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Review

Bioconversion of pentose sugars into ethanol: A review and future directions

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Hemicelluloses, the second most abundant polysaccharide in nature, are well suited for ethanol production due to their enormous availability, low cost and environmental benign process. The major fraction in hemeicelluloses is pentosans and the conversion of pentosans to ethanol is problematic. To get the process economized, the conversion of hemicellulose to ethanol with a satisfactory yield is necessary. In recent years, significant advances have been made towards the technology of pentosans to ethanol conversion. However, there are technical and economical impediments to the development of commercially viable processes utilizing hemicellulosic derived sugars. This article provides an overview of the new insights in pentose sugars conversion into ethanol, pentoses resources, microorganisms and the technology.

Key words: Hemicellulose, bioethanol, pentose sugars, yeasts, fermentation.

INTRODUCTION

Lignocelluloses are the most abundant organic mass in the biosphere, which accounts for approximately 50% of the biomass. In nature the annual production of biomass is estimated to 10 to 50 × 10^9 tons (Chandel et al., 2010a). They get generated in large concentration through activation of agricultural, forestry, fruits and vegetable processing operation as residual waste.

The plant cell wall is composed mostly of the polymers cellulose, hemicellulose, and lignin (Figure 1). Lignocellulosic raw materials, by virtue of their structures, are relatively refractory to direct degradation followed by microbial bioconversion. Hemicelluloses constitute an important fraction of lignocelluloses and the conversion of hemicelluloses into fuel ethanol with utmost yields is the deciding factor for the overall economization of the process (Chandel et al., 2010b). Hemicellulose unlike cellulose is heteropolymer comprised of xylose linked backbone branched with other sugars like arabinose,mannose, galactose and glucose etc (Figure 2).

For bioconversion, pretreatment of such material is necessary. Several approaches including physical, thermal, chemical, biological or combination of these are being explored in order to obtain polymeric components of the lignocellulosic materials in maximum yield and purity (Moiser et al., 2005; Chandel et al., 2007a,b). The resulting hydrolysate after pre-hydrolysis and hydrolysis contain varying amounts of monosaccharides, both pentoses and hexoses and broad range of substances as reaction by-products from sugar and lignin degradation. Many of these substances have an inhibitory effect on the microorganism in subsequent fermentation steps (Balat and Balat, 2009; Chandel et al., 2007c).

The pretreatment process can remove hemicellulose, reduce cellulose crystallinity and increase the porosity of the materials (Saha, 2003; Moiser et al., 2005). Among all the pretreatment methods, dilute acid pretreatment

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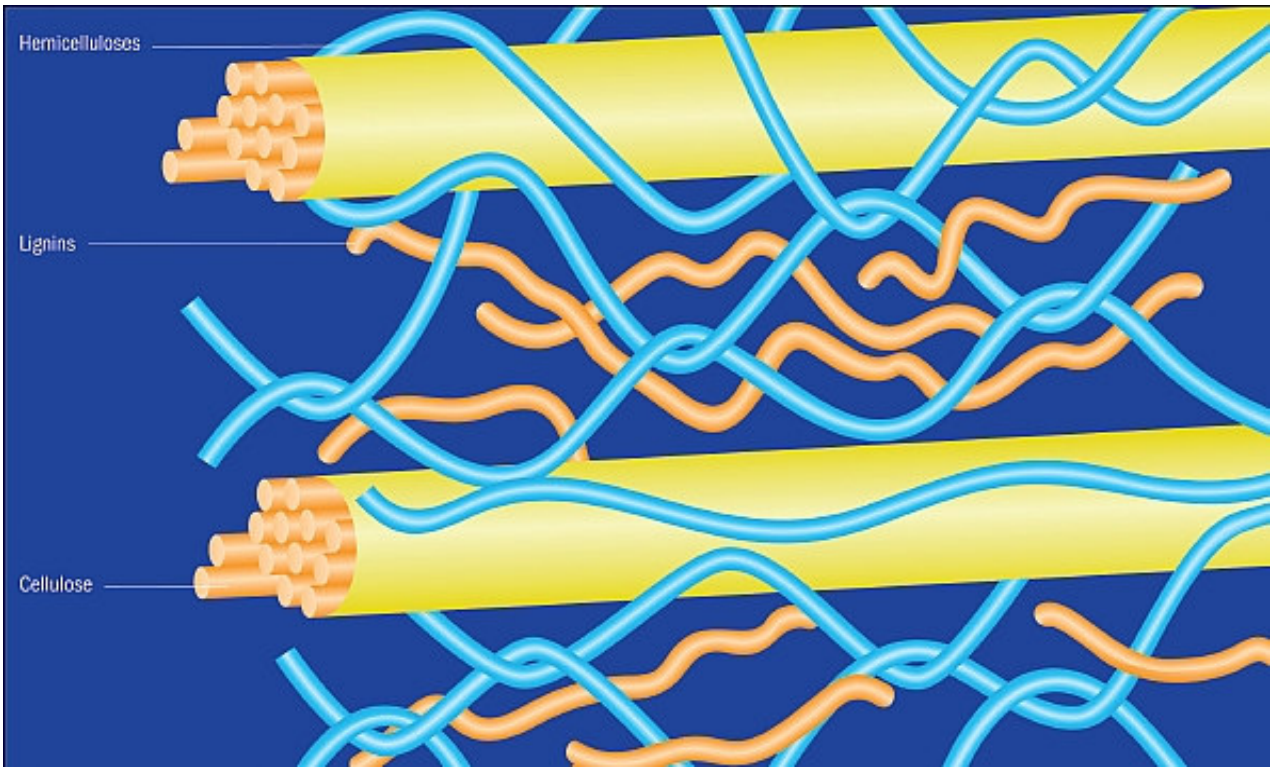

Figure 1. A schematic of a plant cell wall showing cellulose fibrils (brown) laminated with hemicellulose (turquoise) and lignin (orange) polymers (Source: www.scidacreview.org).

