Abstract
This review describes recent research developments on biological conversion of hemicellulosic biomass towards production of xylitol by taking advantage of power of biotechnology. Xylitol is a five-carbon sugar alcohol with established commercial uses in different healthcare sectors and especially as an alternative sweetener for diabetic persons. Xylitol can be synthesized either by chemical hydrogenation of xylose or by fermentation. The precursor xylose is produced from biomass by chemical or enzymatic hydrolysis and can be converted to xylitol primarily by yeast strains which offer the possibilities of economic production by reducing required energy when compared to chemical production. Biomass hydrolysis under an acidic environment is the most commonly used practice and is influenced by various process parameters. Several microbial growth inhibitors are produced during chemical hydrolysis that reduce xylitol production from xylose, a detoxification step is therefore essential. Enzymatic hydrolysis has advantages over chemical conversion although more research is necessary to reduce inhibition due to structural variation from different substrates or plant species. Enzymatic xylitol production is mostly an integral process of microbial species belonging to the Candida genus. Extensive research has been performed to screen for xylitol producing microbial strains as well as to understand microbial metabolism, the xylitol metabolic pathway, cofactor requirements, development of robust recombinant strains, optimization of bioconversion parameters and xylitol production strategies using free and immobilized cells. The imperative role of hydrolysis of xylose containing biomass and subsequent process parameters has major impact on economics of bioconversion. The review identifies ways forward for improved enzymatic xylitol production to compete with current chemical processes.

Key words: Candida, Detoxification, Hemicellulosic material, Hydrolysis, Bioconversion, Xylitol, Xylose.

Introduction
Xylitol is a polyol and a C₅ sugar, also known as wood or birch sugar, obtained from the reduction of xylose. It is a rare sugar that exists in low amounts and is the constituent of many fruits and vegetables, such as raspberries, strawberries, yellow plum, lettuce and cauliflower. Xylitol was first produced from birch trees in 19th century in Finland. It has attracted global interest due to its sweetening power similar to that of sucrose; equivalent to 2.4 kcal.g⁻¹ and laxative nature (145 J.g⁻¹caloric content) (104, 22, 33). Xylitol has applications and potential for at least three types of industries namely food (for dietary especially in confectioneries and chewing gums), odontological (for its anticariogenicity, tooth
rehardening and remineralization properties) and pharmaceutical (for its toothfriendly nature, capability of preventing otitis, ear and upper respiratory infections and its possibility of being used as a sweetener or excipient in syrups, tonics and vitamin formulations). However the major use is for the prevention of dental caries as xylitol inhibits growth of microorganisms responsible for tooth decay (44, 69, 70, 157). In addition, xylitol is accepted for consumption for diabetics and helps in treatment of hyperglycemia as its metabolism is independent of insulin (157). The xylitol market is increasing and at present is estimated to be $340 million yr\(^{-1}\) and priced at $4–5 kg\(^{-1}\).

Currently, xylitol is manufactured at the industrial level by a chemical hydrogenation of the five-carbon sugar D-xylose, in the presence of nickel catalyst at elevated temperature and pressure. This chemical process is laborious, cost and energy intensive. In addition, the process needs expensive refining treatments necessary for xylose production. In order to produce this xylitol in economically and eco-friendly manner, research was initiated for alternative strategies. One of the alternatives is bioconversion of renewable biomass sources which requires hydrolysis followed by bioconversion of xylose from crude hydrolysate to xylitol employing specific microbial strains for fermentation (132, 129, 130).

**Photosynthetic biomass as raw material for xylitol production**

In view of the disadvantages associated with the chemical production of xylitol process such as conversion efficiency, environment impact and energy input parameters research has identified alternative raw materials and production processes. One of the potential alternative raw materials is xylo-oligosaccharides (hemicellulosic materials) from plant biomass; as the annual growth of plant-derived biomass is estimated to be 73.9 terra grams per year on a dry matter basis (54) of which 20-35% is xylose. Biomass material is widespread, abundant, renewable, cost-effective and inexpensive source of polysaccharides which can be used for production of wide variety of biotechnological products including xylitol, these sources include forests, agricultural and agro-industrial residues (Table 1).

**Table 1: Lignocellulosic biomass produced annually in dry mass basis (54)**

<table>
<thead>
<tr>
<th>Crop</th>
<th>Lignocellulosic biomass (Tg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>058.45</td>
</tr>
<tr>
<td>Corn</td>
<td>203.62</td>
</tr>
<tr>
<td>Oats</td>
<td>010.62</td>
</tr>
<tr>
<td>Rice</td>
<td>731.34</td>
</tr>
<tr>
<td>Wheat</td>
<td>354.35</td>
</tr>
<tr>
<td>Sorghum</td>
<td>010.32</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>180.73</td>
</tr>
<tr>
<td>Rye grass*</td>
<td>20.00(^{5})</td>
</tr>
</tbody>
</table>

*Source: Booth et al. (5)

According to estimates, hemicellulose is the second most common polysaccharide available in nature (105) consisting of heterogeneous polymers of hexoses (glucose, mannose and galactose) and pentoses like xylose and arabinose (58). In order to use these materials they must be hydrolyzed into simple monomeric sugars either by chemical or enzymatic methods for fermentation using microorganisms. Several studies on hydrolysis of xylose-rich hemicellulosic materials (Table 2)
have been performed for utilization as substrates for biotechnological xylitol production (78, 129). A variety of plant biomass materials were evaluated as source of raw materials such as corn cobs (129), sugar cane bagasse (14, 129), eucalyptus (146), brewery’s spent grain (12, 78), olive tree pruning (102), soyabean hull (114), palm oil empty fruit bunch fiber (96), and rice straw (65). Residue particle size reduction was performed by grinding for all the pretreatments of lignocellulose residue as it reduces cellulose crystallinity, especially in case of photosynthetic biomass as raw material (134). However, utilization of these resources mainly depends on the degradation of these polymeric materials to simple sugars, with hemicelluloses being important in the overall conversion process (102, 129, 146).

