sugar and sugar alcohols without derivatization and its high separation capacity for acidic, neutral, and amino sugars and sugar alcohols under a single electrophoretic condition.

Rovio et al. (2007) reported a capillary zone electrophoresis (CZE) method for the separation of D-xylitol, D-mannitol, and ten neutral carbohydrates (sucrose, D-fucose, cellobiose, D-galactose, D-glucose, L-rhamnose, D-mannose, D-arabinose, D-xylose, and D-ribose). The alkaline electrolyte solution was prepared with 130 mM sodium hydroxide and 36 mM disodium hydrogen phosphate and the analytes were detected with direct UV detection at the wavelength 270 nm. The proposed UV-absorbing species at the wavelength 270 nm were enediolate ions

and conjugated enol carbonyl structures that were generated as carbohydrate degradation intermediates under the strongly alkaline condition. The source of UV absorbance in sugar polyols such as D-xylitol under such conditions was not presented, but one may speculate that oxidation reaction followed by some kind of β-elimination reaction in polyols could generate α, β-unsaturated carbonyl system with UV absorbance at around 270 nm. The calibration curves were linear in the range of 0.05–3.0 mM and the detection limits for the analytes were 20–50 μM (Rovio et al. 2007). This method was demonstrated in the determination of monosaccharides in lemon, pineapple, and orange juices and in a cognac sample.

In situ derivatization of polyols with boric acid was described for CE analysis of polyols under less alkaline condition (Pospisilova et al. 2007). Boric acid [B (OH) $_3$] reacts readily with a diol forming a borate diester complex $(RO)_2$ BOH, which is readily ionizable and migrates electrophoretically. Thus, capillary zone electrophoresis (CZE) employing on-column complexation with boric acid and indirect UV detection at 215 nm was used to determine D-mannitol, D-sorbitol and D-xylitol (Pospisilova et al. 2007). The separation of the anionic borate-polyol complexes was carried out in a fused silica capillary at 25 kV with optimized background electrolyte of 200 mM borate buffer (pH 9.3) containing 10 mM 3-nitrobenzoate as the chromogenic co-ion. The rectilinear calibration range was 0.2–2 mg/mL for D-mannitol and D-sorbitol while 1 mg/mL of D-xylitol was used as the internal standard. The limit of detection was approximately 30 μg/mL for both D-mannitol and D-sorbitol.

Similarly, the strategy of forming polyol-borate complexes has been utilized in capillary isotachophoresis (ITP) for the determination of D-xylitol in multicomponent pharmaceutical formulations and foods (Herrmannova et al. 2006). The separation was carried out in a capillary tube of 0.8 mm ID and 90 mm effective length made of fluorinated ethylene-propylene copolymer filled with an electrolyte system consisting of 10 mM HCl + 14 mM Tris (pH 7.7, leading electrolyte) and 5 mM L-histidine + 5 mM Tris (pH 8.3, terminating electrolyte). The analysis was performed at a driving current of 200 μA and detected at a current of 100 μA. The aqueous sample solution was treated with boric acid and the substances of polyhydroxyl nature were converted to borate complexes allowing them to migrate isotachophoretically. Using conductivity detection, the calibration curves were linear in the range up to 2.5 mM for all the components analyzed (acesulfame K, saccharine, aspartame, cyclamate, D-sorbitol, D-mannitol, D-lactitol, and D-xylitol). The detection limits were in the range of 24–81 μM. The concentration of D-xylitol was 52 μM. The good precision of this ITP method was evidenced by the favorable relative standard deviation (RSD) values ranging from 0.8 to 2.8 % obtained at the analyte concentration of 1.0 mM ($n = 6$). Simplicity, accuracy, and low cost of analyses made the ITP an alternative procedure to the current methods for the determination of D-xylitol and other ionizable sweeteners.

11.3.3 Biotechnology-Based Methods

11.3.3.1 Indirect Competitive Immunoassay

Recently, Sreenath and Venkatesh (2010) reported an indirect competitive enzyme-linked immunosorbant assay (IC-ELISA) for the detection and quantification of D-xylitol in food. Heptan-specific anti-D-xylitol antibodies (IgG and IgE) were raised against reductively aminated D-xylitol-albumin conjugate as the immunogen and purified through affinity column. Flat bottom polystyrene microtiter wells were coated with high hapten density, D-xylitol-BSA conjugate (32 haptens/mol) at 100 ng per well, followed by the addition of affinity-purified anti-D-xylitol antibodies (4 ng in 50 μL) and different dilutions of food extracts. Indirect competition was created between the D-xylitoyl epitopes of D-xylitol-BSA conjugate coated on the wells of microtiter plate and free D-xylitol present in the food extracts as they both bind to D-xylitol-specific antibodies. With this immunoassay, the limit of detection was 1 ng for D-xylitol and the linear range for D-xylitol quantification was 5–400 ng. The results were in good agreement with the reported values by HPLC and GC methods. This indirect competitive ELISA may serve as a sensitive analytical method to detect and quantify nanogram amounts of D-xylitol in various biological samples and natural/processed foods (Sreenath and Venkatesh 2010).

11.3.3.2 Enzymatic Assays

Biosensor technology is now widely used in the detection, control and measurement of specific compounds in fermentation broths. Takamizawa et al. (2000) reported a D-xylitol biosensor composed of a partially purified D-xylitol dehydrogenase (XDH) from *C. tropicalis* IFO 0618 and a dissolved oxygen meter (electrode). A flow-injection system using the immobilized XDH was evaluated for on-line D-xylitol monitoring for automatic control of D-xylitol production. The partially purified XDH was most active at pH 8.0 and 50 °C but the optimum operation temperature for the biosensor in the flow-injection system was 30 °C. The biosensor favored the use of NAD^+ over $NADP^+$ in the oxidation reaction of D-xylitol to D-xylulose. Correlation studies indicated that the biosensor was applicable for measurement of D-xylitol in the narrow concentration range of 1–3 mM. The biosensor remained active for two days at 5 °C and was re-used three times with reproducible results. The activity later dropped to 30 % of its original value. The biosensor was completely inactive towards arabitol, xylose, glucose, glycerol and ethanol and non-selective toward sorbitol and ribitol, which were also oxidized at 65 % and 58 % of the D-xylitol rate, respectively.

Vermeir et al. (2007) reported the use of a miniaturized and automated enzymatic assays for fast sugar and acid quantification in apples and tomatoes. The detection of D-xylitol was based on the enzyme sorbitol dehydrogenase which

oxidized D-xylitol to D-xylulose, and at the same time NAD^+ was reduced to NADH. The assay was based on an increase/decrease in absorbance at λ340 nm caused by a change in NADH concentration before and after the addition of the substrate, with the analytical sample containing D-xylitol. The change was stoichiometrically related to the concentration of D-xylitol present in the sample being analyzed. The enzyme sorbitol dehydrogenase reacted with both D-sorbitol and D-xylitol, and this made the method non specific for D-xylitol. The key features of this method were its high throughput (due to automation) and micro-scale. The assays were miniaturized from the standard 3 mL assays in cuvettes into assays of 200 μL or lower in 96 or 384 well micro-plates. Limit of detection, linearity of calibration curve, and repeatability of the assays with standard solutions were proven to be satisfactory. The automated and miniaturized assays were validated with HPLC analyses for the quantification of sugars and acids in tomato and apple extracts. This enzymatic assay may serve as a fast, reliable, and inexpensive alternative to HPLC as the standard analysis technique for the detection of D-xylitol in fruits, vegetables and processed food.

Rhee et al. (2002) developed a flow injection analysis (FIA) system with an immobilized D-xylitol oxidase (XYO) cartridge for on-line monitoring of D-xylitol concentrations during a D-xylitol production process. The enzyme D-xylitol oxidase that oxidized D-xylitol to D-xylose was produced in a recombinant *E. coli*, and the isolated enzyme immobilized on a VA-Epoxy Biosynth E3-support. During the oxidation reaction, oxygen was consumed and hydrogen peroxide produced. The concentration of D-xylitol correlated with the amount of oxygen consumed or the peroxide produced. High activity of the immobilized XYO was attained by using potassium phosphate solution (1 M) with 0.5 g/L Triton X-100 adjusted to pH 8.5 as the carrier solution. High concentrations of certain components including arabinose at 20 g/L, D-xylose at 30 g/L and sodium chloride at 30 g/L in the sample had significant inhibitory effects on the response of the XYO-FIA system.

11.4 Conclusion and Future Recommendations

Primary interest in D-xylitol is due to its properties and its potential use as a sugar substitute. It has a sweetness equivalent to sucrose but its slower metabolism makes it useful as a low caloric sweetener. The cool and fresh taste of D-xylitol makes it ideal for candy and medicinal coatings. Incorporating D-xylitol into confectionery products, beverages and other foodstuffs could reduce the incidence of dental carries.

Currently, there are many analytical methods available for the qualitative and quantitative measurement of D-xylitol. Based on chromatography techniques, these are GC, HPLC, and CE methods with different detection options such as UV, RI, ELS, etc. More sophisticated analytical instruments derived from hyphenation of chromatography with structure determination tools such as MS and NMR have

become more accessible in the past decade. Therefore, GC–MS, LC–MS and LC–MS/MS have become routine methods for D-xylitol detection. The coupling of spectroscopic methods such as NMR, and MS to chromatography provides structural information about elemental connectivity and stereochemistry of unknown compounds. Furthermore, immunoassay and enzymatic assay methods are advantageous over other methods of D-xylitol detection in that they are potentially highly specific for D-xylitol and the cost is low.

Development of novel, specific, sensitive and inexpensive biotechnology-based D-xylitol detection methods will go a long way in improving the qualitative and quantitative determination of D-xylitol in fermentation broth, fruits, vegetable sources and cereals. This is a challenge for future research.

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Part IV
Commercial Status of Xylitol Production

Chapter 12
Key Drivers Influencing the Large Scale Production of Xylitol

Zhang Hou-Rui

Abstract The technologies related to xylitol production by fermentation have progressed significantly and become industrialized. To further increase the market competitiveness of fermentative xylitol production on a commercial scale, it is necessary to ascertain the main links affecting the production cost, and accurately formulate the effective strategy to reduce the xylitol production costs. From the aspect of xylitol commercial production, this paper focuses on analyzing the main steps influencing the production costs of fermentative xylitol mass production, and the comparative advantages of the fermentation process are also discussed. Some important sectors, which influence the cost of xylitol fermentation production on a massive scale, are further illustrated. It mainly includes the applicability assessment of raw materials, integration of products purification technologies, etc. Basing on this, the suggestions on how to use the comparative advantages of xylitol fermentation process to establish a highly commercially viable processing strategy are made, and some opinions on how to integrate the xylitol manufacturing unit with the biorefinery are added.

Keywords Xylitol · Hemicellulose hydrolysate · Fermentation · Mass production

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12.1 Introduction

Over the past several decades, technologies related to xylitol production by fermentation have progressed significantly and become industrialized. Some current xylitol production lines have an annual production capacity of several thousand tons. One of the problems with producing xylitol through fermentation is cost competition with chemically produced xylitol. The key drivers influencing the large-scale production of xylitol are the steps in the technical process that affect the cost of mass production. This paper focuses on analyzing the main steps influencing the production costs of fermentative xylitol mass production. We here discuss the characteristics and comparative advantages of the fermentation process, and make some suggestions on how to use those advantages to establish a highly commercially viable processing strategy.

12.2 Xylitol Production: Characteristics and Comparative Advantages of the Fermentation Process

When xylitol is produced on an industrial scale, whether by means of traditional chemical processes or biological fermentation, the first step is to hydrolyze the xylan-rich materials available in large quantitis, and obtain hemicellulose hydrolysate, the main component of which is xylose. Then the two main strategies diverge according to the different technological routes (Fig. 12.1).

Xylan is a hetero-polysaccharide, a polymer composed of various monosaccharides. Its hydrolysate contains xylose and other sugars, including glucose, arabinose, and galactose (Saha 2003; Murzin et al. 2011). Dilute acid hydrolysis is still the main method of preparing hemicellulose hydrolysate. In this process, the highly acetylated native xylan is deacetylated to generate acetic acid; lignin is partially hydrolyzed to generate phenolic compounds; and some simple sugars are dehydrated to produce furan compounds under acidic and high-temperature conditions. These, the saccharides, and any inorganic salt from the plant and pigments produced during hydrolysis are all present in the hemicellulose hydrolysates (Murzin et al. 2011). This is why both the chemical and biological fermentation processes must be complicated procedures, involving specific steps for product segregation and purification. In order to replace the chemical process with fermentation, the latter must present a substantial cost advantage. We here analyze and compare the characteristics of these two processes and of the fermentation process. Analyzing and comparing with the features of these two processes, the comparative advantages of fermentation process can be highlighted, promoting the commercialization of xylitol fermentation.

To produce xylitol by chemical means, the hemicellulose hydrolysates must first be desalinated and decolorized to obtain pure syrup. D-xylose is obtained via purification by crystallization. Next, the D-xylose crystal is dissolved in water and

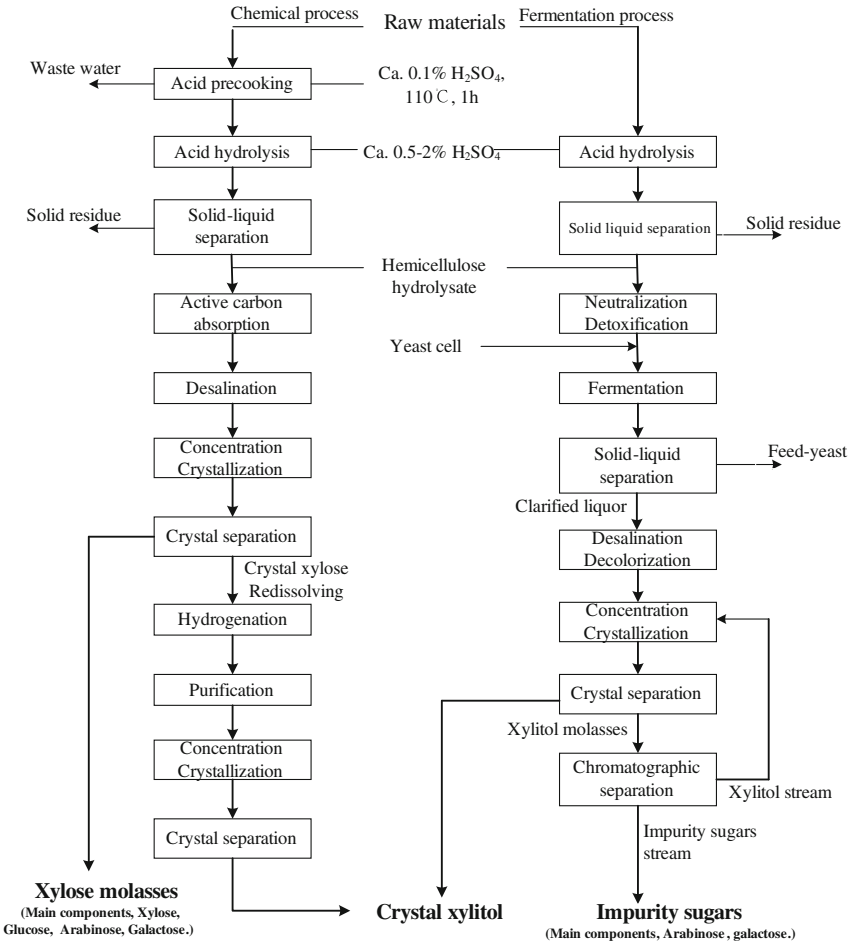

Fig. 12.1 Comparison between chemical and fermentation route for xylitol production

then reduced to xylitol via catalytic hydrogenation using Raney nickel as catalyst under high-temperature high-pressure conditions (Jin shu-ren 2008; Mikkola et al. 2000; Mikkola and Salmi 2001). The hydrogenation liquid is subject to decolorization by activated carbon adsorption, and the inorganic salt is removed via ion exchange. The resulting pure, colorless hydrogenation liquid is then condensed and crystallized into pure xylitol crystal.

The xylose concentration in the hydrogenation is about 50 % and the time for hydrogenation is about 30 min. A 5 m^3 hydrogenation reaction kettle under a hydrogenation pressure of 10 MPa is capable of producing 5,000 tons xylitol crystal per year (Jin shu-ren 2008). Currently, the cost of hydrogenation of xylose to xylitol is about $350/ton, and the cost of xylose production is about $2,300–2,500/tons. This means that the cost of hydrogenation is less than 20 % that of the

total cost of xylitol production, and the cost of xylose crystal production is more than 80 % of the total cost of xylitol production.

In the chemical process, D-xylose must be used as raw material for hydrogenation. This is because if hemicellulose hydrolysates composed of various saccharides are directly hydrogenated, all the saccharides will be reduced to corresponding alcohols. Because the alcohol's solubility is much higher than that of their corresponding saccharides, it is more difficult to purify xylitol via crystallization from a mixture of sugar alcohols than from a mixture of sugars. The main reasons for the high cost of xylose crystal production include the following. First, the composition of the non-sugar components in the hemicellulose hydrolysates is very complicated, and the purification steps required to remove these components are rather tedious. Second, the physicochemical properties of the sugar impurities are fairly similar to those of xylose and can inhibit xylose crystallization. As a result, about 20–30 % of the xylose is retained in the crystal mother liquor. There is currently no economically feasible method of recovering xylose from the mother liquor using crystallization. The complexity of the purification procedures and low product yield further push the cost of producing xylose production to a high level.

In the biological fermentation process, yeast is used to directly ferment detoxified hemicellulose hydrolysates and produce xylitol. In this process, glucose is consumed to provide metabolic energy for the growth of yeast; arabinose and galactose are generally not used by yeast because of the presence of xylose (Mussatto et al. 2006; Silva and Felipe 2006; Sheu et al. 2003), and the small amount that is used does not generate corresponding alcohols (Silva and Afschar 1994). In this way, the fermentation of hemicellulose hydrolysates to xylitol is in essence a biologically selective catalytic process. Using such selective catalysis, the hemicellulose hydrolysates composed of xylose and three impurity sugars (glucose, arabinose, and galactose) become a mixture of xylitol and two impurity sugars (arabinose and galactose). This transforms the product purification process into a segregation process involving the isolation of xylitol from two impurity sugars.