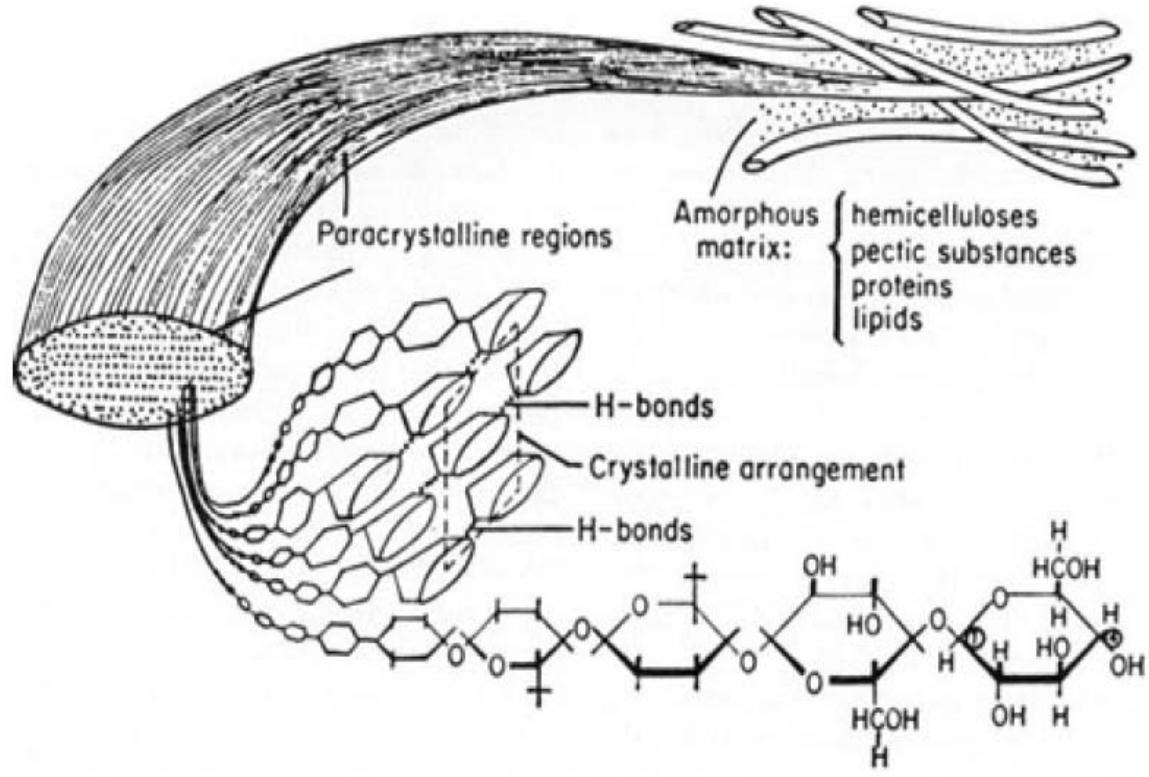

Figure 2. Schematic representation of basic plant cell wall structure showing hemicellulose arrangement (Source: www.uky.edu/~dhild/biochem).

Table 1. Cell wall composition among various lignocellulosic sources considered for biofuel (% of dry material) (Source: Chandel et al., 2010a).

Lignocellulosic source	Cellulose	Hemicellulose*				Lignin	Reference
	Glucan	Xylan	Arabinan	Mannan	Galactan		
Sugarcane bagasse	40.2	22.5	2.0	0.5	1.4	25.2	Neureiter et al., 2002
Wheat straw	32.1	19.5	2.8	0.6	1.1	20	Mani et al., 2006
Corn stover	37.5	21.7	2.7	0.6	1.6	18.9	Ohgren et al., 2006
Switch grass	34.2	22.8	3.1	0.3	1.4	19.1	Mani et al., 2006
Pine wood	44.8	6.0	2.0	11.4	1.4	29.5	Wayman and Parekh, 1990
Aspen wood	48.6	17.0	0.5	2.1	2.0	21.4	Wayman and Parekh, 1990
Spruce wood	41.9	6.1	1.2	14.3	1.0	27.1	Hayn et al., 1993
Saccharum spontaneum	36.81 ± 0.13	21.53 ± 0.04	2.16 ± 0.01	0.16 ± 0.04	0.72 ± 0.01	20.03 ± 0.12	Scordia et al. 2010
Salix wood	41.5	15.0	1.8	3.0	2.1	25.2	Wayman and Parekh, 1990
Douglas fir wood	46.1	3.9	1.1	14.0	2.7	27.3	Robinson et al., 2002

Source: Chandel et al., 2010a *Total hemicellulose amount present in lignocellulosics on the basis of percentage of dry material - Sugarcane bagasse, 27.5; Switch grass, 30; Corn stover, 26.8; Wheat straw, 50; Pine, 26; Aspen, 29; Spruce, 26; Birch wood, 23; Salix wood, 21.7; Douglas fir wood, 20.3

has been widely studied because it is highly active, inexpensive and easy to perform. The dilute acid pretreatment can effectively solubilize hemicellulose into monomeric sugars (arabinose, galactose, glucose, mannose and xylose) and soluble oligomers, thus improving cellulose conversion. The resulting pentose sugars can eventually be utilized for ethanol generation to improve the overall utilization efficiency of lignocelluloses (Moiser et al., 2005).

The bioconversion process would hold more promises of both hexoses and pentose sugars from lignocellulosic materials. The fermentation technology using bacteria and yeast to produce chemicals from hexoses is well known but the information of the ability of these organisms to ferment pentose sugars is somewhat scanty. The ability to ferment pentoses is not widespread among microorganism and most promising yeast species, identified so far, are *Candida shehatae Pichia stipitis* and *Pachysolen tannophilus* (Hahn-Hagerdal et al., 2007; Chandel et al., 2008a). To meet the suitability of these strains at industrial level, many efforts have been made to construct the choice of strain by cloning and further expression of pentose utilizing genes into common hosts such as *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Escherichia coli* etc (Jeffries, 2006; Hahn-Hagerdal et al., 2007). However it still remains a challenging issue to get the suitable strain fulfilling the requirements of ethanol production from lignocelluloses at industrial level (Zhang et al., 2010).