Table 2: Xylan content in different materials

<table>
<thead>
<tr>
<th>Feed stock material</th>
<th>Xylan content (%) dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn stover</td>
<td>22.4</td>
</tr>
<tr>
<td>Corn fiber</td>
<td>16.8</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>21.2</td>
</tr>
<tr>
<td>Switch grass</td>
<td>20.4</td>
</tr>
<tr>
<td>Office paper</td>
<td>12.4</td>
</tr>
</tbody>
</table>

**Hydrolysis methodologies**

Photosynthetic biomass mainly composed of cellulose (34-50%), hemicellulose (19-34%), lignin (11-30%) and smaller amounts of pectin, protein, extractives and ash. Composition of these components differs with the source of plant species, age and growth conditions (4). Among these, cellulose (a homo-polysaccharide of consisting of polymerized D-glucose up to 10,000 or more linked by â-1, 4-glucosidic bonds) forms a skeleton. Hemicellulose is a complex heterogeneous polysaccharide consisting of 200 degree of polymerization composing of glucose, galactose, mannose, xylose, arabinose, glucuronic acid with acetyl side chains. Cellulose is interlinked by hemicellulose to build a structural matrix. This structure is further encrusted with lignin. Lignin, polymer of phenyl propane, is non-polysaccharidic in nature consisting of ñ-coumaryl-, coniferyl- and sinapyl alcohol units bonded by alkyl-, aryl, and combination of both ether bonds. In fact, cellulose, hemicellulose, and lignin are closely associated with covalent cross-linkages, hence, biomass can be regarded as a composite material, in which the lignin serves as a protective layer. In addition, the composition of lignocellulosic materials varies with the biomass material such as hard wood, soft wood and grasses. Because of this, the plant biomass exhibits a remarkable stability against chemical and biological attack and can rarely be converted into simple sugars under normal conditions. Therefore pretreatment is necessary in order to alter the structural integrity, remove the lignin and increase the surface area to make this material available as fermentable sugars (45). Performance of pretreatment depends on selected material harvesting nature, lignin and other components composition, storage type and time, temperature and chemicals used. In general, processes used to produce xylo-oligosaccharides from xylan-rich materials are essentially hydrolytic in nature and can be performed either by chemical means using basic or acidic media, or catalyzed by enzyme sources (78). Since, the scope of this review is limited to xylitol production, detailed information on pretreatment methodologies are dealt very limited.

Chemical hydrolysis is a simple and rapid method for hemicellulosic material however treatment conditions vary with agro-industrial material and with respect to chemical agent type and concentration, incubation temperature and time (129, 134). When aged or fully grown agricultural residues or hardwoods are used as raw materials, xylose is the most abundant sugar for utilization as substrates for biotechnological xylitol production.
in hydrolysates in addition to small fractions of other sugars. For acid hydrolysis different mineral acids such as sulfuric (102, 134, 147), hydrochloric (40), nitric, hydrofluoric (25), acetic acid (17) and phosphoric (27) acids are used at high temperature and pressure (commonly 160°C) and (10 atm). In general, acid hydrolysis performed under concentrated (50–70%) or diluted (below 2%) conditions. Preferences are for diluted acid conditions and high temperatures due to high reaction rates with less microbial growth inhibitors, which is a low cost technology compared to other chemical approaches (46, 101, 102).

The chemical hydrolysis reaction is a complex process (27) that is a multi-step reaction that occurs in following sequence (i) diffusion of protons through the wet lignocellulosic matrix; (ii) protonation of the oxygen of a heterocyclic ether bond between the sugar monomers; (iii) breaking of the ether bond; (iv) generation of a carbo-cation as intermediate; (v) solvation of the carbo-cation with water; (vi) regeneration of the proton with cogeneration of the sugar monomer, oligomer or polymer depending on the position of the ether bond; (vii) diffusion of the reaction products in the liquid phase. All these process steps are influenced by pH of the hydrolysis medium, solid-liquid ratio, incubation temperature and time (65, 129). Sun and Cheng (134) and Cara et al. (10) concluded that acid hydrolysis with the use of concentrated acids is toxic, corrosive and hazardous.

Auto-hydrolysis is an alternative method for the chemical depolymerization of hemicelluloses with limited solubilization of lignin (29) and reduced quantities of sugar derivatives (furfurals and hydroxymethylfurfurals) (78). In addition auto-hydrolysis presents some technical and environmental advantages too as no chemicals (acid or alkali) are used other than water. Auto-hydrolysis performed at mild temperatures yields a high mass of xylo-oligosaccharides without modifying the cellulose and lignin structure substantially, allowing improved recovery during further processing (76, 117). The xylo-oligosaccharides produced are associated with a significant fraction of acetyl and uronic acid groups which has the characteristic of very high water solubility unlike that of chemical hydrolysis. The auto-hydrolysis process efficiency and hydrolysate chemical composition depends on incubation temperature and time, solid to liquid ratio, structural integrity of raw material employed. Nabarlatz et al. (81) working with six agricultural residues namely corn cobs, almond shells, olive stones, rice husks, wheat straw and barley straw as feedstocks for the production of xylo-oligosaccharides by auto-hydrolysis, reported that the yield of xylo-oligosaccharides depended on the content of xylan and its accessibility, and was proportional to the acetyl content of the raw materials. In fact, by regulation of auto-hydrolysis conditions, it is possible to influence characteristics of the xylo-oligosaccharides (the acetyl content and the molar mass distribution), but the nature of the raw material also has an influence (81). Hydrolysate analysis revealed that partially acetylated oligomeric and polymeric xylan fragments were attached with acetyl groups at 2 and 3 positions and some monosaccharides and partially O-acetylated 4-O-methylglucuronoxylan in addition to degradation products were present (80).