Apparently, the advantages of the fermentation method are as follows. First, the purification of xylose by crystallization is not needed. The chemical method of producing xylitol requires two crystallization-drying processes (for xylose and xylitol), but the fermentation method directly transforms the xylose in the hemicellulose hydrolysates into xylitol. The fermentation process therefore requires only one xylitol crystallization-drying step. This shortens the procedure and correspondingly reduces costs. Second, the burden of purifying hemicellulose hydrolysates is alleviated. Although the fermentation liquid does need to be desalinated via ion exchange and decolorized by activated carbon adsorption before the purified xylitol solution can be further condensed and crystallized to obtain the xylitol crystal, the inorganic elements in the hemicellulose hydrolysates, such as N, P, and K, are all used by the yeast as nutrients. Some organic acids and pigments are partially degraded. In this way, the total cost of purifying the fermentation liquid into xylitol crystal is lower than the cost of producing xylose

crystal from hemicellulose hydrolysates. Third, the isolation efficiency and recovery rates of products are increased greatly. Both xylose and xylitol, when the purity is lower than 50 %, are extremely difficult to crystallize. In the chemical process, the mother liquor left behind after xylose crystallization contains xylose at a concentration approximately equal to the sum of each impurity sugar (glucose, arabinose, galactose). While this is also technically true of the mother liquor left behind after xylitol crystallization from fermentation, this liquor contains only two impurity sugars (arabinose and galactose). This makes the separation process much easier after fermentation than after chemical production.

Currently, chromatographic separation is the only method available to the industry to recover target product (xylose or xylitol) from such two crystallized mother liquors. In such separation systems using cation-exchange resin as a chromatographic stationary phase and water as eluent, chromatographic behavior of different sugars in hemicellulose hydrolysate are very similar to each other. The elution order of xylose mother liquor is glucose > xylose + galactose > arabinose. The chromatographic peaks of xylose and galactose almost overlap each other, glucose is eluted faster than xylose, and arabinose is eluted more slowly than xylose. When industrial chromatographic separation is used to recover xylose in the xylose mother liquor, it involves the two steps of separating three components: xylose must be separated not only from the faster glucose but also from the slower arabinose. The efficiency of this type of separation is rather low. The process is also unable to separate xylose from galactose at the same time because their chromatographic peaks almost completely overlap each other. In this way, recovering xylose from xylose mother liquor is of no practical value in commercial applications.

The elution order of the different components in the xylitol mother liquor during liquid cation- resin chromatography is galactose > arabinose > xylitol. The two impurity sugars (galactose and arabinose) are both eluted faster than xylitol. The recovery of xylitol from the fermentation mother liquor via industrial chromatographic separation involves only the one step segregating xylitol from faster components (including galactose and arabinose). It is a two-component separation process from which high-purity xylitol can be obtained with relatively high separation efficiency. Because of this, no usable xylitol remains in the mother liquor, and the total yield of xylitol, based on the raw materials, is substantially higher in the fermentation process than in the chemical process. This is a very important advantage of the fermentation process (Fig. 12.1).

12.3 Technological Process and Production Cost

12.3.1 Raw Material

12.3.1.1 Selecting Good-Quality Raw Materials

In principle, any raw material rich in xylan (main components of hemicellulose) can be used to prepare the hydrolysate for fermentative xylitol production. However, raw materials used in the commercialized fermentative xylitol production must meet the following requirements: (1) The material should be abundant and relatively concentrated so that there is an ample supply within a reasonable transportation radius. (2) The content of hemicellulose must be high and xylan must be the main component of that hemicellulose. In general, not only should the yield of xylose from the hydrolysis of the raw material be high, but the ratio of xylose to arabinose should be greater than 9:1 and the combined amount of xylose and arabinose should make up more than 95 % of the total sugars in the hydrolysate. The fermentation of such hydrolysates allows the relatively high yield of xylitol from the direct crystallization recovery, and the cost of product separation is relatively low. (3) The material should not contain impurities that are difficult to process or isolate. For example, the xylose content of some farming byproducts may be relatively high, but these products contain too many impurities, such as higher content of starch, which increase not only the risk of contamination of the fermentation system, but also the difficulty of isolating and purifying the products.

Currently, the raw materials that have truly been used for xylose production on a large scale, aside from birch wood sulfite pulping liquor, mainly include corncob and bagasse. The xylose content of corncobs (about 28–30 %) (Lee et al. 2011; Wang et al. 2011) is higher than that of bagasse (about 25–26 %) (Branco et al. 2011; Paiva et al. 2009), but corn cobs also have a relatively high level of impurities sugar. The lower cost of refining the bagasse product completely compensates for its lower xylose content.

12.3.1.2 Factors Influencing the Cost of Raw Materials

In addition to the total amount of local resources, transportation radius, storage and transportation conditions, competition between process industries is also a significant factor influencing the price of raw materials. For example, substances that make good raw materials for xylitol production also make good raw materials for furfural production. The competition for raw materials between these two industries is intense. When choosing the site of a prospective xylitol factory, locations near enterprises that may compete for raw materials should be avoided.

The cost of transporting raw materials is also an important component of production costs. Excepting raw materials such as bagasse, which are already highly concentrated and for which transportation costs are not a concern, most

crop straws require collection, packaging, and transportation. For instance, pressing and strapping can increase the efficiency of loading and transportation. Obtaining raw materials in amounts that meet the needs of production within a reasonable transportation radius is an important basic condition for xylitol fermentation factories to gain competitive advantages.

12.3.1.3 Cost of Raw Material Storage

Storing raw materials has two economically significant aspects: the effect of storage on the purity of the hydrolysates and the loss and wastage that may occur during storage.

When fresh straws are used as raw materials for the production of xylose crystal, the concentrations of impurities such as starch, oligosaccharides, and dissoluble monosaccharides are relatively high. During hydrolysis, they are included in the hemicellulose hydrolysates, which make the glucose content in the hydrolysates relatively high and decrease the proportion of xylose in the total amount of sugars, reducing the yield of xylose crystal. For this reason, straw materials are generally retted and stored for 2–3 months before use so that the starch and dissoluble monosaccharides can be consumed by microorganisms. This decreases the glucose content and increases the relative amount of xylose. Although, the microorganisms may consume some xylose during retting storage, the increased xylose purity more than makes up for this loss.

The glucose in the hydrolysates of straws is completely consumed by yeast metabolism during fermentation, so it does not affect xylitol crystallization after fermentation. However, because glucose significantly inhibits xylitol fermentation (Ooi et al. 2002), it increases the risk of introducing undesired microorganisms into the fermentation system, especially when large amounts of glucose are present. For this reason, reducing the glucose content through retting storage of raw materials is beneficial to the xylitol fermentation production process. In particular, for corncobs with high starch content and bagasse with high sucrose content, retting plays a notable role in reducing the negative effects of xylose fermentation.

Straw raw materials, due to their large volume, are usually stored outside to reduce costs. In general, as long as the water content of the raw materials does not exceed a certain threshold (about 20 %), the stack piles are considered safe. However, in high-precipitation regions, rain may lead to considerable rotting. When choosing sites for large-scale xylitol factories, raw material wastage due to the weather must be considered.

12.3.2 Hydrolysis Conditions and the Cost of Hydrolysates

12.3.2.1 Hydrolysis Conditions

Dilute acid hydrolysis is still the most common method of making hemicellulose hydrolysates, and sulfuric acid is the most commonly used catalyst (Saha 2003; Murzin et al. 2011). The volatile hydrochloric acid is rarely used because it requires buildings and equipment with much higher anti-corrosion standards than sulfuric acid. In general, the oxidative nitric acid is not used either because it reduces the service life of ion-exchanging resins.

The main factors influencing the hydrolysis of the raw materials include the acid concentration, saturated vapor pressure (temperature), and duration of hydrolysis (Bustos et al. 2003; Lavarack et al. 2002; Paiva et al. 2009; Rahman et al. 2007). Generally speaking, at higher acid concentrations, the hydrolysis can be performed at a lower temperature over shorter periods of time. At lower acid concentrations, the temperature required for hydrolysis is higher and the duration longer. The acid ions added during hydrolysis must be removed at the end of the process, during the refining of xylitol. Properly increasing the hydrolysis temperature and decreasing the acid concentration can alleviate the burden of removing inorganic ions during the process of refining fermentation liquor.

Although researchers usually use a high liquid-to-solid ratio (about 10:1) to study the optimal conditions for hydrolyzing hemicellulose raw materials (Lavarack et al. 2002; Rahman et al. 2007; Branco et al. 2011; Murzin et al. 2011), in specific commercialized hydrolysis processes, low liquid-to-solid ratios can effectively increase the xylose concentrations of the hydrolysates and thus reduce the costs of evaporation concentration. Decreasing the liquid-to-solid ratio usually decreases the raw material—xylose yield, but the savings on evaporation concentration exceed the loss caused by reduction in yield, causing an overall benefit. Appropriately compressing or shredding the raw materials can increase the specific volume of the material and decrease the liquid-to-solid ratio needed. Dilute acid hydrolysis in steam is one noteworthy way to decrease the liquid-to-solid ratio. Zhao et al. (2003) sprayed diluted acid on the surface of crushed corn cobs, then left them to be soaked for some time before hydrolysis. After the hydrolysis in steam, the extraction of solid–liquid is applied to obtain hydrolysate from the solid residues. The experiment proves that the reducing sugar concentration in the extract liquid reached a high level of 130 g/L with three-step countercurrent extraction.

Sulfuric acid can interact with any carbonate, silicate, or phosphate in the plant materials and decrease the concentration of hydrogen ions. When there is a large amount of excess acid (high liquid-to-solid ratio), metallic salts do not affect the acidity of the medium. When the liquid-to-solid ratio is less than 10, however, neutralization can substantially decrease the acidity of the solution, thus decreasing the rate of hydrolysis and the monosaccharide yield, increasing the oligosaccharide content of the solution. For this reason, the concentration of catalyst in low liquid-to-solid ratio hydrolysis is usually high.

Oligosaccharides are the products of the incomplete degradation of xylan. They cannot be used by yeast, but they can strongly inhibit the crystallization of xylitol. Caution must be taken in controlling the hydrolysis condition to reduce the generation of oligosaccharides. This not only contributes to increasing the xylose yield but also decreases the purification costs of xylitol crystallization.

12.3.2.2 Recovering Xylose from the Hydrolysis Residues

After the hemicellulose in the materials is hydrolyzed, the residues are mainly composed of cellulose and lignin. Recovering the hydrolysate liquor absorbed by the residue is an important way to increase the xylose yield and decrease the cost of fermentation medium. Usually, multistage countercurrent pressed washing or filtered centrifugal washing is used to recover xylose from the residue. These methods require only relatively small amounts of water and have relatively high xylose yield. Currently, the belt filter press is widely used in pressing hydrolysis residues to recover xylose. It has the advantages of low power consumption, high xylose yield, and low liquor content in the residues. It is also convenient to use and maintain.

The xylose content of the hydrolysate liquor recovered by washing the residue is usually relatively low, and the cost of concentrating it for the purpose of fermentation is usually prohibitively high. A more commercially valuable processing strategy might involve replenishing the dilute hydrolysate liquor with concentrated acid so that it can be used in the hydrolysis of the next batch of materials. This increases the xylose concentration of the next batch of hydrolysate liquor.

12.3.3 Cost Factors Affecting Hydrolysate Detoxification

12.3.3.1 Strategy of Hydrolysate Detoxification

If the metabolic physiology of microorganisms is to be used as a detection system, many inhibitors of yeast metabolism can be found in hemicellulose hydrolysate by diluted-acid (Duarte et al. 2006; Luo et al. 2002; Mussatto et al. 2005; Mussatto and Roberto 2004; Olsson and Hahn-Hagerbal 1996; Petersson et al. 2006; Saha 2003). In order to improve the xylitol fermentabilities of hemicellulose hydrolysate, a detoxification process, the removal of these metabolic inhibitors, is necessary. From the perspective of the separating fermentation products, the inhibitors of microorganism metabolism are mainly non-sugar small molecule components and must be removed right after fermentation, if not before. In order to make xylitol syrup that can be crystallized, these components must be removed.

If only from the point of view of isolation and purification, the total amount and cost of evaporation are the same for hydrolysate liquors of the same initial xylose concentration. This is true regardless of whether the hydrolysate liquor is

concentrated before or after fermentation. However, increasing the concentration of fermentation products is crucial to improving the efficiency of the fermentation equipment and decreasing the cost of fermentation. Hence, effort should be made to concentrate the hydrolysates as much as possible before fermentation in order to obtain high-concentration fermentation products. This is an important way to increase the use efficiency of the xylitol fermentation process and decrease costs.

On the other hand, the toxic substances in the dilute acid hydrolysates of the hemicellulose that inhibit yeast metabolism include both volatile substances (such as furfural) and non-volatile substances (such as phenolic compounds). Evaporation and concentration not only remove the volatile toxic substances and increase the xylose content, but also increase the concentrations of non-volatile toxic components in the hydrolysates. When the concentration of toxic substances in the concentrate exceeds a certain value, the yeast cannot grow and therefore cannot perform high-efficiency xylitol fermentation. In this way, the degree of detoxification of the hydrolysate liquor determines the degree to which it can be concentrated (concentration factor). Only hydrolysates that have been sufficiently detoxified can be concentrated into a high-sugar level to which yeast strains can adapt.

Some components can be removed by microorganisms during the fermentation process, but other toxic substances present in the hemicellulose hydrolysates must be adequately removed before the fermentation. This detoxification strategy does not increase the total cost of purifying the hydrolysate liquor for in essence it is a process that transforms the purification of fermentation products to the purification of substrates.

12.3.3.2 Physical and Chemical Detoxification of the Hydrolysate

The composition of the toxic substances in the hydrolysates is rather complex, so it is almost impossible to obtain syrup of a quality suitable for fermentation using only one method of physical or chemical detoxification. The combination of multiple physicochemical techniques, including lime precipitation (Ranatunga et al. 2000; Martinez et al. 2000), vacuum evaporation (Mussatto et al. 2005), activated carbon adsorption (Mussatto and Roberto 2004; Prakash et al. 2011), processing by ion-exchange resin (Villarreal et al. 2006; Zhuang et al. 2009) are not only effective measures of detoxifying the hydrolysates but also good ways to purify fermentation product.

Lime precipitation as an effective detoxification method. It is also an efficient way to remove sulfate radicals. However, if the hydrolysates are subjected to vacuum concentration immediately after lime precipitation, they will form a heavy calcium sulfate scale on the inner wall of the heater, which will inhibit heat transfer. Therefore, in large-scale productions, the neutralized hydrolysates by lime can be subjected to evaporation concentration only, either through decalcification by ion exchange, or the addition of anti-scales. This is crucial to the stable running of evaporation equipment.

Activated carbon adsorption is a widely used and rather effective form of hydrolysate purification and detoxification. Activated carbon not only adsorbs impurities such as pigments, phenols, and organic acids but also colloid impurities that can affect filtering. After powdered, activated carbon makes contact with the hydrolysates for a certain period of time, relatively clear hydrolysates can be obtained by filtering out the carbon and its adsorbed impurities. This guarantees the smooth running of any other purification processes that might follow.

Vacuum evaporation can remove volatile substances effectively. Proper evaporation concentration of the hydrolysates can alleviate the burdens of the subsequent physicochemical detoxification or purification processes. For example, the removal of furfural and partial acetic acid in the hydrolysates by evaporation can significantly reduce the consumption of activated carbon and ion-exchange resin in subsequent steps.

Ion exchange is mainly used to remove the inorganic ions from the hydrolysates. It can also remove many inhibitors of microorganism metabolism. This makes it an effective means of both desalination and detoxification. Anion exchange resins have a rather weak ability to adsorb acetic acid, so it is not economical to remove the acetate from the hydrolysates using anion exchange resins. In the chemical xylose crystal production process, sulfate radicals are mainly removed by neutralization and sedimentation with calcium combined with ion exchange, and organic acids are mainly removed by evaporation and ion exchange. In this way, the chemical strategy involves two separate ion exchange purification processes. The first is the anion–cation exchange performed after using lime or calcium carbonate to neutralize the sulfuric acid in the hydrolysate liquor, with activated carbon for adsorption. The acidic solution produced is subjected to vacuum concentration to partially remove acetic acid, and the second anion–cation exchange is then performed. Then the sugar solution is subjected to vacuum concentration again until the xylose crystallizes.

In hydrolysate ion exchange, certain amounts of inorganic salts, such as phosphate and potassium salt must be retained so that they can be used for yeast cell growth. After the end of the fermentation, they can be removed from the fermentation liquor. If the yeast strain used exhibits rather good tolerance to toxic substances, the complete removal of toxic compounds from the fermentation medium is not necessary (Pereira et al. 2011; Huang et al. 2011), some toxic substances, such as low-concentration acetic acid, may even be beneficial to xylitol production. Determining the appropriate degree of desalination in the ion exchange of hydrolysates can promote reasonable use of inorganic salts, improve detoxification, and so reduce costs.

12.3.3.3 Biological Detoxification

For hydrolysates involved in large-scale xylitol fermentation, a rather noteworthy detoxification method is biological detoxification. Some yeast strains belonging to *Issatchenkia (Pichia)* do not use xylose but exhibit good activity in degrading toxic

substances in the hemicellulose hydrolysates (Zhang et al. 2009; Fonseca et al. 2011). Using these yeasts to ferment the dilute acid hydrolysates of hemicellulose can simultaneously degrade many non-sugar small molecule impurities, such as acetic acid, furfural, phenol, and pigments, etc. This can significantly improve the xylitol fermentative production performance of the hemicellulose hydrolysates (Zhang et al. 2009), and effectively reduce the burden of physical and chemical purification or detoxification process.

Another important function of biological detoxification is to remove the glucose from the hydrolysates. Glucose is seriously detrimental to xylose fermentation. Although a few reports have proposed that glucose-fed batch culture may enhance the xylitol yield from xylose (Silva and Felipe 2006; Yahashi et al. 1996), another study has reported that the presence of glucose inhibits the xylose conversion to xylitol (Ooi et al. 2002). From the practice of large-scale industrial fermentation, one threat brought by the presence of glucose is that it may easily cause the introduction of undesired microorganisms to the fermentation system; but to keep the fermentation system stable over long periods of time it is vital to reducing the costs of large-scale xylitol production. As in the biological detoxification process, the glucose in the hydrolysates is completely consumed, and any the negative effect of glucose on xylose fermentation is eliminated. A properly applied biological detoxification technique can increase the commercial competitiveness of fermentative xylitol production.

12.3.4 Cost Control in the Fermentation Process

12.3.4.1 Strain Selection

Yeast strains, which are valuable in industrial applications for xylitol fermentation, should exhibit good characteristics, including high sugar tolerance and a high yield of xylitol from xylose (g/g), and they should exhibit a high specific rate of xylitol production (g/g h).