Rather than summarizing all of the existing literature on pentose sugar conversion into ethanol, the present article deals with the crucial parameters for developing an indigenous pentose sugars fermentation technology. Here we review the methods of hemicelluloses hydrolysis and the conversion of pentose sugars into ethanol, microorganisms for ethanol production, methods used for strain improvement and the future needs for making this sustainable technology successful at large scale.

HEMICELLULOSES: SOURCES AND STRUCTURE

The name hemicelluloses are given to those polysaccharides, which would be extracted from plants by diluted mineral acids. Hemicelluloses are embedded in the cell walls of plants, sometimes in chains that form a 'ground' - they bind with pectin to cellulose to form a network of cross-linked fibres.

Unlike cellulose, hemicelluloses (also a polysaccharide) consist of shorter chains 500 - 3,000 sugar units as opposed to 7,000 - 15,000 glucose molecules per cellulose polymer (Saha, 2003; Chandel et al., 2010a). In addition, hemicellulose is a branched polymer, which is composed of both linear and branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid (Figure 2). These individual sugars may be acetylated or methylated. Most hemicelluloses from hardwoods have average degrees of polymerization (DP) of 150 - 200. Most

hemicelluloses are based on 1,4-β-linkage of their backbone sugars. The amount of hemicelluloses in wood ranges from 20 to 30% (Abbi, 1996a; Saha, 2003). There are characteristic differences in the composition and structure of hemicelluloses in agricultural residues, soft wood and hard wood. Table 1 summarizes the composition of different hemicellulosic fraction of various lignocellulosics.

CONVERSION OF HEMICELLULOSE INTO FERMENTABLE SUGARS

Hemicellulose is a heteropolymer which has a random, amorphous structure with little strength. It can be de-polymerized into simple form of fermentable sugars by dilute acid and enzymatic hydrolysis (Saha, 2003; Chandel et al., 2010a). A mild pretreatment with dilute acid or base prior to enzymatic hydrolysis could be effective for better hemicelluloses saccharification into fermentable sugars.

This reduces the crystallinity of the biomass and makes it more amenable to further coordinated enzymatic reactions (Howard et al., 2003; Taherzadeh et al., 2007b). In past, various pretreatment strategies with dilute acid, alkali, ammonia fiber explosion, hydrogen peroxide, steam explosion, wet oxidation, liquid hot water, sodium sulfite etc., have been reviewed (Moiser et al., 2005; Taherzadeh et al., 2007b).

Auto hydrolysis and dilute acidic hydrolysis

Auto hydrolysis is a simple method to break down the hemicellulosic backbone into monomeric sugar constituents with fewer by-products (Abbi et al., 1996b; Sun and Cheng, 2003). However, a significant fraction of hemicellulosic may remain with the substrate after the auto hydrolysis. To overcome from this problem, dilute acid hydrolysis is used for maximum degradation of hemicelluloses into monomers (Saha et al., 2005). Compared to other pretreatment methods, dilute acid hydrolysis is more useful for the conversion of maximum hemicellulosic fraction into xylose and other sugars, which can be fermented into ethanol by specialized microorganisms (Chandel et al., 2007a; Saha, 2003). Cell wall structure and components may be significantly different in different plants, which may influence the digestibility of the biomass (Hopkins, 1999). A broad discussion about dilute acid hydrolysis on a variety of lignocellulosics with respective sugar recovery has been reviewed by Taherzadeh et al. (2007a).

Formation of inhibitors during auto/acid hydrolysis and their removal

During acid hydrolysis of lignocellulosics, aliphatic acids (acetic, formic and levulinic acid), furan derivatives and phenolic compounds are formed in addition to the sugars. Furfural and 5-hydroxymethyl furfural (HMF) collectively known as furans are formed by decomposition of pentoses and hexoses respectively (Chandel et al., 2007c). Acetic acid or other weak acids are generated due to the hydrolysis of the acetyl groups or other linkage present in hemicellulosic backbone (Palmqvist and Hahn-Hagerdal, 2000).

To improve the efficiency of hydrolysate fermentation, these inhibitors are required to be removed prior to fermentation. Several detoxification methods such as neutralization, calcium hydroxide overliming, use of ion exchange resins, activated charcoal or tin oxides, enzymatic detoxification such as peroxidase and laccase have showed promising results (Chandel et al., 2007c; Palmqvist and Hahn-Hagerdal, 2000). To make the process more economic, it is necessary to either remove these fermentation inhibitors by less expensive methods or use less severe conditions for hemicellulose break-down such as auto hydrolysis coupled with enzymatic degradation (Chandel et al., 2010b).

Enzymatic hydrolysis of hemicelluloses

Hemicellulases are multi-domain proteins generally containing structurally discrete catalytic and non-catalytic modules (Prates et al., 2001). The most important non-catalytic modules consist of carbohydrate binding domains (CBD), which facilitate the targeting of the enzyme to the polysaccharide, interdomain linkers and dockerin modules. The dockerin modules mediate the binding of the catalytic domain via cohesion-dockerin interactions, either to the microbial cell surface or to enzymatic complexes such as the cellulosome (Prates et al., 2001; Shallom and Shoham, 2003).

The coordinated action of hemicellulases is necessary to obtain the satisfactory yield of pentose sugars from lignocellulosic materials. Therefore, the development of low-cost and high effective hemicellulases is required for cellulosic ethanol production (Howard et al., 2003).