Biological or enzymatic hydrolysis has been proven as an alternative hydrolysis method offers conceptual edges like low chemical and energy use, but depends on enzyme accessibility to the heterogeneous biomass structure. The rate and extent of enzymatic hydrolysis of lignocellulosic biomass is dependent on catalytic properties of enzymes, their loadings concentrations, the hydrolysis period, reaction parameters employed, biomass type, pretreatment method employed and compounds produced during pretreatment process (159). Reduction of
hemicellulosic crystallinity improves the enzymatic hydrolysis rate and time in addition to the enzyme loading. Among all biomass components, lignin is identified as a major deterrent to enzyme attack on cellulose indicating the importance of reducing the structural integrity caused by lignin before hydrolysis. Cellulase and xylanases are the major enzymes employed in most of the pretreatment studies (95, 159). Biomass digestibility by enzymes is found to be regulated by the surface area of the material and an increase in surface area by pretreatment or decreasing particle size improves biomass hydrolysis (95).

Use of xylanase alone may not be sufficient in view of the complex nature of photosynthetic biomass material. Xylanases catalyzes the â-1, 4 bond in the xylan backbone yielding short xylooligomers. They are group of enzymes work synergistically and differ with microbial origin. The selection of critical xylanase blend consisting of xyllosidase, Mannanases, arabinofuranosidases, glucuronidases, esterases (ferulic and cumaric acid, acetyl-mannan, acetyl-xylan, etc.) and hemicellulolytic esterases is one of the important factors for effective production of xylose from hemicellulose fraction. This selection again related with the nature of xylan structure which vary with type of biomass (soft, hard wood, grass, etc). Pre-hydrolysis either by mild chemical treatment at elevated temperatures and/or by other specific enzyme treatment would offer the better hydrolysis process for the efficient production of xylose. Use of non catalytic proteins such as expansins and swollenins decreases the crystallinity structure thereby increases the accessibility to enzymes may be novel approach. However, the applicability and feasibility is yet to require further study. Our laboratory studies indicated that xylanase from certain specific microbial strains could be used as an efficient xylose production from palm seed fibre (95). However, enzyme treatment parameters have to be optimized for maximization of xylose production. Although enzymatic hydrolysis results in high yields in bioconversion of sugars from pretreated photosynthetic biomass, the cost of enzymes is a key aspect and needs to be costed. Use of hemicellulosic hydrolytic enzyme blend is another alternative; however, one has to identify and optimize process environment of the specific enzyme blend for each material. Wet oxidation pretreatment process proven to be efficient for lignocellulosic materials as crystallinity decrease was noticed along with lignin degradation to CO2 and H2O and carboxylic acids. Recently use of ionic liquids such as 1-butyl-3-methylimidazolium caution for biomass pretreatment revealed optimistic results but indeapth studies are essential for its after effects like microbial/enzyme inhibitor production, process environment, etc. In nutshell, upstream to pretreatment, the choice of source material structure is an important in selection of effective pretreatment methodology.

Components of biomass hydrolysate
A range of products such as glucose (mainly from cellulose and hemicellulose), xylose, mannose, galactose and acetic acid (from hemicellulose) and phenolic compounds (from lignin) are produced during the hydrolysis process. In addition, other compounds are also produced during hydrolysis especially when chemical hydrolysis is employed. Without exception, all sugar liquors obtained by chemical hydrolysis contain furan derivatives, aliphatic acids and phenolic compounds. Furan derivatives commonly known as furfurs and hydroxymethylfurfural (HMF) are produced from the degradation of pentoses and hexoses, respectively. Further degradation of furfurs leads to the production of formic acid. HMF is normally produced in less concentration compared to furfurs by hexoses degradation mainly due to the low quantities of hexose in hemicellulose. This is because the conditions employed in the
hemicellulosic material hydrolysis process do not degrade hexoses in large quantities. Acetic acid, the major aliphatic acid present in chemical hydrolysates, is mostly released from the hemicellulosic acetyl groups. During the acid hydrolysis, a minor part of lignin is also degraded to a wide range of aromatic compounds including low molecular mass phenolics (90). With the use of strong alkali solutions, depolymerized xylan may be extracted from lignocellulosics, but the product obtained is completely deacetylated and has very limited solubility in water hence is not the preferred hydrolyzing reagent. In addition other compounds such as acidic resins, tannic, terpene, syringic, vanillic, caproic, caprylic, pelargonic, and palmitic acids are reported to be produced during chemical hydrolysis (6, 78).

**Microbial fermentative inhibitors of biomass hydrolysates**

The major disadvantage of chemical hydrolysis is the reduction of available monosaccharides and production of their derivatives (furans, hydroxymethylfurfurals and other phenolic toxic compounds which are microbial growth inhibitors and hinder further biotransformations (78). However, the type and concentration of microbial fermentative inhibitory compounds mainly depend on raw material as well as the operational parameters. Microbial toxicity is also associated with fermentation variables like microbial physiological growth conditions, dissolved oxygen concentration and pH of the medium. In general, biomass hydrolysate inhibitors can be categorized as sugar or lignin degradation products, derived from lignocellulosic structure and heavy metal ions (78).

Furfurals derived from pentose are the major microbial growth inhibitor compounds present in chemical hydrolysates for xylitol bioconversion. They inhibit the growth of microbe ranging from 25 – 99% relative to the furfural concentration (0.5 – 2.0 g/l) and cell mass yield per ATP by interfering with the respiration process (90, 94). Delgenes et al. (20) and Martinez et al. (71) reported that *Pitchia stipitis* and *Saccharomyces cerevisiae* growth was reduced by 100% when the HMF in the concentration of 1.5 and 1.0 g/l was supplemented in the growth medium indicating the inhibitory effect varies with the type of microbial strain. Presence of low concentrations of these compounds in the fermentation medium showed better microbial growth (94) indicating the microbial strain properties role during bioconversion of hydrolysates. In addition, the antagonistic effect of furfural and HMF along with acetate, formic and levulinic acid on microbial growth was also reported with *P. tannophilus* and *P. stipitis* during xylose fermentation (148).