Increasing the concentration of the fermentation product (xylitol) is one way to increase the efficiency of fermentation equipment. Because the concentration of the product is dependent on the concentration of the substrate, the sugar tolerance of the yeast strain is the critical factor in determining the highest possible xylitol concentration. The genetic properties of the strain are the key factors of the xylitol yield from xylose. The cost of producing xylose in the hydrolysates is not cheap, so whether the strain used has a high xylitol yield affects the cost of xylitol fermentation process significantly.

In large scale fermentation, it is very important to increase xylitol volumetric productivity (g/L h). This is determined by the cell density of the fermentation system and specific rate of xylitol production. Cell density can be increased by improving the fermentation conditions. However, the specific rate of xylitol production, that is the rate of xylitol production per unit cell and per unit time, is

determined by the genetic properties of the strain. Choosing a yeast strain with high specific rate of xylitol production also plays an important role in reducing the cost of xylitol production by fermentation.

An ideal strain should be tolerant to a certain concentration of metabolic inhibitors in the hemicellulose hydrolysates. A strain with these characteristics does not require extensive detoxification of hemicellulose hydrolysate for xylitol fermentation. This not only decreases the risk of contamination of undesirable microorganisms, but also partially combines the detoxification of hemicellulose hydrolysates and the purification of fermentation products, thus reducing the cost of product purification.

At present, strains that have achieved a high yield level and high productivity level of xylitol production mainly belong to *Candida tropicalis* (Kim et al. 2004; López et al. 2004; Kwon et al. 2006; Sheu et al. 2003) and some engineered strains of *Candida tropicalis* (Jeon et al. 2011; Ko et al. 2011), in which the xylitol dehydrogenase gene has been deficient or attenuated, have shown more excellent xylitol productivity.

The acid tolerance of yeast strains must not be ignored for the stability of the xylitol fermentation system. If the yeast strain used can ferment normally at low pH (e.g. pH = 3), the risk of bacterial contamination will be excluded on the whole. In such cases, the system could run stably for a long time even without sterilization.

12.3.4.2 Culture Medium Additives

Without ample ion-exchange desalination, the inorganic salts in the hemicellulose hydrolysates should be able to fully satisfy the needs of yeast growth. What then remains to be added to the hydrolysate culture are mainly some substances that can promote the xylitol production, such as urea, rice bran extract, a small amount of yeast extract, or even some vitamins. Except for substances that can be consumed by yeast cells, all additives must be removed from the fermentation liquor at the end of the process. The additives will increase the burden of purifying the fermentation liquor, and finally become sources of environmental pollution. Therefore, the necessity of each nutrient additive must be evaluated carefully based on the nutritional and physiological features of the strain used, the selected fermentation model, and the method to detoxify the hydrolysates.

12.3.4.3 Fermentation Mode

There are two types of xylitol fermentation: free cell fermentation and immobilized cell fermentation. Although immobilized cell fermentation has many advantages, including less pollution and convenient cell separation and recovery, the mass transfer resistance of dissolved oxygen tends to make it difficult to effectively supply oxygen in cured carriers. The production rate of immobilized

cell fermentation is usually low for xylitol fermentation. Currently, the mainstream mode of xylitol fermentation is still free cell fermentation.

Free cell fermentation can be further divided into two subtypes: single-use cell fermentation and repeated-use cell (cell-recycle) fermentation (Kwon et al. 2006). In single-use cell fermentation, after the fermentation ends, the separated cells are not reused. In repeated-use cell fermentation, after each fermentation, the yeast cells are separated from the fermentation liquor through solid–liquid separation, and the recovered cells are placed in fresh culture medium for the next batch fermentation. The liquid enters the product separation and purification process. For single-use cell fermentation, culturing new cells for any fermentation requires a certain amount of time and consumes a certain amount of xylose, so its xylitol yield and the productivity are usually a bit low. Repeated-use cell fermentation saves some cell culture time, and the xylose consumption required for cell growth is decreased. However, the risk of contamination of the fermentation system with undesired microorganisms is far greater with repeated-use cell fermentation than with single-use cell fermentation.

Repeated-use cell fermentation is very similar to immobilized cell fermentation, differing only in that the yeast cells are intercepted by cured carrier, and those cells are separated from the fermentation liquor. Xylitol is the product of the first step in the yeast xylose metabolic process. As long as the yeast cells remain alive, they continue to metabolize xylose and produce xylitol. In repeated-use cell fermentation, then, yeast cells can be used repeatedly for long periods of time, and the resulting high product yield and high volumetric productivity are superior to those of single-use cell fermentation.

12.3.4.4 Contamination During Fermentation

The contamination of a fermentation system by undesired microbes, also called fermentation infection, is a serious threat to industrial fermentation applications. With xylitol fermentation, mild cases of fermentation infection can decrease the product yield and volumetric productivity; severe cases can completely halt the fermentation process. In order to guarantee the stability of the xylitol fermentation process, contamination must be prevented. In addition to setting up the fermentation equipment in strict accordance with fermentation industrial protocols, the focus of the contamination control strategy of any large-scale xylitol fermentation system should be to prevent infection while minimizing costs on its features.

Sterilization of the culture medium is an important part of controlling costs. In order to improve the competitiveness of commercial xylitol production by fermentation, first a method that can save the cost of culture medium sterilization must be determined based on the characteristics of xylitol fermentation. For example, filter sterilization can replace heat sterilization and a low pH medium can inhibit bacteria, because yeast is tolerant to acid and most bacteria are not. The glucose content of the hemicellulose hydrolysates is usually lower. The glucose is often completely consumed by the xylitol-producting yeast within several hours of

seeding. Because many microorganisms cannot grow without glucose, increasing the amount of yeast in the initial seeding can greatly reduce the probability of contamination.

12.3.4.5 Cell Separation and Recovery

The fermentation liquor must be separated from the yeast cells before the purification process can take place. In both single- and repeated-use cell fermentation, appropriate methods must be chosen to separate and recover the yeast cells. Proper selection of the method of recovering yeast cells must be based on the characteristics of the fermentation mode, costs, and the running stability of the fermentation system. For example, in single-use cell fermentation, contamination during the cell recovery process is not a significant concern, but in repeated-use cell fermentation, special caution must be taken to avoid contamination.

Hollow fiber membrane filtration is the first yeast cell separation and recovery method used in cell recycle fermentation for xylitol production (Kwon et al. 2006). Flocculent precipitation and centrifugation are the commonly used methods in industrial fermentation for separating cells. Use of the self-flocculation properties of certain yeasts to recover cells is also noteworthy, but there have been no reports on its application in xylitol fermentation.

12.3.4.6 Purification of the Fermentation Broth and Xylitol Separation

The purification of the fermentation broth includes the clarification, decolorization, desalination, and deodorization of the solution. Xylitol separation involves two processes: crystallization and the chromatographic separation of xylitol. In large-scale xylitol production, choosing appropriate process schemes for the purification of fermentation liquor and xylitol separation based on the characteristics of fermentation liquor and the physicochemical characteristics of xylitol can decrease purification costs and improve the quality and yield of the xylitol product.

12.3.4.7 Purification of Fermentation Liquor

In order to sufficiently decolorize, desalinate, and deodorize the fermentation liquor and produce purified xylitol liquor, multiple purification processes must be performed together. However, the effects of different processes may correlate or interact with each other. For example, activated carbon exhibits exceptional decolorization by adsorbing large-molecule pigments. It has outstanding clarification and deodorization effects. However, it does not excel in adsorbing small-molecule pigments. Ion exchange resins can effectively remove inorganic salts, and are also excellent in adsorbing small molecule pigments. Ultrafiltration can remove the proteins from the fermentation liquor, which, if not removed, may

denature and precipitate when the pH of fermentation broth changes in the ion exchange resin bed. This clogs the resin flow channels. Ultrafiltration can improve the working performance of ion exchange resins and extend their service life. It can also intercept large amounts of pigment, alleviating the burden of decolorization and saving activated carbon. Therefore, in large-scale operations, the optimal purification processing strategy should be established based on the characteristics of the fermentation liquor, for maximize the efficiency of every process and reducing purification cost.

If there is no desalination involved in the detoxification of hemicellulose hydrolysates, the salinity of the final fermentation liquor will be high. Under such circumstances, the cost of desalination will become a main component of the cost of purification of the fermentation liquor. The large amounts of salinated, colored wastewater produced during the regeneration of ion exchange resins constitute a significant source of environmental pollution. In this case, considering the cost of consuming acid and alkali, treating wastewater can substantially limit the economic viability of a fermentative xylitol factory.

Reducing salinity during preparation of hydrolysates and fermentation media decreases the required regeneration frequency of ion exchange resins and reduces the amount of wastewater. Continuous ion exchange using simulated moving beds can fully utilize the exchange capacity of ion exchange resins (Sitarz et al. 2011) and in this way reduce the amount of resin and water used by over 50 % relative to fixed bed ion exchange. When fermentation liquor is of very high salinity, ion exclusion chromatography may be more advantageous than other desalination processes. This is because, during the regeneration of ion exchange resins, large amounts of acid and alkali are consumed and new pollutants (salt) are produced. However, in ion exclusion chromatography, the xylitol and salts are separated by chromatographic segregation, and no ion exchange is involved. The total amount of pollution is not increased, which makes the treatment of wastewater less burdensome.

12.3.4.8 Concentration Equipment and Costs

Concentrating the hydrolysate liquor and the xylitol solution consumes energy. The method of evaporation concentration must be selected based on the local fuel and electric power supplies. This can reduce the concentration costs. If local electric power is cheap, mechanical vapor recompression may be a relatively economical method. This method can apply heat energy in the secondary steam and thus reduce concentration costs. When using the vapor concentration method, it is vital to use multiple-effect evaporators.

12.3.4.9 Strategy of Controlling Cost of Xylitol Separation and Crystallization

Crystallization not only gives the commercial product of xylitol its necessary physical form but also effectively separates xylitol from impurities. If the purified fermentation liquor meets the requirements of preparing commercial xylitol crystal, it can be concentrated to a supersaturated state. Xylitol can be crystallized from the supersaturated syrup by lowing temperatures. Under certain circumstances, the cost of using crystallization to remove certain impurities may be lower than that of other purification methods. In these cases, crystallization can be used to increase the purity of xylitol before recrystallization to produce the commercial xylitol product.

After the crystallization of xylitol, the concentration of xylitol in the solution decreases, and the impurity content increases correspondingly. The solution eventually becomes unusable for further xylitol crystallization. The impurities contained in the xylitol crystallization mother liquor prepared by fermentation are mainly sugars that cannot be metabolized by yeast, such as arabinose and galactose. They can also include some oligosaccharides obtained via incomplete hydrolysis. If the impurity of the xylitol is lower than 50 %, then usually no xylitol can be further crystallized. The proportion of the non-recoverable xylitol relative to total xylitol is mainly related to the ratio of xylose to impurity sugars in the raw material and to the level of oligosaccharides generated during hydrolysis. In the fermentation liquor of bagasse hydrolysates, about 80–85 % of the xylitol can be crystallized. The impurity sugar content of the fermentation liquor produced using corncob hydrolysates is relatively high, so the amount of xylitol that can be crystallized is much lower. The cost of separation by crystallization is relatively low, so it is better to separate as much as possible of the xylitol in fermentation liquor by crystallization.

According to the difference of raw materials used, about 15–30 % of the xylitol produced by fermenting hemicellulose hydrolysate remains in the crystallization mother liquor with the impurity sugars. Due to the differences in chemical properties, this xylitol can be separated from impurity sugars very effectively using industrial-scale chromatographic separation methods. Industrial-scale chromatographic separation usually involves simulated moving beds equipped with calcium cation exchange chromatography columns. These are used to continuously recover high-purity xylitol, and pure water is used as the eluent. In this way, the xylitol produced during the fermentation process can be fully recovered from the crystallization mother liquor. This is an important advantage of xylitol fermentative production, which chemical techniques cannot match.

Table 12.1 briefly shows the technological requirements of key processes in large scale production of xylitol fermentation. Their effects of pros and cons on chemical or fermentation process are also evaluated.

12.4 Environment Treatment and Biorefinery

12.4.1 Environment Treatment in Large-Scale Production

One key factor that determines whether xylitol fermentative production can be used in large-scale production is whether the process can be made environmentally friendly. In large-scale xylitol fermentative production, proper methods must be established to treat or recycle waste components such as hydrolysis residues, waste steam, condensate water by hydrolysate evaporation, exhaust fumes produced by fermentation, salinized wastewater produced by ion exchange purification, and dead yeast cells.

The main components of hydrolysis residue are the complexes of cellulose and lignin, that is cellulignin, which can be dried using tail gas from boilers and used as fuels. The volatile organic substances contained in the waste steam produced by the hydrolysis process and the condensate water produced during the concentration of the hydrolysates are mainly acetic acid and small amounts of furfural, both of which can be cleaned easily using aerobic fermentation. The exhaust fumes produced by fermentation must be deodorized before discharge. Any components of the salinated wastewater produced by ion exchange purification of the fermentation liquor that have economic value can be recycled. Biologically detoxified yeast cells and yeast cells recovered from the xylitol fermentation process can serve as good-quality single-cell protein in the feed industry. It may also be worthwhile to recover inorganic salts and inorganic acids from cellulose hydrolysates. The combined use of these techniques may facilitate the environmental friendly production of xylitol on an economically viable scale.

12.4.2 Integration of Xylitol Manufacturing Unit with Biorefinery

The dilute acid hydrolysis of hemicellulose can simultaneously break the natural structure of lignincellulose, and enhance the enzymatic hydrolysis of hydrolysic residue (cellulignin). Recovering xylose from hydrolysic residue does not only separate pentose from hexose (cellulose), but also removes acidic acid, furfural and other inhibitors from hydrolysic residue. In other words, most of the environmental pollutants generating in hydrolysis will enter the hydrolysate stream. If the conditions of dilute acid hydrolysis of hemicellulose both meet the demands of xylitol production and enhancement of enzymetic hydrolyses of cellulose, single xylitol manufacturing would be transformed to biorefinery producing multiple chemical products.

In this way (Fig. 12.2), the hemicellulosic fraction of LCM will go for xylitol production after detoxification. Followed by xylitol separation, the impurity sugar molasses can be used for rare sugar production, such as l-arabinose galactose, etc.

Table 12.1 Technological requirements of the key processes influencing the cost of large scale production of xylitol by fermentation

Technological processes	Specification or requirements	Main impacts		The effect on different technology routes	
		Pros	Cons	Fermentation	Chemical
Raw material selection	Choosing materials which have a high xylose content, and a low proportion of impurity sugar	Decreasing the product separation cost	Limiting the raw material availability	The negative effects of impurity sugars are relatively smaller	Impurity sugar level seriously affects the xylose yield from raw materials
Hydrolysis	Minimizing the liquid-to-solid ratio and acid concentration	Saving the energy of hydrolysis and concentration, reducing the desalination burden of fermentation broth	Easily leading to an incomplete hydrolysis, and a generation of oligosaccharide which inhibits xylitol or xylose crystallization	The negative effect of oligosaccharide can be eliminated in a chromatographic step	The negative effect of oligosaccharide cannot be eliminated
Detoxification	Physical, chemical detoxification; Removing fermentation inhibitors	The treatment effect is stable and reliable	Producing large amounts of wastewater; Its running cost is high	Only needing to remove inhibitors, not to purify hydrolysate	Being necessary to fully purify hydrolysate for crystallizing xylose
	Biological detoxification. Removing inhibitors using microorganisms	It is easy, effective, and environmentally friendly	Contaminations may result in xylose molasses	Improving the stability of xylitol fermentation because of glucose removal	–
Fermentation	Batch fermentation	The level of cell activity is high; Contamination is easily controlled, and it has a wide range of substrate compatibility	Xylose is partially consumed by cell growth; the time of cell culture is extended, and the products yield is relatively lower	Fitting for the high glucose hydrolysate	–
	Cell recycled fermentation; the separated and recovered cells are subjected to next batch fermentation	Shorting the time of cell culture, reducing the xylose consumed; products yield and products conversion rate are both relatively higher	Raising the difficulty of contamination control in fermentation system	Only fitting for the low glucose hydrolysate	–

(continued)

Table 12.1 (continued)

Technological processes	Specification or requirements	Main impacts		The effect on different technology routes	
		Pros	Cons	Fermentation	Chemical
Fermentation broth purification	Decolorization and deodorization by carbon absorption	Very effective	It is a higher cost; does not excel in adsorbing small-molecule pigments	Necessary	Also necessary for purifing hydrolysate
	Fixed-bed ion exchange	Investment cost is relatively low	The resin utilization rate is lower; A large amount of waste water is produced, and running cost is high	Fitting to desalt the fermentation broth, the salt content of which is lower	
	Continous ion exchange	Considerably saving acid, alkali and water used for the regeneration of ion exchange resins	Equipment investment is higher than the fixed bed, but running cost is lower	Showing a clear superiority in desalting the fermentation broth with high salt content	The same superiority shows in hydrolysate desalination
	Ion exclusion chromat; Carried out in Simulated Moving Bed (SMB)	Total amount of pollution does not increase because of no ion exchange	Requiring a higher equipment investment; Fermentation broth requires a rigorous pretreatment	Fitting for the fermentation broth with high salt content	–
Chromatographic separation of xylitol	SMB chromatographic separation technology with cation exchange resin as the packing	It is significant to raise the overall recovery of xylitol from fermentation broth	It requires a higher investment	An important technological process to fully show fermentation technology advantages	Not fitting to separate xylose from xylose molasses

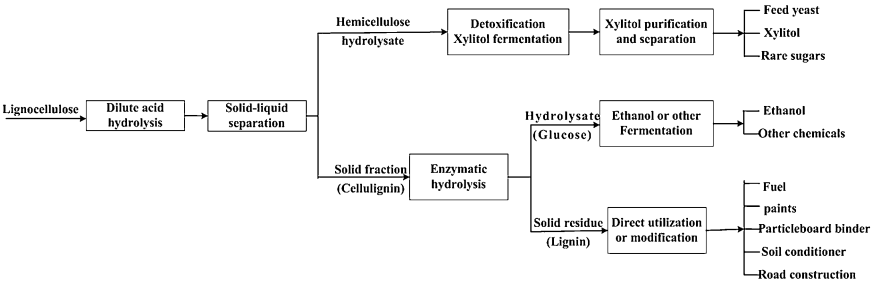

Fig. 12.2 The Biorefinery Integrated with Xylitol Manufacturing Unit

The remaining cellulignin fraction will undergo enzymatic hydrolysis for the subsequent conversion of C 6 sugars into fuel ethanol on a biorefinery concept. Finally, left-over lignin may be applied for several important products such as electricity generation, soil conditioner, road construction, paints and purification of industrial wastes. Furthermore, co-integrated application of basic facilities such as LCM handling and storage, equipment, vessels, and utility operations (boilers, chillers, air compressor) will aid the efficient utilization of machineries, eventually lowering the incurred cost of xylitol and ethanol production.