BIOCONVERSION OF PENTOSE SUGARS INTO ETHANOL

For the conversion of pentose sugars into ethanol, a lot of research reports are available in public domains which decipher the process well at laboratory scale. However, it is still challenging to get the desired yields of ethanol utilizing pentose sugars at large scale (Hahn-Hägerdal and Pamment, 2004). The reason is the unavailability of robust ethanol producing microorganisms from pentose sugars which could withstand against fermentation inhibitors and produce ethanol with high yields and productivities.

Figure 3 systematically shows the metabolic conversion

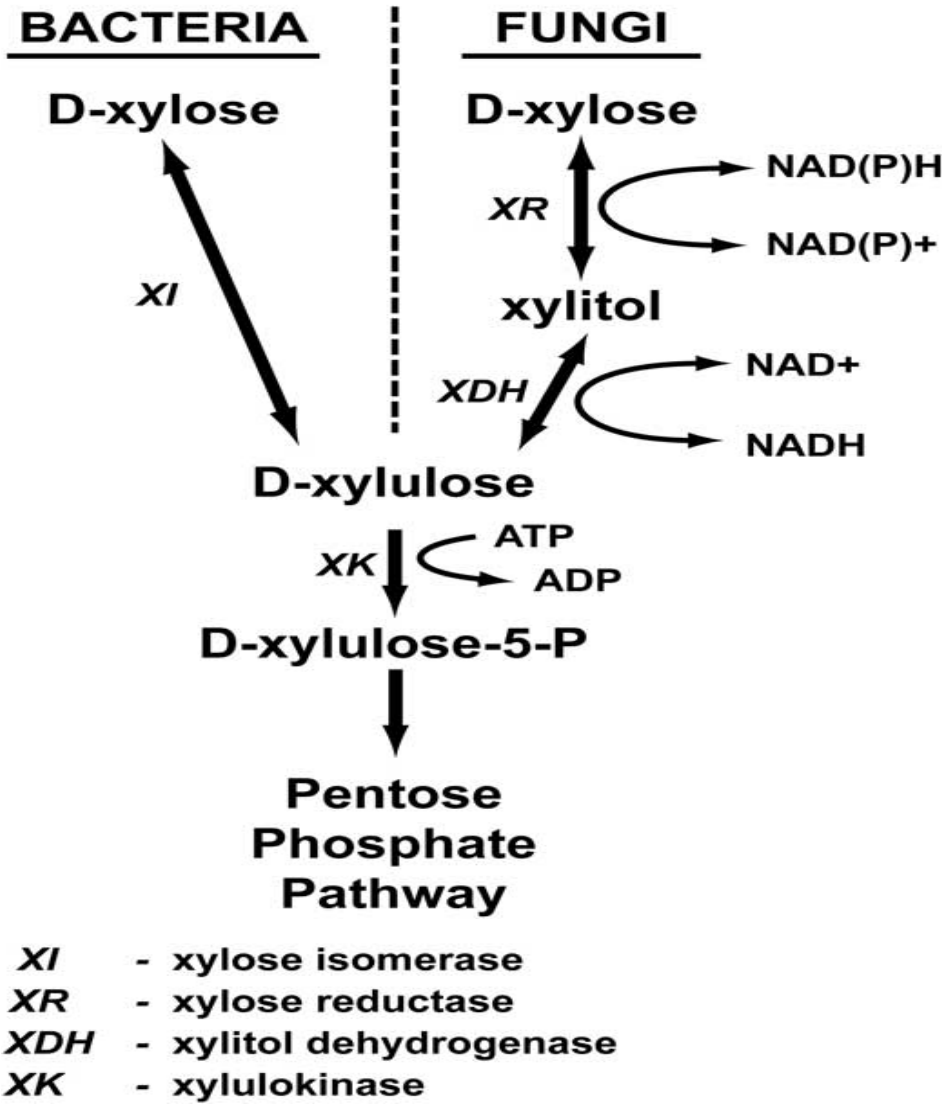

Figure 3. A depiction of xylose metabolic pathways (Source: Hector et al., 2008).

of pentose sugars in bacteria/fungi. D-xylose is first reduced to xylitol by D-xylose reductase (aldose reductase EC 1.1.1.21) and subsequently oxidized to D-xylose by xylitol dehydrogenase (D-xylose reductase, EC 1.1.1.9) to form D-xylose–5-phosphate. Ribolosephosphate-3-epimerase (5.1.3.1) transaldolase (EC 2.2.1.2) and transketolase (EC 2.2.1.1) sequentially convert alpha–xylose-5-phosphate into glyceraldehyde-3-phosphate and fructose-6-phoshate by non-oxidative rearrangement leading to ethanol formation by EMP (Emden-Meyorhoff Pathway). Alternatively, phosphoketolase (EC 4.1.2.9) can split D-xylose–5-phosphate into glycerealdehyde-3-phosphate and acetyl phosphate. Phosphoketolase is known to be important in lipid – producing yeast, especially when they are grown on D-xylose.

In some D-xylose fermenting yeasts, an oxidative pentose phosphate mechanism is present to metabolise D-xylose. Fructose–6-phosphate is oxidized to ribulose–5-phosphate. In this step, CO_2 is released in conjunction with the generation of NADPH. The pathway or its associated steps depend on the metabolic capacities of particular yeast and the growing conditions (Dien et al., 2003). In general, fermentative yeasts possess both aerobic and anaerobic pathways along with adaptive regulatory mechanisms. Some yeasts posses only aerobic pathways. However they can metabolize D-xylose anaerobically also.

D-xylose-fermenting yeasts require oxygen for growth, irrespectively the carbon source employed. This requirement for oxygen could indicate the use of fermentation pathway that does not give rise to sufficient ATP by substrate –level phosphorylation. In fermentation reaction, ethanol can be used as a carbon source after its

Table 2. Growth characteristics of natural pentose-fermenting microorganisms.