A variety of lignin degradation products that included aromatic, polyaromatic, phenolic and aldehydic compounds present in hydrolysate also cause inhibitory effects on microbial growth by integrating into biological membranes and affecting the membrane permeability. Villa et al. (145) reported that phenolic compounds at more than 0.1 g/l concentration affect the xylose consumption, cell growth and xylitol production in *C. guilliermondii*. Acetic acid toxic effect is mainly associated with its pKa property as at this value acetic acid is liposoluble, diffuses across the plasma membrane and discharges protons resulting in cell death due to dropping the internal pH. However, presence of acetic acid at low concentrations (1.0 g/l) in the fermentation medium reported to improve the xylose-to-xylitol bioconversion (24) probably due to more diffusion of internally pooled xylitol during xylose metabolism because of limited acetic acid effect at cell membrane. Heavy metals (iron, chromium, nickel and copper) produced during hydrolysis mainly originate from corrosion of hydrolysis equipment causes cell toxicity by inhibiting metabolic pathway enzymes (93).
Detoxification methodologies

In order to remove the microbial growth inhibitors and increase the hydrolysate fermentability, several detoxification treatments, including chemical, physical and biological methods have been developed. However, the needs for detoxification must be evaluated in each case since it depends on the chemical composition of the hydrolysate and is strain specific. The effectiveness of a detoxification methodology depends on raw material, type of hydrolysis process and microorganism employed (129). Taherzadeh et al. (136) reported four different approaches for minimizing the inhibitory effect of hemicellulosic hydrolysates; (1) use of bioconversion friendly hydrolysis methods; (2) detoxify the hydrolyzate before fermentation; (3) use of inhibitor resistant microorganisms; (4) convert toxic compounds into non-toxic. Since detoxification increases the cost of the process, it is important either to overcome detoxification steps or to develop cheap and efficient methods. Development of a new metabolically engineered microbial species which tolerate inhibitors could be the better option which can eliminate detoxification.

Vacuum evaporation is the best physical detoxification method with limited scope and helps to reduce volatile toxic compounds that include acetic acid, furfural, hydroxymethylfurfural and vanillin. Mussantto and Roberto, (78) reported that more than 90% these compounds are removed from wood, rice straw and sugarcane bagasse hemicellulosic hydrolysates by employing a vacuum evaporation method. However, this process enhances the concentration of non-volatile toxic compounds and reduces volumes of the hydrolysate (61). Neutralization, over-liming, sulfite treatment, extraction with organic solvents, treating with ion-exchange resins and adsorption into activated charcoal or diatomaceous earth reduce the ionization properties of inhibitory compounds by precipitation of toxic compounds. pH adjustment is effective and the most cost-effective chemical detoxification method among available treatments. Calcium hydroxide and sulfuric acid are commonly used for treatment of hemicellulosic hydrolysates for removal of phenolic compounds, ketones, furfurals and hydroxymethylfurfurals (84, 129). Activated charcoal is the other process attracting much attention because of its low cost and a high capacity to absorb pigments, free fatty acids, n-hexane and other oxidation products (98, 129). The effectiveness of activated charcoal treatment depends on different process variables such as pH, temperature, contact time and solid-liquid ratio. Acidic pH favours removal of the neutral or non-ionized phenolic molecules while alkaline pH for organic bases during activated charcoal treatment. Increase of contact time is reported to influence the clarification process. The absorption process increases at elevated temperatures during charcoal treatment basically due to a faster rate of diffusion of absorbate molecules from the solution to the absorbent and temperature induced orientation of charcoal surface (77). Comparative evaluation of different chemical detoxification methodologies indicated that anion exchange resins remove high percentages of toxic compounds such as acetic acid (96%), phenolic compounds (91%), furfural (73%), HMF (70%) in addition to substantial removal of aldehydes and aliphatic acids from hydrolysates compared to cation-exchange resins (78, 129). Grzenia et al. (35) reported use of hollow fibre based liquid extraction system for removal of acetic acid from corn stover hydrolysate using two different LiquiCel Membranes.

Biological detoxification can be done either by using specific enzymes or microorganisms. Laccases and peroxidases are generally employed for detoxification (78). The probable enzymatic detoxification mechanism involved is oxidative polymerization of low
molecular weight phenolic compounds (49) whereas, microbial detoxification of hydrolysate involves utilization of toxic compounds for microbial growth or adaptation of specific microbe for hemicellulosic hydrolysate (129). Schneider (116) reported that acetic acid in the hydrolysate can be removed more than 90% by S. cerevisiae mutant from wood hydrolysate. Silva and Roberto (118) and Sreenivas Rao et al. (129) successfully demonstrated that adaptation of C. tropicalis as an effective and inexpensive approach to alleviate the inhibitory effect of toxic compounds on xylose utilization for xylitol production from rice straw and corn cob hydrolysates.

Sreenivas Rao et al. (129) working on xylitol production from sugarcane bagasse and corn cob hydrolysate reported that various chemical and biological detoxification methodologies i.e., more than one method, were effective compared to single treatment processes. The authors reported pH adjustment followed by activated charcoal and resin treatment only helped up to certain level and adaptation of microbial strain would be the better option for effective and efficient use of sugar compounds from hemicellulosic hydrolysates. In summary, each detoxification method is specific to certain types of compounds. Choosing detoxification methods (more than one) and their sequence was important for improved yields, however identification of inhibitory compounds and their concentrations in the hydrolysate was necessary.

Xylitol producing microbial strains

In the last few decades, several papers published on xylitol production using bacteria (157, 158), fungi (19), and yeasts (55, 62, 119, 126, 132). Among the microorganisms, yeasts are considered as the best xylitol producers (Table 3). Candida strains have been extensively studied for the production of xylitol as they have an advantage over the metabolically engineered S. cerevisiae for being natural D-xylose consumers and maintaining the reduction–oxidation balance during xylitol accumulation.