12.5 Conclusions and Future Recommendations

The crucial elements, which have effects on xylitol production through fermentation on a massive scale, are all also the key factors influencing the cost of xylitol production. Hence, appropriate technology strategies should be chosen in accordance with the comparative advantages of fermentation technology. Based on this, the production cost of every operation unit should be sufficiently decreased. Rules which should be obeyed in the main operation units are the following: (1) choose materials which have high xylose content, and a low proportion of impurity sugar—at present, the materials that have business competitiveness are only corn cob, bagasse and other a few types; (2) raise the initial xylose concentration of hydrolysate through a low liquor-solid ratio hydrolysis which reduces concentration cost, and acid concentrations in hydrolysis should be as low as possible for reducing the costs of icon-exchange in the following processes; these should be known when hydrolysate is being prepared; (3) bio-detoxification degrades inhibitors, while it also cuts the burden of purification of fermentation broth; (4) xylitol in fermentation broth could be efficiently recovered through simulated-moving-bed chromatograph technology due to the selective catalysis of bios in fermentation technology, this is an important measure recommended.

Integrating the unit of xylitol fermentation production into biorefinery is a crucial route of decreasing production cost further and increasing the general benefits of biorefinery in the future. From this point of view, at present, the key

factor is how to provide the solution which hemicellulose hydrolysis production in consonance with the requirements of enzymatic hydrolysis of cellulignin.

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Chapter 13
Overview on Commercial Production of Xylitol, Economic Analysis and Market Trends

Sreenivas Rao Ravella, Joe Gallagher, Steve Fish and Reddy Shetty Prakasham

Abstract The interest in xylitol has increased considerably in recent years, due to many commercial applications in different industrial sectors like food, dental related products, and pharmaceuticals. As industrial biotechnological routes to xylitol are costly they currently represents a small fraction of the marketshare. Therefore, over the past few decades much effort has been devoted to the development of cost-effective and environmentally-friendly biotechnological processes by evaluating cheaper lignocellulosic substrates. In this chapter, xylitol commercial processes, cost and market trends are discussed with a special focus on biorefining and biotechnological methods. Increasing commercial and scientific interest in xylitol has led to a strong demand for this product in the global market, of more than 125,000 tons per anum, with a value that is relatively high (4.5–5.5$/kg for bulk purchase by pharma/chewing gum companies and 12£ or 20$/kg in supermarkets) makes its an attractive proposition for commercialization.

Keywords Xylose · Xylitol · Biorefinery · Biotechnology · Yeast

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13.1 Introduction

Xylitol was identified as one of the top ten chemicals derived from carbohydrates, with potential as a co-product from a plant biomass based biorefinery (Bozell 2008). Xylitol has a number of commercially applicable properties that include: a lower calorie substitute for traditional sweetners such as sucrose, anticariogenicity i.e. prevention of tooth demineralisation, and inhibition of bacteria that cause tooth decay. These properties make xylitol an ideal additive in food and dental-related products.

A common route for the production of xylitol is from plant-based hemicellulosic hydrolysates. D-xylose in hydrolysates is converted to xylitol by catalytic reaction. This method utilizes specialized and expensive equipment to achieve the high pressure and temperature conditions necessary for the process. Danisco (now part of DuPont) is one of the world's largest producers of xylitol using hardwoods and maize as the feedstocks.

The xylitol market continues to experience strong demand and rapid growth worldwide due to an increasingly health conscious consumer and fast growth in chewing gum sales. Xylitol has a 12 % share of the total polyols market with growth projected to increase threefold. Xylitol is approved for food use in over 50 countries and this, combined with increasing commercial and scientific interest in xylitol, has led to a strong demand for this product in the global market.

13.2 Current Xylitol Production

Currently, xylitol is produced from corn cobs (China) and hardwoods such as birch (US). Xylose in hemicellulosic hydrolysate fractions is converted to xylitol by a chemical catalytic reaction. A large amount of waste biomass is generated in the preparation of corn cobs for the food industry and this provides a substrate for xylitol production in China. Because of the purity issues, hydrolysate from birch trees is a popular substrate for xylitol production, especially in the United States. Birch tree hydrolysate is obtained as a by-product of the paper and pulping industry and is therefore amenable for large scale production of xylitol.

Danisco is a major global supplier of xylitol which it produces via catalysis using xylose obtained from hardwood sources. They have recently developed a product (Xylitol XIVIA) using a process which they claim is more sustainable than that used for production of xylitol from corn cob biomass. This is based on 15 environmental impact categories [part of life cycle assessment (LCA)] analysed as part of their study. This is achieved by developing an integrated approach with the pulp and paper industry. However conversion of xylose to xylitol is still achieved via a chemical route.

13.3 Market Trends

Xylitol is the constituent of many fruits and vegetables, such as raspberries, strawberries, yellow plum, lettuce and cauliflower. However it is only present in low concentrations and is therefore not commercially scalable. It has attracted global interest due to its sweetening power, similar to that of sucrose, as well as its low caloric equivalent of 2.4 kcal/g and laxative nature (145 J/g caloric content) (Russo 1977; Emodi 1978; Makinen 2000; Granström et al. 2007). Xylitol has potential applications in the area of odontology (for its anticariogenicity, tooth rehardening and remineralisation properties), pharmaceutics (for its capability of preventing otitis and its use as a sweetener or excipient in syrups, tonics and vitamin formulations) and recently for manufacturing cool fabric e.g. Ice-Fil. However, the major use is in the prevention of dental caries as xylitol inhibits growth of microorganisms responsible for tooth decay (Yilikari 1979; Hyvonen et al. 1982; Makinen 2000). In addition, xylitol is accepted for consumption for diabetics as its metabolism is independent of insulin (Yilikari 1979). In Asia, xylitol is used by gum manufacturers, and it is estimated that 80–90 % of chewing gum sold in the region now has xylitol as the sweetener in their formulations. The China-based Futaste Company alone currently produces approximately 35,000 tons of xylitol per year, as well as 20,000 tons of xylose and other types of sweeteners.

Xylitol produced from Finnish birch, corn cobs and sugarcane bagasse commanded a $200 M market in 2000 and demand in Europe and rest of the world was expected to grow at 15 % (average annual growth rate, AAGR) up to 2007. The xylitol market is increasing globally and at present is estimated to be worth more than $537 million per year. Xylitol prices currently range from US $4 to $5/kg although the price has decreased substantially over the last 10 years. This is as a consequence of economies of scale of production. The raw material for production is hemicellulose, which is abundantly available. During the fast-growing global economy (2006–2007), China's xylitol exports increased; however, following the global financial crisis in quarter4 2008, this has started to decline. Production increased from 80,000 tons per anum in 2006 to 125,000 tons per anum in 2011. Asia produces 50 % of the world's total xylitol, with the rest of the production coming from Europe, the United States and Australia. Table 13.1 shows major xylitol producing companies from China and the United States. The final cost of the product varies and is dependent on raw material cost and transport costs which depend on feedstock mass and location of the manufacturing plant (Kocoloski et al. 2011; Kazi et al. 2010; Koutinas et al. 2007; Lange 2007). A number of xylitol producing companies in China recently went out of business because of the increase in the price of the raw material making the process no longer economically viable. Overall, xylitol production is increasing constantly due to consumer's trend to choose healthy products.

Table 13.1 Major xylitol producing companies in the world (Source: www.made-in-china.com)

Company	Xylitol production in metric tons per year	Source
Futaste pharmaceutical Co., Ltd. China	35, 000	Corncob
Jining Hengda Green Engineering Co., Ltd. China	5, 000	Corncob
Hangzhou Shouxing Biotech-Nology Co., Ltd. China	15, 000	Corncob
Shandong Biobridge Technology Co., Ltd. China	6, 000	Corncob
Tangyin Hung Industrial Co., Ltd. China	2, 500	Corncob
Thomson Biotech (Xiamen) Pte. Ltd. China	10, 000	Corncob
Yucheng Lujian Biological Technology Co., Ltd. China	16, 000	Corncob
Zhejiang Huakang Enterprise Co., Ltd. China	20, 000	Corncob
Shijiazhuang Acid Chemical Co., Ltd	10, 000	Corncob
Shengquan Healtang Biotech Co., Ltd. China	8, 000	Corncob
Xylitol USA, Inc	–	Birch trees
DuPont (Danisco). USA	2, 000	Waste side stream of a pulp and paper plant to extract xylose

13.4 Alternatives to Chemical Processes for Xylitol Production

Biotechnological production of xylitol from renewable sources is a biorefining objective and requires an understanding of the overall process (Clark et al. 2009; Dumon et al. 2011; FitzPatrick et al. 2010; Jørgensen et al. 2007). There are several factors influencing production processes via bio-based or biotech routes, including public concern about environmental pollution and sustainability of natural resources encouraging the manufacturing industries to adopt more environmentally friendly processes. Major pharmaceutical companies now dedicate more than 50 % of their new drug development budget to biotech research and development, a trend away from traditional chemical synthesis (Yang 2007). Commercial production of xylitol via a biotech process is an alternative to a chemical process and depends on the different streams involved such as raw materials, a suitable microbial strain and location of the production unit.

Currently, xylitol is manufactured at the industrial level by chemical hydrogenation of the five-carbon sugar D-xylose in the presence of a nickel catalyst at elevated temperature and pressure. This chemical process is laborious, costly and energy intensive. In addition, the process needs expensive purification treatments

Table 13.2 Main difference between chemical and biotechnological production of xylitol

Process	Source	Catalyst	Downstream process
Chemical	Pure xylose (costly purification step required)	Nickel and hydrogenation	Easy with ion exchange resins
Biotechnological	Lignocellulosic hydrolysate (crude hydrolysate can be used)	Yeasts/bacteria/recombinant microbes or enzymes. This process needs robust microorganisms to withstand inhibitors present in the hydrolysate	Complex downstream process because of different microbial by-products

especially with respect to xylose production when compared to a biotechnological process (Table 13.2). For example, the new Danisco factory, Danisco Sweeteners GmbH, located in Lenzing, Austria, is the latest Danisco investment to come on stream to secure and increase the production of xylose (the raw material for xylitol production) at a cost of €23 million (www.Danisco.com).

In order to produce xylitol in an economical and eco-friendly manner, there has been a drive to explore alternative strategies. One of the alternatives is bioconversion of renewable biomass which involves hydrolysis of the hemicellulose component followed by bioconversion of xylose in crude hydrolysates to xylitol by fermentation with specific microbial strains (Prakasham et al. 2009; Sreenivas Rao et al. 2006b). Bioconversion does not require purification of xylose, the major cost intensive step in the chemical synthesis route; however, downstream processing of xylitol from fermented broth is necessary and needs further research (Faveri et al. 2002; Martınez et al. 2007; Misra et al. 2011).

13.5 Potential Feedstocks for Xylitol Production by Bioconversion in a Biorefinery Context

A number of studies have been carried out looking at a range of biotech processes. One of these studies has investigated the economic feasibility of co-producing xylitol with ethanol from rye straw.

As xylitol has a higher economic value than ethanol, co-production of xylitol from pentose fractions with ethanol production from lignocellulose may increase the profitability of second generation lignocellulosic ethanol plants (Cheng et al. 2010). This is significant because it is critical that such operations achieve economic viability and it is currently speculated that this would require a plant-size capable of processing a minimum of 2,000–4,000 MT per day (Aden et al. 2002). This requires a high level of investment (approx. $200,000,000 for a 2,000 MT/day plant), and value added co-products such as xylitol can make smaller plants feasible thus reducing start up costs.

Table 13.3 Compositions and cost of biomass feed: UK scenario

Raw material	Cellulose (%)	Hemicellulose (%)	Price (£) per tonne	References
Wheat straw	34.20	23.68	56	Adapa et al. (2009)
Perennial ryegrass	30.00	22.50	88	Charlton et al. (2009)
Oat hulls	29.00	34.50	60	Garleb et al. (1991)
Oat straw	37.60	23.34	54	Adapa et al. (2009)
Barley straw	33.25	20.36	66	Adapa et al. (2009)

The feasibility of co-production of xylitol and ethanol from rye straw was evaluated by Franceschin et al. (2011) taking into consideration the different steps involved in the process. These included conversion of C5 sugars to xylitol, pre-treatment using hot water, conversion of C6 sugars to ethanol, and separation and purification of products. The authors based their evaluation on both experimental data and data published in the literature. For example experimental data obtained by Ingarm et al. (2009, 2011), notably on enzymatic pre-treatments, was incorporated into the model. This study employed techniques such as Aspen PlusTM software and Pinch technology for analysis of models and optimization of process energy duties, respectively.

The cost of production of xylose from the hemicellulose fraction of rye straw has been estimated at €59.2 per tonne. This compares favourably with other comparable feedstocks based on a UK scenario (Table 13.3); however, the recalcitrance of the feedstocks will also affect the overall cost of production. The price of xylose from rye straw can be broken down into 30 % for raw materials, 24.4 % utilities, 10.2 % maintenance, 22.9 % depreciation, 6.5 % enzymes, and 6.0 % labour. These figures are based on simulations in which pre-treatment and enzymatic hydrolysis steps are carried out in two reactors, each with a volume capacity of 460 m^3. This model assumes an outlet stream flow rate of 23,104 kg/h, of which 1,048 kg/h is xylose, a subsequent filtration stage and an active charcoal adsorption treatment to remove inhibitors prior to fermentation of xylose to xylitol. Fermentation is carried out in four fermenters (332 m^3 volume each) and it is estimated that 736 kg/h of xylitol can be produced under these conditions (Franceschin et al. 2011). Cheng et al. (2010) has proposed that waste corncob biomass, generated during preparation of corn for the food industry could also provide a suitable lignocellulosic feedstock for the concomitant production of ethanol and xylose by a biotechnological process.

A feasibility study carried out by the Department for Environment Food and Rural Affairs (DEFRA) and the National Non-Food Crop Centre in the United Kingdom shows the potential to obtain value added products from grass grown in UK grasslands. A second study by Charlton et al. (2009) looks at the availability of biomass for biorefining. These authors considered the options for large scale biorefining of high sugar perennial ryegrasses (Figs. 13.1 and 13.2) in Wales, as a model for producing sustainable, bulk quantities of chemicals, including biofuels.

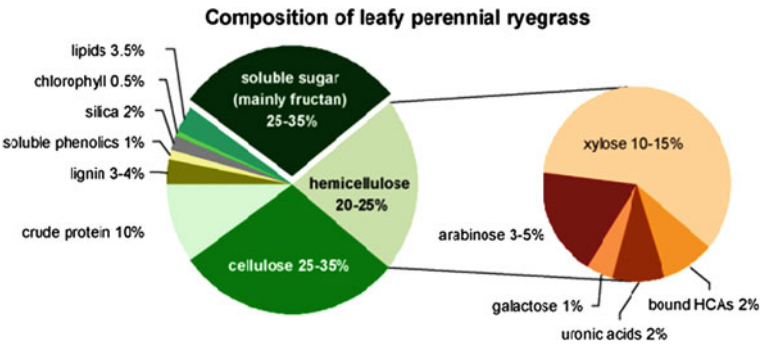

Fig. 13.1 Composition of perennial ryegrass (IBERS, Aberystwyth University)

This feedstock has a relatively high xylose content (Fig. 13.1) and low recalcitrance making it a potential source of xylose for xylitol production.

13.6 Selection of Appropriate Micro-Organisms for the Biotechnological Production of Xylitol from D-Xylose

Biotechnological production of xylitol depends on microbial bioconversion potential of xylose to xylitol. There are several reports on xylitol production by micro-organisms in the literature (Dahiya 1991; Leathers and Gupta 1997; Kim et al. 1997; Silva et al. 2006; Sreenivas Rao et al. 2004, 2007a, b; Yoshitake et al. 1971, 1976). Active bacteria include *Cornynebacterium* and *Enterobacter* species, e.g. *Enterobacter liquefaciens* which produces xylitol from pentose sugars (Yoshitake et al. 1971, 1973, 1976). Fungi and yeasts can also be used for bioconversion of xylose to xylitol. Xylose utilizing microorganisms produce xylitol as an intermediate product in a metabolic pathway involving sequential redox reactions catalysed by two enzymes—xylose reductase (XR) and xylitol dehydrogenase (XDH) in the presence of a cofactor. XR catalyses the initial reduction of xylose to xylitol and XDH catalyses the subsequent oxidation of xylitol to xylulose. A biotechnological process for production of xylitol will require a constant supply of NADPH to restrict further oxidation to xylulose. With this in mind, several scientific researchers have been involved in microbial screening programs to isolate efficient microbial strains for XR mediated xylitol production targeting properties such as a high XR/XDH ratio. Yeast are considered to be the most effective micro-organisms for xylitol production and data compiled from different yeast screening programmes for xylitol are shown in Table 13.4.