Microorganisms	Glu	Xyl	Ara	Man	Cel	Temperature range (°C)	pH range
Filamentous fungi							
Fusarium oxysporum	+	+	+	+	+	28 - 32	5 - 6
Neurospora crassa	+	+	-	-	+	28 - 37	5 - 6
Monilia sp.	+	+	-	-	-	26	5
Mucor sp.	+	+	-	-	-	30	5.4
Yeast							
Saccharomyces cerevisiae	+	-	-	+	-	30 - 35	3 - 7
Klyuvermyces marxians	+	+	+	+	-	30 - 35	3 - 7
Pachysolen tannophilus	+	+	+	-	-	28 - 32	2.5 - 7
Candida shehatae	+	+	+	+	-	28 - 32	3 - 7
Pichia stiptis	+	+	+	+	-	28 - 32	3 - 7
Mesophilic bacteria							
Bacillus polymyxa	+	+	+	+	-	35 - 37	5.5 - 8
Aerobacter hydrophila	+	+	+	+	-	35 - 37	5.5 - 8
Klebsiella pneumonia	+	+	+	+	-	35 - 37	5 - 6
Clostridium acetobutylicum	+	+	+	+	+	35 - 37	4 - 8
Thermophilic bacteria							
Clostridium thermocellum	+	+	+	-	+	65	4 - 8
C. thermohydrosulfuricum	+	+	+	-	-	65	4.7 - 8
C. thermosaccharolyticum	+	+	+	+	-	60	5 - 8
C. thermosulfurogenes	+	+	+	+	-	60	4.5 - 7.5
Thermoanerobacter ethanolicus	+	+	+	+	-	69	4.4 - 9.5

Glu-glucose Xyl-xylose Ara-arabinose Man-mannose cel-cellulose.(Source: Abbi, 1996a).

formation further to yield cell mass, CO_2 and acetate, even in the presence of substantial xylose concentrations (Abbi et al., 1996b). It has been observed that during xylose fermentation in aerobic conditions, ethanol production increases sharply followed by its declination and hike in biomass yields (Abbi et al., 1996b; Chandel et al., 2007c). However, ethanol uptake is not observed in anaerobic xylose fermentation, where the tricarboxylic acid (TCA) cycle is not operational.

PENTOSE FERMENTING MICROORGANISMS: AN ASSET IN BIO-REFINERY

During last decade, a number of laboratories have demonstrated the utilization of pentose sugars using different strains of yeast, molds and bacteria into ethanol alongside the production of acids, polyols and other by-products during bioconversion at diverse pH and temperature conditions (Table 2).

Ethanol production from different lignocellulosic sources depends upon many factors like the initial sugarconcentration in the hydrolysate, strain used for fermentation, presence of the inhibitory compounds and the employed cultivation conditions. Table 3 shows the ethanol production profile from different lignocellulosics using the different pentose utilizing yeasts.

Filamentous fungi

The ability of filamentous fungi to ferment pentose sugars has been known about 70 years. Several fungal species belonging to genera *Fusarium* (Singh and Kumar, 1991), *Rhizopus* (Perlman, 1950), *Monilia* (Gong et al., 1983), *Neurospora* (Deshpande et al., 1986) and *Paecilomyces* (Wu et al., 1986) were found to have potential for fermenting glucose as well as xylose.

In most fungi, the initial conversion of D-xylose and L-arabinose to D-xylulose 5-phosphate proceeds through a series of reduction and oxidation steps involving the cofactors $NAD(P)^+$/NAD(P)H (Hahn-Hagerdal et al., 2007). Among the ethanol producing fungi, *Fusarium oxysporum* has shown more ethanol production than *Neurospora crassa* and *Mucor sp* (Singh et al., 1992). Recently, Xiros and Christakopoulos (2009) explored *F. oxysporum* for ethanol production from brewer's spent grain (BG) under consolidated system. An ethanol yield of 109 g ethanol per kg of dry BG was

Table 3. Ethanol production from pentose sugars solution derived from lignocellulosics.

Lignocellulose material	Hydrolysis conditions	Released sugars (g/l)	Detoxification	Microbial strains	Ethanol yield (g/g)	References
Sugarcane bagasse	(2.5% (v/v) HCl, 140℃, 30 min), # Solid: liquid = 1:10	30.29	Overliming + ion exchanger	*C. shehatae* NCIM3501	0.48	Chandel et al., 2007c
De-oiled rice bran	3.5% v/v H_2SO_4, 120℃, 60 min	38.50±0.45	Overliming	*P. stipitis* NCIM 3499	0.42 ± 0.021	Chandel et al., 2009b
S. spontaneum	1.5% H_2SO_4, 15 min, 160ºC	32.15±0.22	Overliming	*P. stipitis* NCIM 3498	0.36 ± 0.011	Chandel et al., 2010c
Rice straw	Presoaking with 0.5% of H_2SO_4 for 18 h followed by steam heating at 15 bar pressure for 10 min, Solid: liquid = 600 g : 4 liter of 0.5% H_2SO_4	228 g sugar/ Kg of substrate	Overliming	*Mucor indicus*	0.24	Karimi et al., 2006
Wheat straw	(90℃, 1.85% (w/v) H_2SO_4, 18 h), Solid: liquid= 1:20	17.10	Overliming	*P. stipitis* NRRL Y-7124	0.41 ± 0.01	Nigam, 2001
Corn cob	(0.3 M H_2SO_4, 98℃ , 1 h) Solid: liquid = 1:12	45.0	Overliming +ZSM-39 shaking	*P. stipitis*	0.44	Saracoglu-Eken and Arslan, 2000
Eicchornia crassipies	(1% v/v) H_2SO_4, 7 h), Solid: liquid = 1: 8	67.5	Overliming + sodium sulfite	*P. stipitis* NRRL Y-7124	0.35	Nigam, 2002
Pine	Sulfur dioxide (30 min, 160℃ + (225℃, 30 s, HCl equivalent to 1% of dry weight), Solid: liquid = 1: 3	72	Overliming + sodium sulfite	*E. coli* K011	0.43	Barbosa et al., 1992
Willow	Steam [1 bar pressure, soaked with gaseous SO, (1 g SO_2/100 g willow) 6 min, 206 ℃], Solid: gas = 100:1 g	9.0	Overliming + sodium sulfite	*E. coli* K011	0.51	Olsson et al., 1995
Mixed wood	# # Acid hydrolysis	70.4	Electrodialysis + Sodium hydroxide	*C. shehatae* FPL-Y-049	0.48	Sreenath and Jeffries, 2000

Table 3. Contd.