Table 3: Some of the best xylitol producing yeasts

<table>
<thead>
<tr>
<th>Yeast</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida boidinii</td>
<td>106</td>
</tr>
<tr>
<td>Candida guilliermondii</td>
<td>FTI-20037118, 119</td>
</tr>
<tr>
<td>Candida intermedia</td>
<td>28</td>
</tr>
<tr>
<td>Candida maltosa</td>
<td>36</td>
</tr>
<tr>
<td>Candida mogii</td>
<td>122</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>55</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>HXP 2</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>129</td>
</tr>
<tr>
<td>Debaromyces hansenii</td>
<td>107</td>
</tr>
<tr>
<td>Hansenula polymorpha</td>
<td>135</td>
</tr>
<tr>
<td>Pachysolen tannophilus</td>
<td>110</td>
</tr>
<tr>
<td>Pichia caribica</td>
<td>126</td>
</tr>
<tr>
<td>Pichia miso</td>
<td>88</td>
</tr>
</tbody>
</table>

Screening programmes for xylitol production from D-xylose

Xylitol is an intermediate metabolic compound produced in all microbial strains whose xylose metabolism occurs in a sequential catalytic activity of xylose reductase and xylitol dehydrogenase enzymes. Keeping this in view, several scientific researchers have been involved in microbial screening programs to isolate efficient microbial strains for xylitol production. Hiroshi and Toshiyuki (41) tested 58 strains and P. miso emerged as the best xylitol producing strain with an yield of 3.77g of xylitol from 8.50g of D-xylose was consumed. Ojamo (88) screened 30 yeast strains for a xylitol metabolizing pathway and reported that C. guilliermondii and C.
tropicalis were the highest yielding strains. While, Sirisansaneeyakul et al. (122) reported xylitol yield of 0.62 g.g⁻¹ of xylose using C. mogii from 11 strains they tested for xylose utilization. Suryadi et al. (135) tested four methanol-utilizing yeasts for xylitol production from D-xylose. H. polymorpha was found to be the better strain out of 4 strains tested with 43.2 g.l⁻¹ xylitol production from 100 g.l⁻¹ D-xylose after 4 days of cultivation. Whereas, Yablochkova et al (155) tested 13 strains and noticed only 6 strains emerged from Candida genus as the best xylitol producers in the range of 0.50 to 0.65 g.g⁻¹ xylitol production. After screening 274 yeasts for xylitol production Guo et al. (36) selected 5 strains for further production and observed that C. gluilliermondii and C. maltosa were the best xylitol produces. Recently Sreenivas Rao et al. (126) tested a total of 35 yeasts isolated from the gut of beetles collected from Hyderabad city, India. Twenty of these yeasts utilized xylose as a sole carbon source but only 12 of these strains converted xylose to xylitol. The authors also reported that the ability to convert xylose to xylitol varied among the isolates and ranged from 0.12 to 0.58 g.g⁻¹ xylose. Out of these strains Pichia sp. was the best xylitol producer (0.58 g xylitol.g⁻¹ from xylose). In another study, Sampaio et al. (107) tested 270 yeast isolates for xylitol production using xylose as the sole carbon source. The authors reported that D. hansenii UFV-170 was the best isolate with production capacity of 5.84 g.l⁻¹ xylitol from 10 g.l⁻¹ xylose after 24 hours incubation. A report with xylose transport capacity as a screening parameter was reported by Gardonyi et al (2003) to isolate xylose-utilising yeasts.

**Molecular characterization of xylitol producing yeasts**

The approach to yeast identification has significantly changed in just a few decades due to the rapid increase in basic biological knowledge, increased interest in the practical applications and biodiversity of this important microbiological group, and technological advances. The development of molecular techniques has significantly widened the tools available for understanding and documenting species designations and phylogenetic relationships. Analyzing ribosomal DNA (rDNA) is now standard in molecular techniques and have made it possible to construct phylogenetic trees of all known species, with the capacity to better understand interspecific and intergeneric relationships. As a result, it is a common practice to deposit the sequences of key molecular regions, such as the 600-nucleotide variable region D1/D2 of LSU (large subunit) (26S) rDNA and the ITS1 and ITS2 (internal transcribed sequences) of 18S rRNA, with database servers such as Genebank. From D1/D2 sequence analysis, greater than a 100 species have been assigned to the genus *Pichia* and *Candida* which are distributed across the *Saccharomycetales* (59) and there is a specific distinct clade that contains xylose utilizing species (The xylose-fermenting clade). Molecular characterization studies help to understand the relationship between xylose utilizing yeasts that fall in this specific clade. Sreenivas Rao et al. (126), reported that phylogenetic analysis helped to characterize the xylitol producing yeasts (Table 4).

The best xylitol producer YS54 based on their D1/D2 domain sequence, showed similarity with *Pichia caribbica* and this strain is identified within the xylose utilizing clad in the phylogenetic tree. Suh et al. (133) isolated several xylose utilizing strains and demonstrated that the LSU rDNA sequence data helped to identify the xylose fermenting yeasts and noticed that they also in to specific xylose fermenting clade. Similar trend was also observed by Nguyen et al. (83) who isolated two yeasts which ferment xylose, and...
based on molecular characterization the authors reported that these strains belong to novel species and named as *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov.

**Construction of recombinant yeasts for xylitol production**

Screening of different xylitol producing microbial strains confirmed that xylitol production metabolic process is mostly associated with yeast in general and particularly with the *Candida* genus. Among different species in this genus, *C. tropicalis* is the best strain for xylitol production due to its high xylose uptake rate and xylitol production capacity (33, 132) and has application potential at industry level. In addition, this genus has an advantage, due to the lack of sexual stage (33), for further development of recombinant strains with high xylitol production potential. In fact, the major genetical differences of *Candida* and *Saccharomyces* genera are that the latter species is more tolerant in terms of their xylose fermentation, toxicity and growth tolerance in the presence of inhibitors of hemicellulosic hydrolysates. This has created new horizons to develop recombinant strains of *Saccharomyces* sp. with *Candida* sp. XYL1 gene for improved biological production of xylitol (82, 151). In order to make *S. cerevisiae* an efficient xylose-utilizer for the production of xylitol, an efficient enzyme

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**Table 4: Tentative identification of xylitol producing yeasts from insect guts based on D1/D2 domain sequence of the 26S rRNA gene (126)**