Most studies on xylitol production by yeast have been carried out with strains belonging to *Candida* and *Pichia* genera (Sreenivas Rao et al. 2004, 2007a, b; Silva et al. 2006; Leathers and Gupta 1997; Kim et al. 1997) and several *Candida* sp

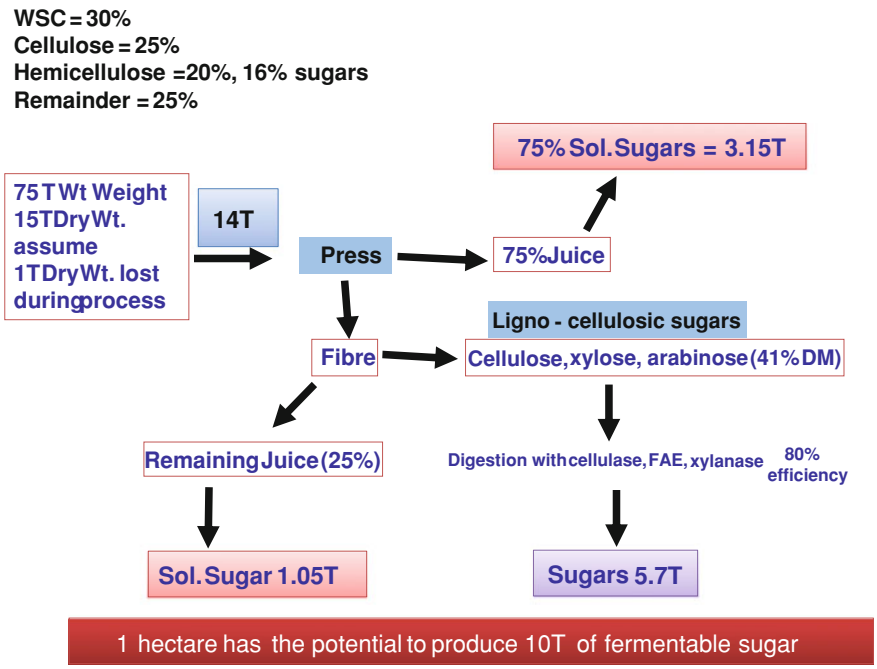

Fig. 13.2 Sugars from grass for potential grass biorefinery

Table 13.4 Data from screening studies on yeast strains for xylitol production from xylose

Number of yeast strains tested	Species	Maximal xylitol production	Reference
58	*Pichia miso*	3.77 g of xylitol from 8.50 g of D-xylose	Hiroshi and Toshiyuki (1966)
30	*Candida guilliermondii* and *C. tropicalis*		Ojamo (1994)
11	*C. mogii*	0.62 g/g of xylose	Sirisansaneeyakul et al. (1995)
4	*Hansenula polymorpha*	43.2 g/L xylitol production from 100 g/L D-xylose	Suryadi et al. (2000)
13	*Candida* sp	0.50–0.65 g/g of xylose	Yablochkova et al. (2003)
5	*C. guilliermondii* and *C. maltosa*		Guo et al. (2006)
35	*Pichia* sp	0.58 g xylitol/g xylose	Sreenivas Rao et al. (2007a)
270	*Debaryomyces hansenii* UFV-170	5.84 g/L xylitol from 10 g/L xylose	Sampaio et al. (2008)

(West 2009; Wang et al. 2011) have been extensively studied (Table 13.4). These microbial strains have an advantage over genetically engineered strains of *Saccharomyces cerevisiae* for the production of xylitol as part of a biotechnological process because they are adapted to cope with the oxido-redox conditions required for xylitol production.

Numerous studies have demonstrated that *Candida* species are potential sources of xylitol producing strains for application in a biotechnological process. Ojamo (1994) identified a strain of *Candida guilliermondii* and *Candida tropicalis* respectively as the best xylitol producing strains from a screen of 30 yeast strains. Further screening studies demonstrated a yield of 0.62 g/g of xylose with a strain of *Candida mogii* (Sirisansaneeyakul et al. 1995). This compares with the findings of Yablochkova et al. (2003) who identified six strains of *Candida* sp with xylitol production levels of 0.50–0.65 g/g xylose. In an extensive study, Guo et al. (2006) screened 274 yeasts for xylitol production and concluded that *C. guilliermondii* and *Candida maltosa* were the best xylitol producers. Screening studies on xylitol production by yeast have not been restricted to members of the *Candida* genus. In one study in which 58 strains were tested, a *Pichia miso* proved to be the highest xylitol producing strain with a yield of 3.77 g of xylitol from 8.50 g of D-xylose consumed (Hiroshi and Toshiyuki 1966). Suryadi et al. (2000) observed xylitol production levels of 43.2 g/L xylitol production from 100 g/L D-xylose with a strain of *Hansenula polymorpha* in a study of xylitol production by four methanol-utilizing yeasts. In a novel study, Sreenivas Rao et al. (2007a, b) screened yeast isolates from the gut of beetles collected from Hyderabad city, India, for xylitol production from xylose. Twenty of the 35 isolates utilized xylose as a sole carbon source and 12 of these converted xylose to xylitol. A high producing strain of *Pichia sp*. was identified with xylitol yields of 0.58 g xylitol/g xylose. In a more recent study, Sampaio et al. (2008) screened 270 yeast isolates for xylitol production using xylose as the sole carbon source and reported high production levels with *Debaryomyces hansenii* UFV-170 with a yield of 5.84 g/L xylitol from 10 g/L xylose after 24 h incubation. Studies described above show conclusively that the potential for conversion of xylose to xylitol is dependent on the genetic nature of the microbial strain; however, physiological as well as metabolic growth parameters have an impact on the ratio of the two xylose metabolizing enzymes. In addition to XR/XDH ratio the rate of regeneration of reduced cofactor may be considered another limiting factor in xylitol production. Sreenivas Rao et al. (2007b) clearly demonstrated that cloning of the XR gene resulted in improved xylitol production and that co-substrates improve co-factor regeneration in recombinant strains.

A number of studies on yeast xylitol production have been reported with high yielding wild-type and mutant yeast strains (Saha and Bothast 1997; Silva et al. 2006; Gong et al. 1981; Gardonyi et al. 2003; Kim et al. 1997; Sreenivas Rao et al. 2006a; Suryadi et al. 2000; Sampaio et al. 2006; Sánchez et al. 2004).

13.6.1 Enzymatic Production of Xylitol from Xylose

Owing to the limitations of microbial conversion of xylose to xylitol such as high dilution rates and residence time, a more efficient biotechnological approach may be to add XR enzyme directly to a bioreactor. Application of immobilization technology where the XR enzyme is immobilized on a suitable carrier material with a substrate/product separating membrane system could provide an alternative microbe-free system. This would require the development of a metabolism independent cofactor regeneration system. This is likely to be achieved through the use of regenerative oxido-reductive chemical/biochemical systems (Prakasham et al. 2009).

13.6.2 Recovery of Xylose from Hemicellulosic Material

Hemicellulosic material offers an economic and renewable source of xylose (Sreenivas Rao et al. 2006b). Indian Institute of Chemical Technology, Hyderabad, India working on pilot level xylose production from sugarcane baggase. Xylose production from biomass is impeded by the lack of species-specific hydrolyzing enzymes for selective hydrolysis of xylan from renewable biomass, the inhibitory effect of several hydrolysis-associated compounds (e.g. cell-wall phenolics) and xylose utilization in the presence of other monomeric sugars. Isolation of micro-organisms with species-specific enzymatic systems for release of xylose from hemicellulosic fractions could facilitate development of an industrial process. Production via a direct enzymatic route may also overcome some of the limitations described above.

13.6.3 Industrial Research and Development of Biotechnological Processes for Xylitol Production

There are several companies that are pursuing development of a biotechnological process for production of xylitol from xylose and other five-carbon sugars. These include Thomson biotech and zuChem Inc. Thomson Biotech is an international company that is at the forefront of developing biotechnology routes to produce five-carbon sugar products with more than 30 patents to their name. Thompson Biotech has recently invested over 300 million RMB to develop a manufacturing base in China for the production of 10 k tonnes of xylitol and arabinose using biotechnological processes. They have developed a process for microbial bio-conversion of xylose to xylitol utilising a yeast strain of *C. tropicalis* (US patent application 20120021467). ZuChem is a US company which uses bioprocesses to produce a range of sugars with application in the food and Pharma industries. The company has been awarded grants by the US Department of Energy (SBIR Phase I

and II, 2007 and 2008) to develop methodology for the production of xylitol from biomass. They have recently been granted a patent for the fermentative production of xylitol entitled 'Methods for production of xylitol in microorganisms' (US patent 7960152).

The zuChem process, based on recombinant bacterial strains, was developed by zuChem in collaboration with the U.S. Department of Agriculture (USDA) and the Biotechnology Research and Development Corporation (BRDC). zuChem is the exclusive licensee for the process. The authors claim that recombinant bacterial strains offer advantages over yeast strains in terms of: (i) utilizing a mixed sugar stream in lignocellulosic hydrolysates, (ii) the absence of polyol contamination in fermentation broth which facilitates the purification process, (iii) tolerance to feedstock variability, (iv) high volumetric productivity compared to yeast strains and (v) the ability to utilise L-arabinose as well as xylose for xylitol production.

Other strains may convert arabinose to arabitol which is very difficult to separate from xylitol using a cost-effective process. zuChem have demonstrated xylitol production using this specially designed microbe up to pilot scale level, and they are now aiming to produce xylitol on a commercial scale and to license this technology. zuChem has reported that several companies have agreed to produce xylitol on a commercial scale and that agreements are also in place for the purchase of bulk xylitol. This process may provide a route for the production of co-products from waste streams in a biorefinery. An obvious candidate is the second generation ethanol producing industry where conversion of five-carbon sugars in lignocellulosic hydrolysates to xylitol may provide a more economically viable option than fermentation to ethanol as discussed earlier in this chapter.

Furthermore, a multi-million pound biorefining project (BEACON) http://beaconwales.org aims to develop biorefinery processes for the conversion of plant biomass into higher value products. This Aberystwyth University-led initiative in collaboration with Bangor and Swansea Universities will explore a variety of techniques and methodologies that include: mechanical processing, steam explosion, membrane filtration, enzymes, microbes and combined fermentation approaches with the objective of converting a variety of plant feedstocks such as ryegrass, Miscanthus, oat hulls and macroalgae into, e.g. transport fuels, bulk chemicals, fine chemicals and new biocomposites. One of the target molecules within this matrix is the production of xylitol and developing a better appreciation of the conversion process and the economic modelling.

This initiative focuses on research that goes beyond the laboratory and is potentially scalable for commercial application. By doing this the aim is to operate in the scientific 'valley of death' that is the space between pure research and technology deployment. This approach has been adopted to maximise the applicability of the research to the commercial sector, both in Wales and beyond. By doing this the aspiration is to drive scientific discovery into commercial development by enhancing 'market pull.'

A key component within BEACON is LCA and process modelling. Only by undertaking these activities can we fully appreciate the implications of varying processes and input feedstocks with their impact on production economics

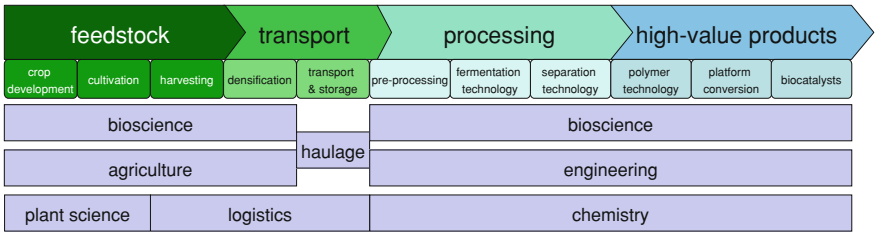

Fig. 13.3 Biorefinery Supply Chain (from the Feasibility Study)

(Fig. 13.3). Delivering sound economics in association with scientific and technical delivery will allow the conversion of plant biomass into, e.g. xylitol within a commercial context whilst maximising co-product value.

Other centres that have capacity associated with Biorefining within a UK context include the Biorenewables Development Centre based in York http://www.biorenewables.org, the Biorefinery Centre at the Institute of Food Research (IFR) Norwich http://www.ifr-extra.com/Biorefinery.aspx, and The Centre for Process Innovation (CPI) http://www.uk-cpi.com/contact at Wilton.

13.7 Future Perspectives and Conclusions

Changing traditional technology from using corncobs as a source of xylose production to advanced technologies using side streams from wood pulp processing for xylose production is important for sustainable production of xylitol. Continual developments in research starting from hemicellouse hydrolysis for pure xylan/xylose to downstream conversion will improve the economics of the process and will help drive the consumer market upward. In addition, constant education of the population especially in under-developed and developing countries will help to expand the xylitol market. The main impediment in this context is availability of product or raw material to fulfil the world market demand. Finding new xylitol applications and educating consumers on xylitol use as a healthy choice will improve the xylitol market.

One of the biggest disadvantages of biotechnological production of xylitol compared to chemical technology is in the diversity of technologies required to obtain the end products; these include:

- Achieving sustainable production of agricultural biomass for feedstock
- Chemical treatments with acids and alkalis/enzymatic digestion
- Inhibitors present in the hydrolysates
- Robust microorganisms.

In addition, the variation in feedstock is potentially very large; the biorefinery systems developed will need careful optimisation in relation to the input material.

Numerous studies over the last 20–30 years have been carried out on the biotechnological production of xylitol with regard to improving the economics of the process. Comparisons between the relative technologies need to be demonstrated at pilot scale to access the suitability for large-scale production. Typically, the manufacturing costs have been split into two major components, namely, the feed and the processing costs. The former covers the cost of purchasing the feedstock. The latter covers the cost of installing and running the manufacturing plants, i.e. the cost of the plant and the labour, as well as the energy and chemicals consumed. For biotechnology processes the second component has to be scaled up and must be optimized.

Biorefinery-based products appear to be important for strategic advantages and a sustainable agriculture-based economy (Wright and Brown 2007). Development of the robust strain having the ability to convert to xylose to xylitol from lignocellulose hydrolysates with maximum xylitol yields/productivities with minimum by-products will help biotechnological production of xylitol. Integration of process operations like hydrolysis, detoxification, fermentation, development of enzyme bioreactor and purification is an effective strategy to maximize the efficient utilization of renewable substrates (Prakasham et al. 2009). Initial incentives from governments for biotechnological production of xylitol may encourage companies to switch over from chemical processes to biotech processes.

The available chemical technology for xylitol production from corncobs had some environmental impact, therefore, an alternative process to convert different suitable feedstock to xylitol is advisable. Recommendation to evaluate the sustainability of xylitol production using different substrates full life cycle analysis (LCA) of both attributional and consequential types will need to be completed for each substrate and processes.

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Part V
Applications of Xylitol

Chapter 14
Application of Xylitol in Food Formulations and Benefits for Health

Solange Inês Mussatto

Abstract Sugar substitution by any sweetener in the food industry is usually made taking into account the calories of the sweetener, the possibility of using it in diets for weight reduction or control, and the degree of similarity between its flavor and the traditional sugar. Among the existent alternative sweeteners, xylitol has attracted the attention of food manufacturers since it has sweetening power similar to sucrose but with lower caloric value, and can be consumed by diabetics. Nowadays, a variety of products containing xylitol in the formulation can be found in the market. Most of them are especially formulated for people with insulin-deficiency. Another important advantage of xylitol ingestion when compared to the traditional sugar is that it promotes several benefits for human health, acting both on the prevention and/or treatment of diseases. Due to these important properties, the use of xylitol in food products is a market in great expansion. The current applications of xylitol in food formulations are summarized in this chapter. The benefits to health promoted by its ingestion are also presented and discussed.

Keywords Xylitol · Health benefits · Medical applications · Food · Feed

14.1 Xylitol: Characteristics and Properties

Xylitol is a natural compound of the type pentitol (molecular formula $C_5H_{12}O_5$), also known as polyol or polyhydroxy alcohol acyclic (Mäkinen 2000). The name "polyol", as well as "sugar replacer", "sugar alcohol" or "hydrogenated carbohydrate"

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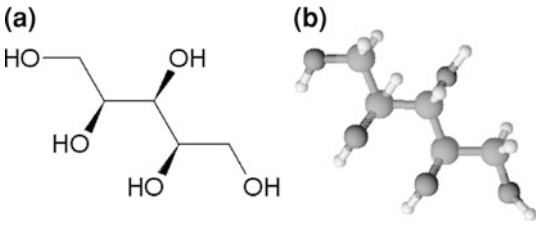

Fig. 14.1 Planar (**a**) and three-dimensional (**b**) chemical structure of xylitol

corresponds to a class of carbohydrates. However, polyols are not considered sugars (Livesey 2003). Xylitol is considered a "sugar-free" sweetener and is a member of the polyols family that includes other common dietary sweeteners such as sorbitol, mannitol, and maltitol.

The xylitol molecule has an opened structure with five hydroxyl groups (OH), each one of them connected to a carbon atom (Fig. 14.1) (Mäkinen 2000). The odd number of carbon makes this structure more difficult to be attacked by microorganisms in order to extract energy.

Pure xylitol is a white crystalline powder that looks and tastes like sugar. Table 14.1 summarizes the main characteristics and physicochemical properties of this compound. Among the physicochemical properties, the sweetening power is similar to sucrose but with only two-thirds of the caloric value (2.4 vs. 4.0 cal/g). Xylitol has also 75 % fewer carbohydrates than sucrose, and produces no aftertaste. Additionally, xylitol syrups are less viscous than sucrose syrups, and xylitol does not caramelize if heated to temperatures near the boiling point (216 °C) for several minutes.

Solid xylitol dissolves quickly in the mouth and produces a noticeable cooling sensation due to its high endothermic heat of solution (34.8 cal/g), which gives an excellent taste and cooling sensation particularly advantageous with certain spicy, herbal, and mint flavors.

The xylitol molecule has strong affinity for water. Its solubility is similar to sucrose at room temperature and higher at elevated temperatures, which is an important advantage since it allows the formation of very high solids content solutions, a property that is beneficial in hard coating procedures (Bond and Dunning 2006). On the other hand, xylitol is only slightly soluble in alcohol: 1.2 g/100 g solution of 96 % ethanol, and 6.0 g/100 g of 96 % methanol.

The xylitol stability is not affected by pH, i.e., it can be used across a broad pH range (1–11). Because of the high chemical and microbiological stability, xylitol works, even at low concentrations, as a preservative in food products providing resistance to microbial growth and prolonging the shelf life of these products (Bar 1991).

14.2 Natural Occurrences

Xylitol is a natural constituent in many fruits, vegetables, lichens, algae, and mushrooms (*Psalliota campestris*), but it is found only in small amounts (less than 1,000 mg/100 g) in these sources. Some of the natural sources of xylitol are listed

Table 14.1 Characteristics and physicochemical properties of xylitol

Properties	Characteristics/values
Empirical formula	$C_5H_{12}O_5$
Chemical name	1,2,3,4,5 Pentahydroxy pentane
Synonyms	Hydrogenated xylose; Xylite
Molecular weight	152.15 g/mol
Saccharide class/sub-class	Monosaccharide/hydrogenated monosaccharides
Generic form	Pentitol
Appearance	Crystalline powder
Color	White
Taste	Sweet
Odor	Odorless
Melting point	92–96 °C
Boiling point	216 °C (1 atm)
pH (in aqueous solution 10 %)	5–7
Density	1.03 g/ml (aqueous solution 10 %) 1.23 g/ml (aqueous solution 60 %)
Solubility in water at 20 °C	64.2 g/100 g solution
Solubility in methanol	6.0 g/100 g solution methanol 96 %
Solubility in ethanol	1.2 g/100 g solution ethanol 96 %
Viscosity at 20 °C	1.23 cP (aqueous solution 10 %) 20.63 cP (aqueous solution 60 %)
Hygroscopicity (in high humidity)	More than sucrose and less than sorbitol
Solution heat (endothermic)	34.8 cal/g
Relative sweetness	Similar to sucrose, greater than sorbitol or mannitol
Caloric value	2.4 cal/g
Stability	Stable at 120 °C (does not caramelizes)
Refractive index at 25 °C	1.35 (aqueous solution 10 %)
Specific rotation	Optically inactive
Heat of combustion	16.96 kJ/g

in Table 14.2. Xylitol can be found in relatively high quantities (300–935 mg/100 g dry solids) in plums, strawberries, and cauliflowers, the quantities varying according to the season and plant variety.