Paja brava	Pre-steamed, dilute sulfuric acid (0.5% or 1.0% by wt),at temperatures between 170-230°C for 3 and 10 min. Solid: liquid = 1: 10	22.2	No Detoxification	*P. stipitis* CBS 6054	0.20	Sanchez et al., 2004

(Source: Chandel et al., 2010a) # Solid: liquid (Lignocellulose substrate: dilute acid solution) ## Details are not available.

obtained with alkali-pretreated BG under micro-aerobic conditions (0.01 vvm), corresponding to 60% of the theoretical yield based on total glucose and xylose content of BG.

Few fungal strains have been identified that ferment not only glucose and xylose but also other complex natural cellulosic substrates which is an advantage to get the maximum yield of ethanol (Singh et al., 1992). However, some critical factors such as their long generation and fermentation time, low tolerant to substrate and products and secretion of organic acids make them unattractive for ethanol production.

Bacteria

In bacteria, D-xylose and L-arabinose are isomerized to D-xylulose and L-ribulose, respectively (Chen, 1980) followed by their phosphorylation to D-xylulose 5-phosphate (Anderson and Wood, 1962) and to L-ribulose5-phosphate (Lee and Bendet, 1967), respectively. It is then epimerized to D-xylulose 5-phosphate (Lee et al., 1968), linking with the central metabolism through the pentose phosphate pathway (PPP). Watanabe et al. (2006) deciphered the alternative arabinose utilization pathway in bacteria. A review written by Senthilkumar and Gunasekaran (2005) describes ethanol production ability of wild and genetically engineered bacterial strains for ethanol production from cellulosic substrates under integrated process configurations.

Unlike yeast and filamentous fungi which do not ferment pentoses anaerobically, bacteria can readily convert xylose to ethanol under anaerobic conditions (Rosenberg, 1980; Sommer et al., 2003). These include *Bacillus macerans, Bacillus polymyxa, Kiebsiella pneumoniae, Clostridium acetobutylicum, Aeromonas hydrophila, Aerobacter* sp., *Erwinia* sp., *Leuconostoc sp* and *Lactobacillus* sp. (Gong et al., 1983; Detroy and Bolen, 1983; Abbi, 1996a). These microorganism convert pentose sugars into ethanol along with undesired by-products like acetic acid, lactic acid, 2, 3-butanediol and CO_2.

In addition to mesophillic bacteria, thermo-tolerant bacterial species such as *Clostridium thermocellum*, *Clostridim thermo hydrsulfurium, Clostridim thermosaccharolyticum, Clostridim thermosulfurogenes* and *Thermo anaerobacteretahnolicus* (Rosenberg, 1980; Sonneitner, 1983) can also produce ethanol. Sommer and colleagues (2003) carried out a mass screening to get thermophillic anaerobic bacteria for their ability to produce ethanol from paper pulp mills, hot springs and brewery waste water.

Due to low substrate and ethanol tolerance, pentose sugars utilizing ethanologenic bacteria are getting less attention. Though they grow fast relatively to fungi and yeast, but due to their low substrate specificity they are not considered right choice for ethanol production at large scale from pentose sugars (Senthilkumar and Gunasekaran, 2005).

Yeast

Yeasts are the most suitable microorganisms for ethanol production. They have been the center of attraction for bioethanol research since the dawn of biorefinery principle. They can grow on variety of sugars with high substrate and ethanol tolerance. However, they grow preferably on hexose sugars but few strains are also capable of utilizing pentose sugars and conversion into ethanol (Dien et al., 2003; Hahn-Hagerdal et al., 2007; Zhang et al., 2010). Among the pentose utilizing yeasts, microorganisms such as *P. tannophilus*, *C. shehatae*, *P. stipitis* and *Kluveromyces maxianus* have been discussed in detail for the ethanol production. Other yeast investigated for their xylose-fermenting ability include *Brettanomyces*, *Clavispora*, *Schizosaccharomyces*, other species of *Candida* viz., *Candida tenius*, *Candida tropicalis*, *Candida utilis*, *Candida blankii*, *Candida friedrichii*, *Candida solani* and *Candida parapsilosis* and species of *Debaromyces* viz., *Debaromyces nepalensis* and *Debaromyces polymorpha* (Abbi, 1996a). Gong et al. (1983) have tested 20 species of *Candida*, 21 strains of *Saccharomyces* and 8 strains of *Schizosaccharomyces* for their ability to ferment D-xylose. Xylitol was produced in the range of 10 - 15% by *Candida* strains with arabitol as the second major product. Ethanol was the major product with most of the *Candida* stains.

Schizosaccharomyces strains have also been reported to produce ethanol but the

concentrations are low (1-5 gl). Maleszka and Schneider (1982) screened 15 yeast strains for their ability to utilize D-xylose, D-xylulose and xylitol for ethanol production under aerobic, microaerobic (low aeration) and anaerobic condition using rich undefined or defined media. In almost all cases, ethanol production by *P. tannophilus* and species belonging to *Candida* and *Pichia* was better on rich media under microaerobic conditions. Majority of yeasts are not able to ferment D-xylose directly. It has been observed that instead they utilize D-xylulose, an isomer of D-xylose, both oxidatively and fermentatively. The best xylulose-fermenting yeasts so far identified are species of *Btrettanomyces*, *Candida*, *Hansenula* and *Torulospora* (Skoog and Hahn-Hagerdal, 1988). Pentose utilizing yeasts also convert xylose into xylitol in addition to ethanol (Jeffries, 1982, 1984). Xylitol, for example, is generally a poor substrate, though it is an essential intermediate in D-xylose catabolic pathway. Pentiols are the major by-products of pentose fermentation with *C. shehatae*. However, important yeast, *P. stipitis* apparently produces no xylitol during pentose sugar fermentation (Nigam, 2001) which is a unique quality of this microorganism.