<table>
<thead>
<tr>
<th>Yeast isolate</th>
<th>Accession no. of D1/D2 domain</th>
<th>Identification</th>
<th>Isolated from</th>
<th>Xylitol yield (g⁻¹ of xylose)</th>
<th>XR activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YS 5</td>
<td>AM159103</td>
<td><em>Issatchenka</em> sp. <em>Euetheola</em> sp.</td>
<td></td>
<td>0.14</td>
<td>1.5</td>
</tr>
<tr>
<td>YS 6</td>
<td>DQ358865</td>
<td><em>Candida</em> sp. <em>Nicrophorus</em> sp.</td>
<td></td>
<td>0.30</td>
<td>4.4</td>
</tr>
<tr>
<td>YS 21</td>
<td>AM159101</td>
<td><em>Candida</em> sp. <em>Strategus</em> sp.</td>
<td></td>
<td>0.54</td>
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</tr>
<tr>
<td>YS 24</td>
<td>AM159108</td>
<td><em>Candida</em> sp. <em>Diplotaxis</em> sp.</td>
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<td>YS 43</td>
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<td>DQ358868</td>
<td><em>Candida</em> sp. <em>Copris</em> sp.</td>
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<td>YS 54</td>
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<td>AM420304</td>
<td><em>Candida</em> sp. <em>Anoplophora</em> sp.</td>
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<td>AM420306</td>
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<td>AM159103</td>
<td><em>Issatchenka</em> sp. <em>Euetheola</em> sp.</td>
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<td>YS 6</td>
<td>DQ358865</td>
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<td><em>Candida</em> sp. <em>Strategus</em> sp.</td>
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system for the conversion of xylose to xylitol should be introduced into the *S. cerevisiae*. Cloning of XYL1 gene from *C. tropicalis* to *S. cerevisiae* improves the latter yeast for utilization of xylose from hemicellulosic material and conversion of xylose to xylitol (82, 130).

Several investigators have cloned the necessary genes responsible for xylose metabolism in *S. cereviceae* and constructed the recombinant strains for production of xylitol (16, 38, 39, 73, 138). In the construction of a xylose metabolizing *S. cerevisiae* the gene encoding XR was cloned from the xylose metabolizing yeasts and transferred to *S. cerevisiae*. The authors of this review observed that these transformants could not produce xylitol for prolonged periods due to an imbalance of the redox potential in the cell (130).

One of the main possible limitations of utilization the XYL1 gene recombinant strains during continuous production of xylitol was the lack of reducing cofactors for the xylose to xylitol catalyzing enzyme, NADPH. The redox balance on substrate uptake in the yeast xylose metabolism has therefore been studied (38, 48). Different co substrates were evaluated, as generators of reduced cofactors for xylitol production by recombinant *S. cerevisiae* expressing the XYL1 gene, encoding xylose reductase. Glucose, mannose, and fructose, which are transported with high affinity by the same transport system as xylose inhibit xylose conversion rates by 99, 77 and 78 respectively. Competitive inhibition of xylose transport was indicated and xylitol yields varied widely with different co-substrates (48). Galactose as co-factor generator gave the highest xylitol yield, 5.6 times higher than that for glucose. This may be attributed to the observed difference in redox metabolism of glucose and galactose and subsequent enhanced availability of reduced cofactors for xylose reduction with galactose (130). Granstrom et al. (33) evaluated formate as a co-substrate to increase the intracellular concentration of NADH and based on the results the authors have hypothesized that excess NADH would result in higher oxygen and xylose consumption and correspondingly increase xylitol production by inhibiting xylitol dehydrogenase enzyme.

In this context, addition of cofactor in the growth medium may be a possible solution. Experimental evidence of 25% enhanced XR activity in galactose supplemented xylose media further supported that cofactor limitation is an important drawback for enhanced production of xylitol in recombinant strain studies (130). Similar observations are also noticed by Granstrom et al. (33) where the authors reported the metabolism (Metabolism Flux Analysis (MFA)) of xylose by *C. tropicalis* in oxygen-limited chemostat conditions. Furthermore, in vitro enzyme assay indicated that glycolytic and gluconeogenetic enzymes are expressed simultaneously, facilitating cofactor recycling. Moreover, enhancing the redox imbalance by co feeding of formate increased xylose and oxygen consumption rates and ethanol, xylitol, glycerol and CO₂ production rates at a steady state. MFA indicated that fructose 6-phosphate is replenished from the pentose phosphate pathway in sufficient amounts without contribution of the gluconeogenetic pathway (33). Overall, the observed enhanced XR activity in galactose supplemented xylose medium by transformant *S. cerevisiae* suggested the cofactor availability importance for xylose metabolism in recombinant strain and improved xylitol production.

**Metabolic pathways for xylose utilization**

In 1960, Chiang and Knight found that the filamentous fungus *Penicillium chrysogenum*
converted D-Xylose to D-xylulose through a two-step reduction and oxidation and noticed xylose utilizing enzyme in the bacteria was different. This finding, as well as some further investigations (15) led to the conclusion that the two-step conversion of D-xylose to D-Xylulose is specific for yeasts and fungi, whereas in bacteria the same conversion is catalyzed by xylose isomerase in a single step. The detection of xylose isomerase in the yeasts *Rhodotorula* (42) and *C. boidinii* no. 2201 (149) is one of the few exceptions to this generalization.

In xylitol producing yeasts, xylose is reduced to xylitol either by NADH- or NADPH-dependent xylose reductase (aldose reductase EC 1.1.1.21). The produced xylitol is either secreted from the cell or oxidized to xylulose by NAD- or NADP-dependent xylitol dehydrogenase (EC 1.1.1.9). These two reactions are considered to be limiting for D-Xylose fermentation and xylitol production. The ratio of xylose reductase and xylitol dehydrogenase in addition to cofactor regenerating system is the major metabolic regulator for xylitol production. However, certain strains of yeast are known to utilize xylose as a carbon source via the phosphorylation of xylulose to xylulose-5-phosphate which is catalyzed by xylulokinase (EC 2.7.1.17) (60, 124). A detailed study of biochemistry and physiology of the yeasts metabolizing xylose was published by Hahn-Hagerdal et al. (37). In fact, the conversion of D-xylose to xylitol in yeasts cannot be separated from the conversion of D-Xylose to other metabolic products such as carbon dioxide, ethanol, acetic acid and polysaccharides.