Since the content of xylitol in fruits and vegetables is usually low, the extraction of this sweetener from fruits and vegetables for application in industrial products is not an economic process. Therefore, xylitol is produced on an industrial scale by a chemical process that consists of the reduction of D-xylose recovered from raw materials containing hemicellulose, such as birch and other hard wood trees. Xylitol produced by this process is a product similar in structure and properties to the natural substance.

Xylitol is also an intermediate product during the mammalian carbohydrate metabolism. A human adult produces up to 15 g of xylitol per day during normal metabolism in the liver, and the concentration of xylitol in the blood is in the range between 0.03 and 0.06 mg/100 ml (Pepper and Olinger 1988).

Table 14.2 Xylitol content in some natural sources

Product	Xylitol (mg/100 g dry solids)
Plum (*Prunus domestica* subspecies *italia*)	935
Strawberry (*Fragaria* var.)	362
Cauliflower (*Brassica oleracea* L. var. *botrytis*)	300
Raspberry (*Rubus idaeus* L.)	268
Endive (*Cichorium endives* L.)	256
Bilberry (*Hippohae rhamnoides*)	213
Aubergine (*Solanum melongena* L.)	180
Lettuce (*Lactuca sativa*)	131
White mushroom (*Boletus edulis* Bull.)	128
Spinach (*Spenacia oleracea* L.)	107
Pumpkin (*Cucurbita pepo* L.)	96.5
Onion (*Allium* cepa L.)	89
Carrot (*Daucus carota* L.)	86.5
Banana (*Musa sapientum* L.)	21

From Washüttl et al. (1973)

14.3 Food Applications

Xylitol has been mainly used as a sugar substitute in foods due to its sweetening power similar to sucrose. However, it presents also important advantages when compared to this traditional sugar. One of them is the high chemical and microbiological stability, which allows its use as a preservative for food products, extending their shelf life (Bar 1991). Another advantage is that, due to the absence of aldehyde or cetonic groups in its molecule, xylitol does not participate in reactions with amino acids ("Maillard" reactions). This means that it does not undergo non-enzymatic browning reactions, which reduce the nutritional value of proteins. Thus, it is recommended for use in the manufacture of products where Maillard reactions are undesirable, such as infant foods obtained by drying. On the other hand, the exclusive use of xylitol is not desired in products where browning reactions are of interest, such as bakery products, for example, since these reactions are responsible for the appearance and flavor characteristics of these products (Manz et al. 1973). Other positive aspects of xylitol when compared to sucrose is that when continuously supplied in the diet, xylitol limits the tendency to obesity, which does not occur with diets based on sucrose.

Xylitol also presents advantages when compared to other sweeteners. Due to its sweetening power similar to sucrose, it can be used alone to substitute this sugar, while other polyols like sorbitol, arabitol, and mannitol have lower sweetening power and must be used in combination with other sweeteners to obtain the sweet taste desired (Gliemo et al. 2008).

In terms of application, crystalline xylitol can be sprinkled on cereal or fruit, mixed in salad dressings, or used in cooking and baking. In commercialized products, xylitol has been used as a food additive and sweetening agent since the 1960s, but in recent years, the number and types of products containing this

sweetener in the formulation have increased at a rapid rate. Nowadays, a variety of products containing xylitol in the composition can be found in the market, including chewing gums, confectionery, jams, chocolates, frozen desserts, cookies, puddings, ice creams, and soft drinks.

Due to the high endothermic heat of solution (34.8 cal/g) xylitol has been used to provide a pleasant cooling effect in several products, such as biscuit creams, fillings, fondants, and chewing gums. In chewing gums, particularly, xylitol is largely used for three main reasons: (1) promoting a cooling effect, (2) promoting sweetness similar to sucrose but with lower caloric value, and (3) providing novel texture, forming more flexible products. Although xylitol can be used as the only sweetener in chewing gums, it is usually used in mixture with other intense sweeteners in order to enhance flavor and sweetener longevity (Bond and Dunning 2006).

In confectioneries, xylitol is not necessarily used for the production of sugar-free products, but it is commonly used as a sweetener to promote a cooling effect or as engrossing material. Among the numerous applications of xylitol in confectionary, the most common include candies, jellies, pastilles, toffees, lozenges, tablets, and mini-mints.

Xylitol is also used to replace sucrose in chocolates. Although high levels of xylitol may be linked to a slightly "scratchy" aftertaste in chocolates (effect usually common when using monosaccharide sugars in chocolate), it has been used in this kind of food because it negates the need for the addition of intense sweeteners (Bond and Dunning 2006). However, when sucrose is replaced by xylitol in chocolates, some slight changes must be made in the production process, mainly because of the lower viscosity of the xylitol.

The addition of xylitol (alone or in combination with other sugars) in yoghurts, jams, and frozen desserts improves the texture, color and taste of these products, providing stability for longer periods. Xylitol can also act as an antioxidant, moisturizer, stabilizer, cryoprotectant, and freezing point reducer. When used in ice creams, for example, xylitol may act as a bulking agent, sweetener, and crystallization inhibitor (Maia et al. 2008). Xylitol ice cream has also a considerably softer consistency than sucrose ice cream at the same temperature.

Xylitol is also an effective humectant when used in cakes and muffins, since it binds the moisture within the product, improving the texture and shelf-life of baked products. When used to totally replace sucrose in sponge cakes, xylitol leads to products with similar characteristics and sensory profile to that manufactured with sucrose (Ronda et al. 2005).

14.4 Safeties and Market

The xylitol incorporation in food products is legally permitted. In 1983, the Joint Expert Committee on Food Additives (JECFA) of two United Nations agencies (FAO and WHO) recommended unlimited "Acceptable Daily Intake" (ADI) for

xylitol consumption based on the safety of this compound. This means that it can be consumed in the levels necessary to achieve the desired effect. However, xylitol is extremely well tolerated by humans when ingested in interspaced doses of up to 20 g each, and up to a maximum daily intake of 60 g. The ingestion of higher doses can result in temporary gastrointestinal effects, such as bloating, flatulence, and diarrhea, due to the osmotic imbalance in the large intestine caused by its low rate of assimilation (Culbert et al. 1986). These effects must be taken into account when xylitol is used in food products normally ingested in large quantities, such as soft drinks, for example. Even producing these undesirable effects when consumed in elevated doses, xylitol is more easily tolerated than other sweeteners like mannitol and sorbitol, and has a very low toxicity via all routes of administration.

The use of xylitol in industrial products has been approved in over 50 countries worldwide, and industries that use this sweetener are, by order, of food, drugs and cosmetics. In Scandinavia and other parts of Europe, xylitol has been widely used in these three industries for over 30 years. Nowadays, the Danish company Danisco is the largest xylitol manufacturer in the world, and several other suppliers are Chinese companies (Franceschin et al. 2011). In Asia, xylitol is particularly used in chewing guns, and in this region, about 80–90 % of the commercialized chewing gums contain this sweetener in their formulation (Annies 2012).

Although the xylitol application in human food is well described and legally accepted, the xylitol ingestion by animals, particularly dogs, has not been encouraged. The consumption of xylitol by dogs in amounts above 0.1 g/kg bodyweight may cause hypoglycemia (low blood sugar), which can be life-threating. Low blood sugar can result in a loss of coordination, depression, collapse, and seizures in as soon as 30 min (Dunayer 2004). Moreover, the intake of very high doses of xylitol (above 0.5 g/kg bodyweight) may cause acute liver failure in dogs, which can be fatal (Dunayer 2006). In rats, monkeys, and horses, the insulin level increase after ingestion of xylitol is negligible when compared with the consumption of glucose. In rabbits, baboon, cattle, and geese, the use of xylitol can be compared with the ingestion of a dose of glucose. In dogs, however, the xylitol ingestion may cause a 2.5 to 7.0-fold increase in the level of insulin when compared with the same amount of glucose consumed. This increase in the insulin level after the ingestion of xylitol may cause severe hypoglycemia, elevated levels of liver enzymes, and hepatic necrosis in dogs (Dunayer and Gwaltney-Brant 2006; Todd and Powell 2007).

14.5 Benefits to the Health

In view of the growing number of people who have some type of metabolic disorder and need, therefore, to reduce or even stop their consumption of sugar, many efforts have been directed to find a sugar substitute that, at the same time, is nutritious and beneficial to health, acting in curing or preventing of diseases. Xylitol is a compound that satisfies these requirements with additional advantages,

since it is tolerated by diabetics and has several clinical applications, being indicated for treating hemolytic anemia, renal and parenteral lesions, as well as to prevent dental caries, acute otitis media, osteoporosis, respiratory infections, and inflammatory processes (Mussatto and Roberto 2002). The medical and nutritional uses and effects of xylitol ingestion to health are summarized in Table 14.3 and discussed in detail in the next sections.

14.6 Treatment of Diseases

14.6.1 Diabetes

One of the main advantages of xylitol when compared to sucrose is that it can be consumed by diabetics. In people with this metabolic disorder, i.e. with deficiency in the metabolism of carbohydrates, it is of great importance to control the rate of glucose in the blood in order to avoid problems such as hyperglycemia, disturbances in lipid metabolism, and symptoms such as excessive thirst and hunger. Unlike conventional sugars, xylitol does not depend on insulin to be metabolized by the organism. Therefore, it is well tolerated by people with diabetes mellitus type I or type II (Manz et al. 1973; Pepper and Olinger 1988; Bar 1991).

None of the two main routes of absorption of xylitol (liver and gut) is mediated by insulin. Although xylitol can penetrate in almost every cell of the body, those of the liver are particularly permeable and contain a huge amount of enzymes able to quickly metabolize it and turn it into energy. On the contrary, the absorption from the small intestine is considerably slow, and most of it is then fermented in the large bowel. All the D-glucose derived from the metabolism of xylitol is firstly stored as glycogen in the liver being released gradually. As a consequence, its concentration in the blood does not undergo abrupt changes as caused during the metabolism of sucrose and glucose. Therefore, xylitol is considered a sweetener suitable for diabetics (Ylikahri 1979). In terms of daily dose, xylitol can be safely consumed by diabetic patients at doses up to 60 g per day without promoting significant increases in blood glucose concentration.

This characteristic allows the application of xylitol in products that can be consumed by people that have this metabolic disorder and need a controlled diet. However, xylitol can also be consumed by people that do not have this deficiency but that are concerned with their health and well-being.

14.6.2 Hemolytic Anemia

Glucose 6-phosphate dehydrogenase (G6PDH) is an enzyme of great importance for survival of the cells, since it is responsible for maintaining an adequate level of NADPH (a reduced coenzyme important in oxidative biological processes).

Table 14.3 Medical, nutritional uses and effects of xylitol

Uses of xylitol
Non- and anticariogenic sweetener
Alleviation of xerostomia; generally recognized owing to saliva stimulation
Decrease of *Pseudomonas*-based biofilm (with lactoferrin)
Prolonging of chlorhexidine effect on *Streptococcus mutans*
As a sweetener in diabetic diets; generally recognized
Energy source in infusion therapy
Promotion of endogenous fat mobilization and oxidation
Studies as an antiulcer agent
Resuscitation from diabetic coma
Prevention of adrenocortical suppression during steroid therapy
Increase in auditory threshold values in patients with Ménière's disease
Therapy of adenosine deaminase deficiency in a form of adult myopathy
Therapy of glucose 6-phosphate dehydrogenase deficiency in red blood cells (anemia)
Restoration of heart muscle adenine nucleotide levels
Increase in the levels of retinol-binding proteins
Reduction in the incidence of liver and bile duct disorders
Stimulation of the mixed-function oxidase system
Treatment of ketonemia
Prevention of experimental osteoporosis; improvement of collagen and bone properties
Prevention of some diabetic complications
As a protein-sparing and thiamine-sparing agent; stimulation of enteral vitamin synthesis
Preservation of red blood cells
Amelioration of drug-induced hemolysis
Prevention of acute middle ear infections in infants
Antibacterial effect on pneumococcal nasal colonization
Alleviation of cystic fibrosis condition
Stimulation of cytokine induction (rat bladder cell lines)
Reconstitution of integral membrane transport proteins
Skin care with farnesol (atopic dry skin; *Staphylococcus aureus*)
Prevention of cardiac arrhythmias
Stimulation of pancreatic enzyme secretion
Anti-tumor effect (increasing host cell metabolism)
Beneficial effect on the growth of broiler chicks
Prevention of phenylenediamine-induced hepatotoxicity
Wound care (inhibition of wound biofilm formation)
Inhibition of fish oil oxidation (fish flavor suppression)
Inhibition of food spoilage microorganisms
As a sanitizer (food safety; kitchen hygiene; with free radicals)
Removal of KL-6 mucin (around cell surface; carcinomas)
Prevention of cattle ketosis; improvement of udder health; lowering of piglet mortality

From Mäkinen (2011)

In humans, deficiency of the enzyme G6PDH sets up a condition known as hemolytic anemia. This deficiency promotes a reduction of erythrocytes due to the inability of cells to regenerate NADPH (Vieira-Neto et al. 1999). This pathology is

the most common human enzymopathy and affects about 400 million people worldwide (Luzzatto and Mehta 1995). Clinical manifestations such as hemolytic anemia induced by drugs or infection, favism, neonatal jaundice, and chronic congenital hemolytic anemia are associated with deficiency of the G6PDH enzyme.

Xylitol can be used as a therapeutic agent (maximum dose of 1 mM/L of blood) by people with G6PDH deficiency, since this enzyme is not required for its metabolism. Xylitol supplies NADPH to the cell by oxidation of L-xylulose, and helps to maintain the integrity of the membrane of red blood cells (van Eys et al. 1974; Ylikahri 1979).

14.6.3 Renal and Parenteral Lesions

The use of xylitol in parenteral nutrition (daily dose up to 6 g/kg bodyweight) is recommended for two reasons: (1) xylitol does not react with amino acids as glucose reacts; therefore, the production of infusions containing both compounds is facilitated; and (2) tissues can use xylitol under postoperative or post-traumatic conditions. Patients in postoperative or post-traumatic conditions present an excessive excretion of "stress" hormones (cortisol, catecholamins, glucagon, and growth hormones, among others), which cause resistance to insulin absorption and prevent the efficient use of glucose by the organism. Treatment of such people with xylitol produces a limited increase in the levels of glucose and insulin in the blood, benefiting their health (Ylikahri 1979).

14.7 Prevention of Diseases

14.7.1 Dental Caries

Caries is the result of an infectious process caused by bacteria in dental plaque. Bacteria of the genus *Streptococcus*, found in the oral flora, are highly cariogenic, since, under acidic conditions, they produce a large amount of lactic acid and synthesize extracellular polysaccharides, which increase the adhesion of the plaque to the tooth surface (Kandelman 1997). The sooner these colonies appear on the teeth and the greater the quantity in the oral flora, the greater the risk of developing caries.

The anticariogenicity is one of the most important properties of xylitol, and is determined mainly by its non-fermentability by bacteria of the genus *Streptococcus*, whose proliferation in the oral flora becomes limited. By reducing the concentration of *Streptococcus mutans*, the amount of insoluble and soluble polysaccharides is decreased and increased, respectively. As a consequence, a less adherent plaque of

easier removal by the usual brushing of the teeth is formed (König 2000; Gales and Nguyen 2000).

In brief, xylitol helps the oral health in four different ways:

(1) It reduces the incidence of caries and stabilizes the formed ones. A study demonstrated 85 % reduction in the incidence of dental caries when the usual diet of sucrose was replaced by xylitol over 2 years (Mäkinen et al. 1998). In another study, 3–5 units of chewing gum containing xylitol or sorbitol were consumed per day by a population of high risk for development of caries, over 40 months. Chewing gums containing xylitol were able to reduce up to 63 % of the caries in these patients, while those containing sorbitol reduced only 30 % (Gales and Nguyen 2000).
(2) It stimulates the formation of saliva and stabilizes calcium and phosphate ions in saliva (Autio 2002; Loveren 2004). The pleasant taste of xylitol stimulates salivation without increasing the acid production in dental plaque. Once the amount of saliva is increased, the amount of minerals present therein is also increased. Some of these minerals, especially calcium and phosphate ions, promote remineralization of the teeth and, as a consequence, the reversion of dental caries at an early stage.
(3) It reduces the growth of *Lactobacillus* and *S. mutans* in the saliva. The effects of a daily consumption of 5.2 g of saliva stimulants containing xylitol or erythritol under the control of dental plaque and *S. mutans* were evaluated in a study. The results revealed, that for those consuming xylitol, the weight of the total plaque (collected over the surface of teeth after a period of 3 min) and the amount of *S. mutans* present in the plaque and saliva have been reduced significantly, while no effect was observed in those consuming erythritol (Mäkinen et al. 1998).
(4) It controls the pH of the plaque and the buffering capacity of the saliva after the consumption of sucrose. Oral washing with xylitol solution prevents the pH drop from the tooth surface (one of the causes for the caries arising). Such effect occurs because the increase in salivary flow increases the pH of the plaque, which neutralizes the acids produced by other fermentable carbohydrates that have been consumed. As a consequence, the levels of some enzymes are also increased, increasing the buffering capacity and the bacteriostatic activity of the saliva. Then, the oral environment becomes less favorable for the development of bacteria (Kandelman 1997).

It is worth mentioning that the formation of caries in children can be prevented from birth. During pregnancy, mothers have high levels of the bacterium *S. mutans* in the saliva, which is then transmitted to the children by talking, kissing, etc. The contamination usually occurs in children between 6 and 30 months of age, a period when the first teeth appear. Babies under 6 months do not run that risk, because the surface of the teeth is required as habitat for this bacterium. Söderling et al. (2000) conducted a study with mothers of infants 3 months old, subjecting them to regular use of chewing gum containing xylitol. After about 2 years, when comparing these

children with others of the same age, whose mothers did not participate in this study, the authors noted that the levels of *S. mutans* in the children of users of xylitol were much lower, which demonstrated that xylitol inhibited transmission of the bacteria, thereby reducing the risk of dental caries formation. Isokangas et al. (2000) followed this same group of children until the change of their primary dentition (around 5 years old) and observed that, even with secondary dentition, these children had levels of *S. mutans* about 70 % lower than those of children whose mothers had not performed treatment.