STRAIN IMPROVEMENT FOR PENTOSE FERMENTATION

Microorganisms have excelled at producing primary and secondary metabolites from a variety of raw carbohydrates for billions of years under varying cultivation processes. Todaythe results of studying the giant "microbial libraries" currently in vogue for microbial conversion of alternative carbohydrates into value-added products can be applied to ethanol production from cheap and surplus lignocelluloses. In addition, timely interventions, such as strain improvement through mutagenesis, gene cloning and expression and optimization of potential fermentation parameters can enhance the production of ethanol from pentose sugars (Chandel et al., 2008b).

Mutation

Although improving the yeasts strains by mutation is an old process. It has been quite successful at laboratory level. The major drawback with this method is to get the stable ethanol titers from mutagenic strains after every fermentation reaction. In past, multiple studies have shown progress toward strain improvement by mutation (Gong et al., 1983; Jeffries, 1984, 1985; Maleszka et al., 1983).

Candida sp. was mutated by UV mutagenesis that produced five times more ethanol than the native strain due to increased activity of xylulokinase and xylitol dehydrogenase (Gong et al., 1983). Later, Jeffries (1984) observed increased ethanol production from xylose by a mutant of *P. tannophilus* under aerobic conditions.

Based on single gene addition or deletion, various aneuploid and polyploid strains were developed to improve ethanol production from pentose sugars (James and Zahab, 1983; Maleszka et al., 1983a). Ingram et al. (1987) developed the mutants of *E. coli* that over-produced native alcohol dehydrogenase showing the high levels of ethanol.

Protoplast fusion

Protoplast fusion is a conventional but a successful technique for strain improvement. It enables a characteristic advantage of promoting high frequencies of genetic recombination between organisms which are genetically uncharacterized (Rajesh and Tauro, 1992). This technique is considered to be of exceptional importance in the construction of yeast strains, because sexual processes are frequently not available or not effective enough for efficient transfer of genetic information (Yoon et al., 1996).

In the past, protoplast fusion in bacteria and fungi has been reviewed by Peberdy (1980), Hopwood (1981). During the protoplast fusion, a hybrid state of microorganism develops showing the restoration of the genome on chromosomes which lead to further genetic recombination. Thermotolerant *S. cerevisiae* hybrids were developed by Kida et al. (1992) using protoplast fusion. Heluane et al. (1993) developed a protoplast fusant between *P. tannophilus* and S*. cerevisiae* which exhibited the improved utilization of xylose. Recently, protoplasts of thermotolerant *S. cerevisiae* VS_3 and mesophilic, xylose-utilizing *C. shehatae* were fused by electrofusion (Pasha et al., 2007). The fusants were selected based on their growth at 42°C and ability to utilize xylose. The mutant fusant CP11 was found to be stable and showed an ethanol yield of 0.459 ± 0.012 g/g, productivity of 0.67 ± 0.15 g /l/ h and fermentation efficiency of 90%.

Genetic engineering of pentose fermenting yeasts

A strong "degeneration" in selective strains can occur upon the storage of conidial material; therefore, molecular biology-based modern genetics and protein engineering have put forth new avenues to create genetically engineered microorganisms (GEMs) that can function as "booster biocatalysts" (Chandel et al., 2008b; Zhang et al., 2010). Despite the development of numerous industrial recombinant yeasts, GEMs have not been used commercially for ethanol production. Using GEM that can convert xylose and other pentose sugars along with cellulosics derived sugar constituents to ethanol can greatly improve the ethanol production efficiency and reduce the cost of the production.

Zhang et al. (1995) made the construct for xylose assimilation for the bacterium *Z. mobilis* for the effective ethanol yield. Further, Dien et al. (2000) developed a recombinant strain of *E. coli* with the genes from *Z. mobilis* for the conversion of pyruvate into ethanol. The recombinant plasmids with xylose reductase and xylitol dehydrogenase genes from *P. stipitis* and xylulokinase gene have been transformed into *S. cerevisiae* for the co-fermentation of glucose and xylose (Ho et al., 1998). Ingram and his colleagues has done extensive work on the development of efficient recombinant *E. coli* strains for ethanol production, eliminated the dependence on host of alcohol dehydrogenase (ADH) activity by combining *adh* B (coding for ADH II) and *pdc* genes of *Z. mobilis* to form a portable, plasmid borne operon for ethanol production (Ingram et al., 1987; Ingram and Conway, 1988; Beall et al., 1991). A genetically modified strain of *S. cerevisiae* containing the genes for pentose utilization and ligninolytic degradation could be the suitable strain for hemicellulosics derived sugars fermentation. Such strain can be directly used for hemicelluloses hydrolysate fermentation without detoxification by saving the cost of detoxification (Jonsson et al., 1998). Detailed information regarding the development of GEMs has been reviewed by Dien et al. (2003), Hahn-Hagerdal et al. (2007) and Matsushika et al. (2009).

Although bioethanol production from pentose sugars has been greatly improved by newly developed GEMs, there are still challenges that need further investigations. These challenges include maintaining a stable performance in commercial scale fermentation operations (Dipardo, 2000).

IMPROVED METHODS FOR PENTOSE FERMENTATION

To get the desired yields of ethanol from pentoses, essentially improved methods need to be used. Pentose fermentation is a tedious process unlike hexose or molasses fermentation. The choice of strain and other associated approach have an important role for the pentose sugars fermentation to get the desired yields and productivities. A suitable microorganism must be able to survive over a wide range of complex substrates at higher levels of carbohydrate with little or no toxicity of the formed product. Routinely applied methods in fermentation such as use of immobilized cells, adaptation of microorganisms with the lignocelluloses hydrolysates, recycling of free and immobilized cells in fermentation have shown promising results and can be used at industrial level also.