**Coenzyme specificity**

The first two enzymes, D-xylose reductase (XR) and xylitol dehydrogenase (XDH), of xylose utilization in xylitol producing microbial strain, are regulated by the ratio of cellular pools of NAD(P)H/NAD(P). These two enzymes require pyridine nucleotide cofactors and their specificity which differ with different yeast strains. It was reported that XR from, e.g. *Candida utilis* can utilise only NADPH (8), the XR from *Pachysolen tannophilus* CBS4044 and *Pichia stipitis* can use either NADH or NADPH as a cofactor (143, 144). The dual cofactor dependence of XR on NADH and NADPH may prevent a complete regeneration of NAD+ which is needed for the XDH reaction (47, 57), and hence xylitol is secreted into the medium. Xylitol may also be formed due to the action of unspecific reductases, like *GRE3* (139).

Under anaerobic or oxygen-limited conditions, the difference in the cofactor requirements of these enzymes causes a redox imbalance which influences xylitol production in yeasts. In general xylitol formation is favored under oxygen-limited conditions because of the NADH accumulation and subsequent inhibition of NAD-linked xylitol dehydrogenase. Cell growth depends on some of the above metabolic products and it is also necessary that the cofactors be regenerated through different steps in the metabolic pathway. Therefore, for obtaining 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 (130).

**Process regulatory factors on xylitol production**

Bioconversion of xylose to xylitol using microbial strains is generally influenced by, nutritional composition (substrate, nitrogen source and micro nutrients and their concentrations), culture and process conditions (temperature, pH, aeration, inoculum concentration, immobilization and reactor conditions) as well as genetic nature of the microorganisms (native isolates, mutants and recombinant strains).
Impact of nutritional composition

Among all nutritional parameters xylose concentration (51, 107, 108, 125, 132) yeast extract, urea, corn steep liquor, casamino acids, hydrolysate composition play a vital role on cellular metabolism and subsequent xylitol production. In general, in the presence of glucose, xylose utilization was strongly repressed and glucose followed by xylose sugar utilization was observed.

Kim and Oh (56) demonstrated a chemically defined medium with urea (5 gl⁻¹) as a nitrogen source and various vitamins supplementation as a substitute for a complex medium containing yeast extract (10 g l⁻¹) in the production of xylitol by C. tropicalis. C. gluilliermondii VTT-C-71006 growth on rare pentoses and their implications for production of pure xylitol was studied by Granstrom et al. (33) and observed that this yeast strain grew on all the tested pentoses like L-arabinose, L-ribulose, D-ribose and D-xylose and gave the fastest growth. Suryadi et al. (135) working with methanol-utilizing yeasts reported that H. polymorpha produces 43.2g/l xylitol from 100g/l D-xylose after 4 days of cultivation with 1% (v/v) methanol supplementation and further additions of urea, (NH₄)₂SO₄, and NH₄NO₃ proved to be effective for an increase of xylitol yield this yeast. The effect of different nitrogen sources on xylitol production from D-xylose by Candida sp.L-102 was also reported by Lu et al. (67) and maximum xylitol production of 87% was obtained with urea as the nitrogen source. Yeast extract at a maximum concentration of 10 g/l was found to be optimum for xylitol production by C. tropicalis DSM 7524 and concentrations higher than 15 g/l blocked the conversion of D-xylose to xylitol (120).

Increase in concentration of yeast extract from 5 and 10g/l increased the biomass production but a sharp decrease in xylitol productivity was identified for C. gluilliermondii FTI 20037 (121). Similarly the addition of yeast extract and peptone to the chemically defined medium enhanced cell growth of C. mogii ATCC 18364 but had little impact on the yield and specific productivity of xylitol (122). However, in some yeasts, special nutrient supplementation improved xylitol production. Lee et al. (63) reported that high-biotin containing medium favored ethanol production over that of xylitol with P. tannophilus NRRL Y-2460, while in C. gluilliermondii FTI 20037, xylitol formation was favored under similar conditions.

Role of temperature and pH on xylitol production

In general, the most suitable temperature for xylitol production in yeasts is 30°C. However, the xylitol yield was temperature-independent when the yeast was cultured in a temperature range between 30°C and 37°C but above 37°C the xylitol yield decreases sharply (120). Exceptions to this were observed by Sreenivas Rao et al. (132) where a variation of 3°C influenced (27%) on xylitol production in C. tropicalis. No variation in xylitol formation in C. guilliermondii FTI 20037 was noticed in temperature range of 30 and 35°C but decreased when the temperature increased to 40°C (Barbosa et al., 1988). The conversion of D-xylose to xylitol by Candida sp. B-22 was relatively constant over the temperature range of 35-40°C and further increase in temperatures to 45°C and higher, the conversion was sharply reduced (Cao et al., 1994). This was probably due to loss of the activities of both NADPH and NADH-dependent xylose reductase associated with the temperature increase (123). Sampaio et al. (108) reported a significant observation, that xylitol production with D. hansenii UFV-170 was hardly affected either at lower (10–20 °C) or higher (40–45 °C) temperatures. Wilkins et al., (2008) reported higher xylitol production at above 45°C with thermotolerant yeasts.
The yeasts are generally cultivated at pH values between 4 and 6. However, variation also reported in literature. For example, *C. parapsilosis* ATCC 28474 (86) and *C. guilliermondii* NRC 5578 (75, 86) revealed the maximum growth at pH 6.0 while, *Candida guilliermondii* ATCC (122) and *P. stipitis* NRRL Y-7124 show optimum at pH 5 and 5.5, respectively whereas, pH 4 was optimum for *C. tropicalis* IFO 0618 (43). In general, the optimum initial pH value for the best xylitol yield in *C. boidinii* was 7.0 (142, 150), whereas under controlled conditions, a pH of 5.5 (142). Batch culture of *C. parapsilosis* ATCC 28474 (64) showed higher performance in xylitol production at pH 6 while for continuous culture a pH of 4.5 was found to be effective (26). Variation of pH from 4.5 to 5.5 did not show any influence on xylitol production by isolated *C. tropicalis* (130, 132). In contrast *Silva* and *Afschar* (120) reported that *C. tropicalis* DSM 7524 was not very sensitive to pH and attained a maximum xylitol yield at pH 2.5. Increasing the pH from 2.5 to 4.0 led to an increase in xylitol productivity but a decrease in xylitol yield. *Sampaio* et al (108) noticed that the percentage of xylose consumed for xylitol production progressively increased with pH and decreased reaching nearly constant values at pH 4.0. This process is associated with both biomass growth and catabolic reaction through the TCA cycle.