14.7.2 Acute Otitis Media

Acute otitis media, the second most common infection in children, is caused by bacteria of the nasopharynx that enter in the middle ear via the Eustachian tube (Erramouspe and Heyneman 2000). Xylitol is active in preventing or combating this disease because it inhibits the growth of the bacterium *Streptococcus pneumoniae*, the main cause of sinusitis and middle ear infections (Kontiokari et al. 1995). A daily dose of 8.4 g of xylitol given in the form of two pieces of chewing gum (chewed for 5 min each) to children is effective to combat this disease, reducing by 40 % the incidence of this infection (Uhari et al. 1996). Xylitol is also well tolerated by children when in the form of syrup, being effective in the prevention of otitis and reducing the need for antibiotics (Uhari et al. 1998).

Xylitol is able to inhibit the growth of *S. pneumoniae* and *S. mutans* bacteria because when ingested, it is phosphorylated to xylitol-5-phosphate. Since these bacterial species do not have the enzymes responsible for the xylitol-5-phosphate metabolism, an intracellular accumulation of this compound occurs. Once accumulated within the cell, the xylitol-5-phosphate becomes toxic, causing inhibition of glycolytic enzymes and of the bacteria growth, whose survival time is reduced (Kontiokari et al. 1995; Trahan 2000). It is important to mention that the inhibitory effect of xylitol on the growth of *Pneumococci* is eliminated by fructose, which is required by the fructose phosphotransferase system. For this reason, products suitable for preventing acute otitis media cannot contain fructose in mixture with xylitol (Tapiainen et al. 2001).

14.7.3 Osteoporosis

Osteoporosis is an illness caused by the decrease of calcium deposition in bones, which suffer loss of volume and biomechanical properties. This disease primarily affects the femur that becomes more brittle, breaking easily. Xylitol combats osteoporosis stimulating calcium absorption by the intestine and facilitating passage of blood to the bone. The content of calcium in bones is then increased, reducing the need for resorption. Such effects occur because during the

metabolism of xylitol, there is an increase in the NADH/NAD ratio, and changes in the NADH/NAD ratio are closely related to the calcification process. The increase in the concentration of NADH enhances the transport of Ca^{2+} ions through the cell membrane, activating the calcification process of the bones and cartilage, and increasing the collagen synthesis (Mattila et al. 1996).

In studies with animals, xylitol promoted an increase in bone mass, preserving their minerals and preventing the weakening of their biomechanical properties. When the animals' diet was supplemented with 10–20 % of xylitol in the feed formulation, it was verified that, the greater the amount of xylitol in the feed, the better the results in combating osteoporosis. Xylitol was also demonstrated to be able to stimulate collagen synthesis in both normal and diabetic rats (Knuuttila et al. 2000). In humans, a daily dose of approximately 40 g of xylitol in the diet of individuals of middle age was suggested to prevent osteoporosis, besides being an amount well tolerated by the organism (Mattila et al. 1998).

14.7.4 Respiratory Infections

The inner surface of the lungs is coated with a thin layer of liquid containing antimicrobial substances able to remove bacteria constantly sucked or inhaled, thereby preventing pulmonary infections. Increasing the salt concentration in this liquid that coats the interior of the lungs inhibits the antimicrobial activity of these substances, causing spread of the bacteria and production of chronic infections as a consequence. On the other hand, reducing the salt concentration in the liquid may increase the antimicrobial activity and prevent infection.

The efficiency of the xylitol in the treatment of respiratory infections is given by the low transepithelial permeability of this sweetener. As a consequence, it is not metabolized by most of the bacteria and can reduce the salt concentration in the liquid that wrap the inner surface of the lungs. *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria (main causers of pulmonary infections) do not use xylitol for growth, which causes a decrease in the salt concentration in the liquid and increases the natural antibiotic activity of the lungs. In brief, xylitol strengthens the natural defense system of the lungs by delaying or preventing the establishment of bacterial infections, including pneumonia (Zabner et al. 2000).

Patients with respiratory problems usually present congestion in the nose, difficulty in breathing and lung problems, with nasal irrigation recommended to keep the respiratory ways clean, thus facilitating breathing. Xylitol applied in the form of nasal spray reduces the bacterial load and increases the local defense mechanisms (Zabner et al. 2000). It also inhibits the growth of *S. pneumoniae* and *Haemophilus influenzae* bacteria, which cause sinusitis and respiratory infections, and decreases the adhesion of these bacteria to epithelial cells, reducing the incidence of infection processes (Tapiainen et al. 2001).

14.7.5 Inflammatory Processes

The use of xylitol as a food supplement (6–15 % of feeding) has shown good results with respect to induced acute inflammatory processes. Takahashi et al. (1999) performed studies with birds up to 12 days of age, feeding them for 10 days with a diet containing 15 % xylitol. After this period, antigens were injected in the birds to induce inflammation and it was observed that xylitol alleviated the growth retardation and anorexia caused by the infection. Treatment with xylitol also prevented further weight loss of the birds, without affecting or changing any other part of their system. In further studies, the authors reduced the percentage of xylitol in the diet of the birds to 6 % and for only 1 day feeding period prior to induction of inflammation. The same beneficial effects were observed using this shorter time and at a lower cost of treatment (Takahashi et al. 2000).

14.7.6 Colon Diseases

Xylitol is a low-digestible carbohydrate and therefore, it is only partially absorbed in the upper intestine. Approximately 50 % of the ingested dose passes to the lower intestine, being available for fermentation by beneficial commensal bacteria in the colon, such as *Bifidobacterium* and *Lactobacilllus* species. The result of this fermentation is a reduction in the intestinal pH, which has been closely linked to improvements in epithelial function, maintenance of a healthy colonic epithelium, and a decrease in the risk of diseases of the colon. Xylitol ingestion has been reported to influence the colonic and faecal microflora in humans and animals (Bond and Dunning 2006).

14.8 Conclusion and Future Recommendations

Xylitol is a compound of great interest for use as an ingredient in the food industry since it confers specific characteristics to the products, with sweetness similar to sucrose but with a lower caloric value. Additionally, xylitol can be used in a large variety of food products, including those specially formulated for diabetic people. For all these reasons, the xylitol incorporation in diets has been adopted by many nations. In the last decade, particularly, a rapid growth in the worldwide market of xylitol has been observed since consumers are becoming increasingly aware of the health. It is well known that besides being a suitable sweetener to replace sucrose, xylitol is also beneficial to human health acting on the treatment or prevention of diseases. Therefore, xylitol incorporation in diets brings benefits not only for those who need a controlled diet, but also for those who, although having no metabolic disorders, are concerned with their health and well-being. It is expected that in the

near future, xylitol will be most widely sold on the market. The main problem to be overcome in order to extend the xylitol commercialization is its higher price when compared to sucrose. In this sense, great efforts have been directed in order to find a lower cost technology suitable for its production. The production by biotechnological process appears to be an alternative with potential to reduce the costs involved in the chemical process currently performed on industrial scale. However, studies are still necessary in order to define a suitable biotechnological route for the production of this sweetener with high yields and low cost.

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Chapter 15
Medical Applications of Xylitol: An Appraisal

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Abstract Xylitol is a sugar alcohol, normally produced by mammal metabolism, which has a great (and increasing) applicability in food and pharmaceutical industries due to its intriguing and proper characteristics. The most explored area is oral health, due to its non- and anti-cariogenic properties, followed by its use as a sweetener. Moreover, some other applicabilities in the medical area can be found in literature and patents, such as the use in the treatment and/or prevention of acute otitis media, respiratory diseases, parenteral nutrition, atopic dermatitis, wound repairing, gastrointestinal infections, osteoporosis, anti-aging and inflammatory processes. Market usage of xylitol for over 20 years, accompanied by toxicological studies, corroborated its safety for medical purposes. A great number of recent studies demonstrate that xylitol can be used to develop pharmaceutical products. Especially because of its activity against microorganisms, xylitol medical applications are still an important point for studies, with potential for novel medical applications in the upcoming years.

Keywords Xylitol · Medical applications · Antimicrobial action · Dietetic usage

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15.1 Introduction

The great number of publications, patents and products highlight the medical importance of xylitol in the prevention and treatment of human health. Its more consolidated application is in oral health since its efficacy has already been proven in reducing caries and increasing teeth remineralization rate. Xylitol has also been successfully employed in the treatment of acute otitis media and respiratory infections, mainly due to the fact that nasopharynx and oral microbiota bacteria species did not have the enzymes responsible for the metabolism of xylitol-5-phosphate (a compound formed by phosphorylation of xylitol into the cell). Another important clinical application of xylitol is on its use by people with diabetes (Ylikahri 1979). There are also less known reports on other possible applications of xylitol, as its probable use in atopic dermatitis, gastrointestinal disorders and inflammation, which will also be discussed in this chapter.

Xylitol use is approved in over 50 countries worldwide, including members of the European Union, Switzerland, Brazil, Argentina, Australia, Japan, South Korea and China. The approval allows the use of xylitol for all categories of food, dietary supplements, pharmaceuticals, cosmetics and oral hygiene products (Kruger 2005). Xylitol incorporation in foods is allowed because it is generally recognized as a safe (GRAS) additive recognized by the Food and Drug Administration (FDA) (Saulo 2005).

According to the World Health Organization (WHO), oral acute toxicity tests performed with animals indicated that xylitol had very low toxicity. Conventional test results for the teratogenicity and embryo toxicity effects of xylitol were negative, as well as in vitro and in vivo tests of mutagenicity and clastogenicity (WHO/FAO 1997). In 1996, the Joint Expert Committee on Food Additives (JECFA), a North American institution linked to WHO and Food and Agricultural Organization (FAO), included xylitol in the safest category in which a food additive can be included, based on previous categorization studies according to an acceptable daily intake (ADI) (Calories Council Control 2005).

The results obtained from different studies with xylitol allow a global analysis on the advantages of its administration in patients with different pathologies (Zabner et al. 2000; Ferreira et al. 2009a; Silva et al. 2010a, 2011; Souza et al. 2011). The economic and social importance of xylitol is mainly due to its high sweetening power and anti-cariogenic property, and this usage makes xylitol a high added-value compound. However, new clinical applications for xylitol have been studied and should be highlighted.

15.2 Oro-Facial Usage

Diseases such as acute otitis media, sinusitis, nasopharyngeal congestion and inflammations associated with respiratory complications can be prevented and treated with a saline xylitol nasal application (Sawaguchi et al. 2001). The administration of

xylitol in solid preparations (chewing gum, tablets and powder, for example), as well as in liquid preparations, may help in the treatment of mammal respiratory infections, especially acute otitis media in humans (Uhari et al. 1996; Leiras 2000).

15.2.1 Acute Otitis Media

Uhari et al. (1996) verified in a double-blind trial conducted with 300 children that xylitol action on acute otitis media management referred to its presence and not to a mechanical function, for example, chewing gum containing xylitol and sucrose used in tests. Previosly, Kontiokari et al. (1995) had already reported the antimicrobial activity of xylitol on the *Streptococcus pneumoniae* bacteria. These authors observed a reduction of 35 and 72 % of this bacterium growth when it was cultivated with 1 or 5 % xylitol, respectively, for 2 h.

Later, Kontiokari et al. (1998) evaluated the adherence of microorganisms associated with nasal colonization and otitis media in three situations: using epithelial cells exposed to 5 % xylitol, using bacteria exposed to 5 % xylitol, and using bacteria + epithelial cells exposed to 5 % xylitol. It was found that xylitol prevented the *S. pneumoniae* adherence, and in a lesser proportion, the *Haemophilus influenzae* adhesion. However, xylitol did not significantly prevent the adherence of *Moraxella catarrhalis*.

Uhari et al. (2001) concluded that xylitol is an attractive alternative for acute otitis media treatment, since it inhibited *S. pneumoniae* growth and prevented the binding of pneumococci and *Haemophilus influenzae* in nasopharyngeal cells. A reduction of approximately 40 % in children affected by the infection was observed when chewing gum containing xylitol was administered and approximately 30 % when syrup containing xylitol was administered (8.4–10.0 g/day divided in five doses).

Tapiainen et al. (2004) exposed *S. pneumoniae* in a medium containing 0.5 and 5.0 % xylitol. After 2 h of contact, a decrease in the diameter of capsule and dissolution of its contents was observed. This modification can affect the bacterium adherence and virulence, which may explain the clinical efficacy of xylitol in preventing acute otitis media.

Recently, Danhauer et al. (2010) conducted a meta-analysis to verify if there was evidence regarding xylitol use as a prophylaxis for acute otitis media in children. These authors found an overall risk ratio of 0.68 when compared to the control group, which corroborated the positive xylitol effect in the prevention of this disease. Moreover, in another meta-analysis study, Azarpazhooh et al. (2011) also found a reduction in 25 % of acute otitis media occurrence in children up to 12 years of age. Kurola et al. (2011) suggested that changes in biofilm formation due to different sugar compounds may partly explain the efficacy of xylitol in preventing acute otitis media in clinical trials, based on the results observed in *S. pneumoniae* biofilm formation and gene expression in vitro. In a study using rats infected with *S. pneumoniae*, Renko et al. (2008) observed that dietary xylitol

enhanced oxidative killing in neutrophilic leucocytes and prolonged the survival of rats suffering from sepsis.

15.2.2 Respiratory Diseases

Xylitol efficiency in treating respiratory diseases (Zabner et al. 2000; Tapiainen et al. 2001) is attributed to its low transepithelial permeability. Therefore, xylitol is not metabolized by most bacteria and therefore decreased the salt concentrations in the fluid that coats the inner surface of the lungs (Zabner et al. 2000).

Sajjan et al. (2004) observed that xylitol (60–80 mg/mL) inhibited 65 % of *Burkholderia cepacia* complex growth, whose absence is closely related to success of lung transplantation.

Cystic fibrosis is a lung disease characterized by bacterial colonization and chronic infection. Several microorganisms can be present, but *Pseudomonas aeruginosa* and *Staphylococcus aureus* stand out. As observed by Welsh and Zabner (2004) and Zabner et al. (2000), xylitol administered in powder form and/or osmotic aerosol had osmotic features that allowed it to act in control and/or treatment of cystic fibrosis, reducing the ionic strength and thus enabling the endogenous antimicrobials activation. Durairaj et al. (2007) showed that xylitol was safe and well tolerated, being administered by means of nebulization in patients with cystic fibrosis. Domenico and Lavecchia (2002) found that 30 % xylitol can stabilize blood through preventing erythrocytes lysis due to this same property.

Souza et al. (2011) studied in vitro the antimicrobial activity and the anti-adherent property of xylitol (0.5, 2.5 and 5.0 %, w/v) on two *P. aeruginosa* strains (ATCC 9027 and a clinical strain). The results showed that xylitol had no antimicrobial activity on these strains. However, bacterial adherence inhibition was observed in photomicrographies obtained by scanning electron microscopy (SEM). These results indicated that xylitol could be a future alternative to combat bacterial colonization.

Durairaj et al. (2004) verified that 5 % xylitol solution in water, which is equimolar to 0.9 % sodium chloride, was as safe as a vehicle when mice were exposed for 150 min in an exposure chamber. This result was corroborated in a sample of ten health volunteers. There was no change in spirometry, laboratory tests results (serum glucose, sodium, bicarbonate and anion gap levels; osmolarity), and bronchoalveolar lavage cytokine levels after xylitol exposure (Durairaj et al. 2004).

15.2.3 Caries and Teeth Demineralization

The chemical structure of xylitol is characterized by a threo sequence of hydroxyl groups (-OH). This chemical configuration can be responsible for the formation of stable complexes with ions such as calcium (Mäkinen 2000). Xylitol is believed to

have the ability to carry and to form complexes with calcium ions, and therefore is able to penetrate the tooth enamel (Amaechi et al. 1998; Sano et al. 2007).

Xylitol is not fermented by most oral cavity microorganisms, exercising a cariostatic effect (Silva et al. 1994; Tamanini and Hauly 2004). This action was observed on *Streptococcus mutans* bacterium, the main cause of caries. The non-cariogenic effect is observed because the microorganisms which form bacterial plaque did not ferment this sugar and it was not a suitable carbon source. Since xylitol stimulated salivation and was not consumed, it did not cause a decrease in pH (local acidification), which would contribute to the growth of *S. mutans* and development of caries (Iwata et al. 2003).

Mäkinen (2000) reinforced xylitol's anticariogenic activity by studying newborns whose parents chewed gum containing xylitol. It was found that this action prevented the babies from developing caries, since aerosols from the mother would be the most common transmission way of *S. mutans*. This is possible because xylitol prevents bacterial adhesion on teeth, reducing the viable cell count and also the chance of transmission. The xylitol mechanism of action in this case can be explained by the ability of pentitols molecules to change the adherence associated with the surface of pathogens structures.

Amaechi et al. (1998) demonstrated in an in vitro experiment that teeth surface erosion caused by the consumption of acidic drinks like orange juice can be minimized due to the additive effect of xylitol and fluoride. Xylitol forms a complex with calcium ions, therefore retarding demineralization by lowering their diffusion coefficient.

Silva et al. (2010b) verified the in vitro influence of xylitol in the teeth demineralization process, evaluating its preventive and/or corrective performance, in comparison with fluoride action, the standard drug used in cases of dental erosion. Bovine teeth were exposed to lemon juice, xylitol (10, 25 and 40 %) and fluoride (500 ppm) in different ways. Microscopic characteristics of the enamel by SEM showed that xylitol (10 and 25 %) was not effective enough to prevent the microstructural changes of the dental enamel and, consequently, to prevent the dental erosion process in the prevention and correction studies. However, 40 % xylitol showed protective and correction effects on the erosive process, similar to that one adopted as standard, i.e. 500 ppm of fluoride. This study highlighted the importance of substance knowledge which can prevent progression or treating the erosive process, and that 40 % xylitol may be an appropriate replacement substance for fluoride.

A number of clinical trials illustrating xylitol efficacy on the prevention and treatment of oral diseases have been successfully completed (Campus et al. 2011, 2009; Hildebrandt et al. 2010; Bader et al. 2010; Nakai et al. 2010; Milgrom et al. 2009). Table 15.1 summarizes a few of these studies.