Immobilization of cells

In industry, application of immobilized cells during microbial fermentation is the most important parameter for obtaining the required yields and economizing the process (Williams and Munnecke, 1981; Kourkoutas et al., 2004). Immobilized cell systems are now used industrially for production of fructose syrups from glucose, ethanol and the production of stereo specific amino acids (Chibata and Tosa, 1977). A higher cell mass per unit fermentation volume can be achieved than with batch, continuous or cell recycle system, resulting in a corresponding increase in ethanol production. The risk of contamination is also reduced due to fast dilution rates and high cell densities (Norton and D'Amore, 1994; Chandel et al., 2009a).

A variety of synthetic and natural matrices are available for cell immobilization, synthetic materials such as polyacrylamide, poly urethane foam (PUF) may toxic to the cells, though they exhibit strong mechanical strength. Natural matrix such as alginates and carrageenan, yeast biocapsules and lignocellulosic materials (loofa sponge, pine apple cannary, sugarcane bagasse, *Saccharum spontaneum* pith etc) are non-toxic to cells and were used for yeast cell immobilization for ethanol production (Ogbonna et al., 2001; Peinado et al., 2006; Chandel et al., 2009a). However, their low mechanical strength and increased leaching of cell makes natural gels unsuitable for industrial application (Norton and D'Amore, 1994).

The immobilization of cells of xylose-fermenting yeasts has led to improved ethanol yields. *P. stipitis* cells entrapped in nylon net showed an ethanol productivity of 1 g/l/h compared with an average of 0.22 g/l/h obtained by free cells when grown on 50 g/l xylose (Linko et al., 1986). Immobilization of *P. tannophilus* in calcium alginate beads led to a high ethanol yield of 0.45 g/g initial xylose (Maleszka et al., 1982). Immobilized *C. shehatae* showed an ethanol yield of 0.5 g/g initial xylose from 1% xylose when compared with 0.48 g/g initial xylose obtained with free cells (Abbi, 1996b). Slininger et al. (1985) has reported continuous conversion of D-xylose to ethanol by *P. tannophilus* immobilized in alginate beads.

Recycling of cells

Cell recycling and retention offer important advantages when the process micro-organisms are either slow growing or strongly affected by product inhibition (Nishiwaki and Dunn, 1977). Systems with cell recycling are finding increasing use in ethanol production to increase the productivity of the fermentation process. Cysewski and Wilke (1976) first proposed the use of yeast cell recycling to increase ethanol productivity. Recycling of cells can be performed by means of continuous ultra filtration and microfiltration (Hoffman et al., 1985).

Kargupta et al. (1998) used recycled cells in the case of a continuous membrane ferment or separator with

continuous removal of product by per-evaporation. Abbi et al. (1996b) used the free cells of *C. shehatae* NCIM3501 for ethanol production from rice straw auto-hydrolysate and observed a constant ethanol production (0.44g/g) up to 5 batches.

Adaptation of microorganisms

Often, microorganisms can adapt to a variety of fermentation media. However, multiple essential nutrients along with suitable carbon and nitrogen sources are required for any fermentation reaction (Chandel et al., 2008b).

Adaptation of microorganisms to lignocelluloses hydrolysates is an important parameter to get the improved ethanol yields (Hahn-Hagerdal et al., 1991). It is evident that lignocelluloses hydrolysates contain a variety of inhibitory products (furans, furfurals and weak acids) along with the sugars. Adaptation of fermenting biocatalyst to these hydrolysates prior to using them in fermentation of lignocellulose hydrolysates will be yielding much improved ethanol productivities (Parawira and Tekere, 2010). Another important aspect of the adaptation of microorganisms is to get rid of detoxification step by saving the total cost incurred for ethanol production.

Adaptation of strains of *P. stipitis, C. shehatae* and *P. tanophillus* is expected to overcome such inhibition and improve ferment ability of xylose-containing hydrolyzates (Tran and Chambers, 1986; Parekh al., 1986). Parekh et al. (1987) used an adapted strain of *P. stipitis* on a steam-stripped hardwood hydrolysate which yielded 46% more ethanol, with over 90% xylose utilization than steam-unstripped hardwood hydrolysate. Nigam (2001) used *P. stipitis* NRRL Y-7124 adapted on wheat straw hemicellulosic hydrolysates which showed improved ethanol production (yield, 0.41 ± 0.01 g/g) equivalent to 80.4 ± 0.55% theoretical conversion efficiency.

CONCLUSIONS AND FUTURE PERSPECTIVES

The conversion of hemicelluloses into ethanol with utmost yields is crucial to get the best utilization of lignocelluloses in biorefinery. However, the lack of industrially suitable microorganisms for converting biomass into fuel ethanol has been a major issue for the success of bioethanol industries. The maximum recovery of pentoses from lignocelluloses with less inhibitor concentration and susequently their conversion into ethanol with desired yields and productivities are important issues to make bioethanol program successful at industrial scale. Both the issues have been addressed by scientists in past and research is going on currently in many laboratories in order to get the outcome of the said issues.

To get the maximum hydrolysis of hemicellulosics backbone with less inhibitors concentration, an optimized thermochemical pretreatment having less severity is required. A fine enzyme cocktail comprising hemicellulases, xylanases, pectinases and other ancillary enzymes is necessary to get the maximum sugars in quick time. The most significant issue in front of molecular biologists is to develop the robust strain having the ability to produce ethanol from all the sugars available in lignocellulose hydrolysates with maximum ethanol yields/productivities with minimum cultivation times.

The greatest successes have been in the engineering of pentose utilizing *S. cerevisiae*, *E. coli, Klebsiella oxytoca,* and *Z. mobilis.* Future development for these microorganisms will continue to emphasize increasing inhibitor tolerance, reducing growth factors and improving ethanol productivity by utilizing micro-array and robotic-aided directed evolution technologies.

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