Table 15.1 Randomized clinical trials evaluating xylitol efficacy on oral diseases

Study design	Patients	Duration	Xylitol dose and administration route	Hypothesis	Outcome	Reference
Double-blind, interventional study	120 Health adults with high risk of caries	37 days	Chewing gum with 30 % xylitol, 5 min, 3 times/day. Daily intake: 2.2 g	Is xylitol alone or with *Magnolia* bark extract (MBE) effective in preventing caries and gingivitis in this sample?	Both preparations were efficient when compared to a non-sucrose chewing gum, but MBE increased the efficacy	Campus et al. (2011)
Double-blind, controlled study	105 Adults with high salivary ($>10^5$ CFU/mL) *Streptococcus mutans* content	3 months	1. Chewing gum, daily intake: 4.3 g 2. Aqueous solution, daily intake: 4.4 g	Does the use of xylitol decrease *S. mutans* content?	Both preparations trended to lower *S. mutans* in a month	Hildebrandt et al. (2010)
Multi-center, placebo controlled, double-bind study	691 Adults with at least one coronal or root surface cavitated carie lesions	3 years (still in progress)	1.0 g of xylitol as the sweetener in each lozenge dose; 5 doses/day	Does the use of xylitol lozenges reduce dental caries incidence in active adults caries?	It is a protocol study. The primary outcome will be the increment of cavitated lesions	Bader et al. (2010)
Controlled and blinded study	107 Pregnant woman	25 months	Chewing gum, daily intake: 5–10 g	Does the mother's use of xylitol prevent caries in the baby?	Control group children acquired *S. mutans* 8.8 months earlier than those in xylitol group	Nakai et al. (2010)
Controlled and longitudinal study	176 School children at high risk of caries	9 months	Chewing gum, daily intake: 11.6 g	What is the effect of a daily xylitol high dose on carie prevention in this sample?	Non-sucrose chewing gum long-term use is beneficial for carie prevention, and xylitol greatly improved the results	Campus et al. (2009)

(continued)

Table 15.1 (continued)

Study design	Patients	Duration	Xylitol dose and administration route	Hypothesis	Outcome	Reference
Double-blind, controlled study	100 Children ages 9–15 months	Until primary tooth eruption	Syrup divided in 2 (4 g of xylitol per dose) or 3 (2.67 g of xylitol per dose) daily doses. Daily intake: 8 g	Does xylitol pediatric topical oral syrup is effective in reducing the incidence of dental caries in very young children?	Oral xylitol administration 2 or 3 doses daily (total dose of 8 g) was effective in preventing early childhood caries	Milgrom et al. (2009)

15.3 Dietetic Usage

15.3.1 Parenteral Nutrition

The use of xylitol in parenteral nutrition (daily dose of up to 6 g/kg body mass) is recommended because there is no reaction between amino acids and xylitol, which facilitates the production of infusions containing both, also because the tissues can use xylitol under postoperative or post-traumatic conditions (Ylikahri 1979). Patients under such conditions have an excessive excretion of stress hormones (cortisol, growth hormones, etc.) which cause insulin absorption resistance and prevent efficient glucose usage by the body. Thus, treatment with xylitol is recommended as it produces only a limited increase in glucose and insulin levels in the blood (Ylikahri 1979).

Georgieff et al. (1991) studied parenteral nutrition of rats with burns and observed when xylitol was used there is no protein degradation, which happened when such a solution was prepared with glucose. Thus, a new formulation consisting of an aqueous solution with amino acids and xylitol is to be used for parenteral nutrition and has been patented for the treatment of patients with severe stress or renal complications. Such preparation reduced nitrogen loss and accelerated gluconeogenesis (Georgieff and Oehmke 1990).

15.3.2 Low Calories

In 1987, Shafer et al. observed that an ingestion of 25 g xylitol in nine health volunteers prolonged the gastric emptying of the solid phase of the meal and suppressed food intake.

In healthy volunteers, King et al. (2005) observed that an association between xylitol and polydextrose can be effective as an appetite control and a result of their lower energy content and appetite suppression. In rats, it was recently demonstrated that xylitol included in an isocaloric diet can reduce visceral fat percentage, decrease insulin and lipid levels, and increase the gene expression of peroxisome proliferator-activated receptor α (PPAR-α), adiponectin, hormone sensitive lipase, and adipose triglyceride lipase, all related to obesity (Amo et al. 2011). Also in a rodent study, Islam (2011) observed that xylitol reduced glucose levels and improved glucose tolerance when compared to sucrose. Moreover, xylitol also lowered food intake and body weight gain when compared to a control group (conventional food) or glucose group. The results suggested xylitol application as an appetite-suppressing sugar substitute for overweight and obesity-related metabolic diseases patients.

15.3.3 Diabetes

Hämäläinen and Mäkinen (1982) failed to find an atiketogenic effect in streptozotocin-diabetic rats through xylitol consumption, although an increase in serum glucagon was observed as well as a decrease in total liver ascorbic acid levels, when compared to glucose and/or fructose consumption.

Xylitol metabolism practically did not contribute to adipose tissue formation and is independent from insulin, which enables its use as a sweetener in the diabetic diet. Xylitol has a low intestinal absorption when compared to sucrose and, therefore, can be recommended for patients with diabetes, since it does not produce rapid changes in blood glucose (Tamanini and Hauly 2004).

Valero et al. (2011) compared the efficacy of a glucose solution to a glucose: fructose: xylitol solution (2:1:1) in 138 septic and nonseptic diabetic patients as a total parenteral nutrition. It was found that the solution containing xylitol could be beneficial in attaining glycemic control in nonseptic patients with diabetes, although it did not present any significant difference in septic patients. Therefore, glucose is still the first choice as the carbohydrate substrate in total parenteral nutrition until more studies are performed.

15.4 Dermatological Usage

15.4.1 Atopic Dermatitis

Katsuyama et al. (2005a, b) studied patients with atopic dermatitis, an inflammatory skin disease in which *S. aureus* bacterium is present in 95 % of cases. They found that xylitol inhibited the glycocalyx formation of this microorganism and acted synergistically with farnesol (an abundant sesquiterpene commonly found in essential oils with antimicrobial potential) in controlling the disease. The adjuvant action of xylitol is important, since glycocalyx is the primary structure responsible for bacterial drug resistance.

Through in vitro assays, Ferreira et al. (2009a) observed the anti-adherent property of xylitol. It was found that this polyol had no antimicrobial activity on *S. aureus* (ATCC 25923), but the adherence inhibition is most probably the xylitol mechanism of action on this bacterium. Furthermore, Ferreira et al. (2009b) observed dermal toxicological assays performed on rodents that suggested xylitol could be an appropriate biomolecule to be used in atopic dermatitis treatment, since it was not skin irritating. However, this sugar presented a phototoxicity action and, therefore, should be concomitantly administered with sunscreen.

15.4.2 Leishmaniasis

Ferreira et al. (2008), through in vitro studies, observed that 2.5 and 5.0 % xylitol was able to induce nitric oxide production by J774A.1 macrophages after 72 h of incubation, while it can inhibit nitric oxide production by *Leishmania amazonensis* after 48 h of incubation. When both cells were incubated in the same environment, it was observed that xylitol inhibited macrophage infections on an expressive level, which indicated its applicability in this neglected disease control.

15.4.3 Wound

Ammons et al. (2009) evaluated the xylitol and lactoferrin action in vitro, alone or in combination, in reducing the viability of a chronic wound-derived *P. aeruginosa* biofilm growth. Combined lactoferrin and xylitol treatment disrupted the structure of *P. aeruginosa* biofilm and this synergistic action resulted in few bacterial cells adhering to the growth surface. Results obtained indicated that xylitol acted in disrupting the overall biofilm structure. Recently, Ammons et al. (2011) observed that the xylitol and lactoferrin combination in a commercial silver wound dressing had a significant reduction in biofilm viability when compared to a commercial wound hydrogel, which corroborated with the efficacy of xylitol in wound dressing.

Korponyai et al. (2011) observed that xylitol presented an anti-irritant action, since it suppressed the sodium lauryl sulfate-induced skin irritation, evaluated by absent transepidermal water loss.

15.4.4 Anti-Aging Property

Mattila et al. (2005) observed in rats that a long-term dietary xylitol supplementation protected against aging due to a markedly lower collagenase-soluble fraction in the skin, when compared to the control group.

15.5 Gastrintestinal Usage

Naaber et al. (1996) found a dose-dependent inhibition of *Clostridium difficile* adherence to Caco-2 cells, using 1.0, 5.0 and 10.0 % xylitol. The authors were unaware of the action mechanism involved, but suggested that it could occur, in vivo, in intestinal cells.

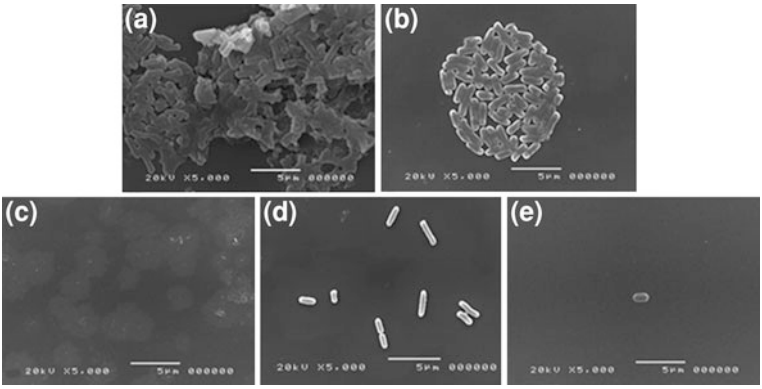

Fig. 15.1 Scanning electron microscopy microphotographs showing the effect of xylitol on the adhesion of clinical *Escherichia coli* strain. **a** and **b** Treatment with 5.0 % glucose (positive control). **c** Treatment with 0.5 % xylitol. **d** Treatment with 2.5 % xylitol. **e** Treatment with 5.0 % xylitol. *Scale bar* 5 μm (Other images from this study can be viewed in Silva et al. 2011)

Silva et al. (2010a) evaluated the anti-adherent and antimicrobial in vitro properties of xylitol (0.5, 2.5 and 5.0 %) on *Shigella flexneri* ATCC 12022, clinical (isolated from patients) *Shigella flexneri* and clinical *Salmonella enterica* serotype Typhimurium. Xylitol did not have an antimicrobial influence on the tested strains. However, the photomicrographies obtained by SEM showed the inhibition of microbial adherence on slides treated with xylitol as a probable action mechanism for this compound. These results gave xylitol a greater added-value, making it an appropriate biomolecule to be used as an adjuvant therapeutic, alternative to conventional antibiotics for recurrent gastrointestinal tract infection treatment.

Following the same line of study, Silva et al. (2011) evaluated antimicrobial and anti-adherent activities of xylitol (0.5, 2.5 and 5.0 %) on *Escherichia coli* (ATCC 8739) and on another clinical enteropathogenic strain, *E. coli* (EPEC), in vitro. It was found that xylitol did not have antimicrobial effects on these strains. The SEM demonstrated that the slides treated with xylitol had a significant reduction in the number of bacilli, and inhibition of microbial adhesion was probably the xylitol's action mechanism. Thus, xylitol could be a possible alternative in the control of *E. coli* infections. Figure 15.1 illustrates the evaluated glass slides, showing the xylitol probable anti-adherent mechanism. More details and information can be found in Silva et al. (2011).

15.6 Medical Device Application: Protesis

Sanróman et al. (1991) observed that xylitol is a suitable compound to prepare ramified polyesters such as hydroxyl-propyl-xylitol, in which mechanical and thermo-physics properties were similar to those found in polyurethane, with the

advantages of being less deformable and absorbing less water than the conventional materials used in traumatology.

Recently, Bruggeman et al. (2010) observed that an elastomer containing xylitol presented an increased biocompatibility when compared to an elastomer containing L-lactic-*co*-glycolic acid in rat assays.

15.7 Hemolytic Anemia

A long-term experiment performed using rabbits proved that 5 % xylitol infusion could be protective from acute hemolysis induced by acetyl-phenylhydrazine, while it also conserved and reduced glutathione levels (Wang et al. 1971; Ukab et al. 1981). Moreover, the plasma and whole blood glucose was not altered for 6 h after a single xylitol injection (0.5 g/kg body weight) in patients with glucose-6-phosphate dehydrogenase (G6PD)-deficiency. In patients with G6PD-deficiency, this enzyme may also be decreased in other organs and cells. The NADP-linked xylitol dehydrogenase has been found in almost all mammal organs. Therefore, xylitol's potential applicability cannot be limited to the erythrocyte (Wang et al. 1971). Xylitol's anti-hemolytic effect is likely accomplished through NADPH generation, which maintained glutathione (GSH) level and protected hemoglobins and other structural and functional proteins against peroxidative damage.

Chronic congenital hemolytic anemia or anemia induced by drugs or infection is associated with G6PDH enzyme deficiency. The enzyme G6PDH is responsible for maintaining an adequate reduced coenzyme NADPH level and is required for glucose metabolism. A deficiency of this enzyme promotes the reduction of erythrocytes. Xylitol is a therapeutic agent that can be used by people with G6PDH deficiency, since this enzyme is not required for its metabolism. Therefore, xylitol supplied the cell with $NADPH_2$ through L-xylulose oxidation, maintaining the erythrocyte membrane integrity (Ylikahri 1979).

15.8 Osteoporosis

Another xylitol application would be in osteoporosis treatment or prevention. Xylitol acts by promoting a bone mass increase, preserving the minerals contained then and preventing its biomechanical properties attenuation. It occurs through xylitol's ability to stimulate calcium absorption through the intestine and its ease of passage from blood to bones. Bone calcium is increased, reducing the resorption need (Mattila et al. 1998, 1999). Mattila et al. (1999) supplemented rat diets with 10 and 20 % xylitol and observed that dietary xylitol supplementation in rats improves bone biomechanical properties and increases trabecular bone volume dose dependently.

Despite the studies performed, which until now have only been conducted in animals, Mattila et al. (1998) suggested that a daily dose of approximately 40 g of xylitol in middle-aged people's diets can promote an osteoporosis-preventing effect, besides being well tolerated by the body.

15.9 Inflammatory Processes

Takahashi et al. (1999) observed that xylitol was effective in preventing body weight loss after immunological stimulation without affecting some immune responses such as plasma interleukin-1 activity when chicks were fed with 15 % xylitol for 10 days ad libitum prior to immunological stress. Additionally, these authors (Takahashi et al. 2000) observed that an administration of 6 % xylitol 1 day before the immunological challenge was also effective in the maintenance of immunological factors such as nitric oxide levels.

Ackermann et al. (2004) observed that the administration of a saline solution containing xylitol as an osmotic agent suppressed the immune system mediators m-RNA expression, such as interleukin-8 and defensins (SBD-1 and SBD-2), in sheep lungs infected with *Mannheimia haemolytica*.

Han et al. (2005) demonstrated that xylitol inhibited lipopolysaccharide (LPS)-induced gene expression and protein synthesis of tumor necrosis factor alpha and interleukin 1 beta in immunological cells. Xylitol also inhibited LPS-induced nuclear factor kappa B activation in RAW 264.7 cells stimulated with *Porphyromonas gingivalis* LPS.

Recently, Ferreira et al. (2011) observed that 5 % xylitol can reduce J774A.1 macrophage adhesion to a glass surface in vitro. Macrophage adhesion to a tissue could be a desired situation, e.g., in wound healing. However, when an inflammatory process takes place, macrophage adhesion to a tissue leads to a deleterious action.

15.10 Other Medical Applications of Xylitol

Polyols such as xylitol, sorbitol, mannitol and inositol, or combinations of them, can be used in ophthalmic products to lower intraocular pressure (by topical application or solution) (Franz et al. 1994). Franz et al. (1996) patented a topical solution containing xylitol to reduce intraocular pressure. Moreover, xylitol is the osmotic agent of a therapeutic preparation for antimicrobial treatment for the eye area (Merck Sharp and Dohme 1993).

An antipyretic composition being orally administrated or by another route, containing an antipyretic compound (2–100 mg) and xylitol (0.5–15 g) was patented (Wills and Bernard 2006). The role of xylitol is to help in body temperature maintenance after its reduction through antipyretic action. Xylitol was also patented for its use in fever control in nonhuman mammals, such as baby sheep (Jerry and Alonzo 2005).

Table 15.2 Summary of xylitol medical applications

Xylitol medical application	References
Acute otitis media	Uhari et al. (1996), Kontiokari et al. (1995, 1998), Danhauer et al. (2010)
Cystic fibrosis	Welsh and Zabner (2004), Durairaj et al. (2007)
Caries prevention and treatment	Amaechi et al. (1998), Iwata et al. (2003), Sano et al. (2007), Silva et al. (2010b), Hildebrandt et al. (2010)
Parenteral nutrition	Ylikahri (1979), Georgieff and Oehmke (1990), Georgieff et al. (1991)
Calories controlled diet	Shafer et al. (1987), King et al. (2005), Amo et al. (2011), Islam (2011)
Diabetes	Valero et al. (2011)
Atopic dermatitis	Katsuyama et al. (2005a, b)
Leishmaniasis	Ferreira et al. (2008)
Wound	Ammons et al. (2009, 2011), Korponyai et al. (2011)
Anti-aging property	Mattila et al. (2005)
Gastrointestinal usage	Naaber et al. (1996), Silva et al. (2010a, 2011)
Protesis	Sanróman et al. (1991), Bruggeman et al. (2010)
Hemolytic anemia	Wang et al. (1971), Ukab et al. (1981), Ylikahri (1979)
Osteoporosis	Mattila et al. (1998, 1999)
Inflammatory process	Takahashi et al. (1999, 2000), Ackermann et al. (2004), Han et al. (2005), Ferreira et al. (2011)
Intraocular pressure reduction	Franz et al. (1996)
Antipyretic action	Jerry and Alonzo (2005)

15.11 Summary of Medical Applications of Xylitol

The wide variety of xylitol applications presented in this chapter, discovered over the past 30 years, demonstrates its potential in the clinical area. Table 15.2 summarizes a few of them. Xylitol seems to be safe and effective as indicated in many studies. Maybe it is due to its chemical structure or maybe it is due to its physical properties—it is not known yet. However, because of the continuous scientific interest in xylitol, it can be expected that in the next few years other new medical uses will be attributed to xylitol.

Considering the above, through a great number of studies already completed, it can be observed that xylitol may provide benefits to human health and, therefore, it can be used to develop medications/products in pharmaceutical and medical areas. These types of studies should being conducted in order to make xylitol an appropriate biomolecule to be used for prevention and/or treatment of a series of diseases.

15.12 Conclusion and Future Recommendations

Xylitol medical applications should be constantly studied, since it can be used in a variety of important diseases. It is a safe, non-immunogenic compound, which can be obtained by a fermentative process using low-cost raw material. Especially due

to its microbial anti-adherent property, xylitol could be incorporated in a great variety of microorganism growth control products, such as antibiotics, antiseptics and disinfectants, that are suitable in products for hospital cleaning and infection control and therapeutics.

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