**Inoculum**

Conflicting reports are available in the literature on xylitol production versus inoculum loading. On studying the effect of initial cell concentration of *Candida sp. B-22* on xylitol production from D-xylose, *Cao* et al. (9) reported that the rate of xylitol production was linear and the fermentation time was dramatically reduced over an initial biomass concentration range of 3.8 to 26 g.L⁻¹. The authors noticed 210 g.L⁻¹ of xylitol with an initial yeast cell concentration of 26 g.L⁻¹ and using 260 g.L⁻¹ D-xylose indicating a high initial cell mass concentration is beneficial for xylitol production by *C. boidinii* NRRL Y17213. In another study, *Vandeska* et al., (142) reported a doubled xylitol yield and specific productivity with the increase of inoculum level from 1.3 to 5.1 g.L⁻¹ using initial D-xylose concentration of 50 g.L⁻¹. Use of very high inoculum observed to improve the xylitol formation under nitrogen limitation environments. In addition, xylitol formation was simultaneously influenced by the physiological state of the culture and the concentration of biomass (109). However limited variation in xylitol production was observed by *C. tropicalis* with the use of inoculum concentration in the range of 6%-10% (132).

The effect of inoculum size on the microbial production of xylitol from hemicellulose hydrolysates was also investigated. A high initial cell density did not show any positive effect when *C. guilliermondii* FTI 20037 when grown on rice straw hemicellulose hydrolysate since increasing the initial cell density from 0.67 g.L⁻¹ to 2.41 g.L⁻¹ decreased biomass formation, xylose utilization and xylitol accumulation (99). On the contrary *D. hansenii* NRRL Y-7426 grown on wood hydrolysate produced more xylitol at higher initial cell densities (91). Overall, the relationship between biomass and xylitol production was observed to be dependent the microbial strain physiological growth and metabolic properties.

**Aeration**

With respect to aeration, the oxygen supply rate is a key parameter for D-xylose metabolism in xylitol producing yeasts and determines whether D-xylose will be fermented or respired. It is very important, therefore, for an effective process to determine the oxygen flux that will enable balanced utilization of carbon both for growth and xylitol production.

Xylitol production by yeasts is always associated with micro aerobic conditions. Several authors reported aeration and agitation effects
on yeast growth and xylitol production (7, 14, 132, 152). In general, under strict aerobic and anaerobic conditions, xylitol is not produced extracellularly (115). Kastner et al. (50) reported that the growth of the xylitol producing organism, *Candida shehatae*, is drastically affected when the culture was incubated under anaerobic conditions and a step change from aerobic to anaerobic improved product formation. Walther et al. (152) reported that oxygen limitation and initial xylose concentration had considerable influences on xylitol production by *C. tropicalis* ATCC 96745. Under semi-aerobic conditions, the maximum xylitol yield was 0.62 g·g⁻¹ substrate, while under aerobic conditions, the maximum volumetric productivity was 0.90 g·l⁻¹·h⁻¹. Granstrom et al. (33) studied the metabolism of xylose by *C. tropicalis* in oxygen-limited chemostat and reported glycolytic and gluconeogenetic enzymes are expressed simultaneously facilitating substrate cycling based on an *in vitro* enzyme assay. The authors were able to enhance the redox imbalance by co-feeding of formate which increased xylose and oxygen consumption. Santos et al. (112) working with immobilized cells of *C. guilliermondii* on porous spheres reported xylitol production in fluidized bed reactor using sugarcane bagasse hemicellulose hydrolysate and reported a maximum xylitol (17.0 g·l⁻¹) yield with an aeration rate of 75 ml/min.

To determine the specific oxygen uptake rate at which *C. boidinii* NRRL Y-17213 begins to produce xylitol, Winkelbausen et al. (154) cultivated yeast continuously under oxygen-limited conditions and noticed that xylitol secretion was triggered at 0.91 mmol·h⁻¹. No xylitol production was observed at specific oxygen uptake rates above this value. Upon a shift to lower specific oxygen uptake rates, as expected, xylitol production rates and yield increased more rapidly than those of ethanol. Branco et al. (7) studied the influence of the aeration on *ca* alginate immobilized *C. guilliermondii* cell concentration and reported the highest conversion efficiency (41%) using 1.33vvm aeration rate and 40% immobilized system. Whereas, Roseiro et al., (103) reported a combinatorial influence of substrate concentration and aeration rate on xylitol formation in yeasts. The authors noticed a maximum xylitol productivity of 2.67 g·l⁻¹ when the initial *k₅a*, D-xylose and yeast extract concentrations were 172, 21 g·l⁻¹ and 452 l·h⁻¹, respectively.

Reports are also noticed in the literature on relationship between co-factor generation and aeration. The general characteristic of most xylose-fermenting yeasts is that their xylitol dehydrogenase uses predominantly NAD and very rarely the NADP cofactor (30, 31, 60, 64). The varying ratio of NADH- to NADPH-linked D-xylose reductase activity with aeration conditions was first found in *P. tannophilus* and similar variations were observed in the yeasts *C. parapsilosis* ATCC 28474 (86) and *C. boidinii* NRRL Y17213 (141). It has been noticed that oxygen may lower the ratio of NADH linked D-Xylose reductase and NAD-linked xylitol dehydrogenase activities and consequently minimize xylitol accumulation in D-xylose-fermenting yeasts (123). This was also observed in *C. boidinii* NRRL Y-17213 (141). The NADH/NAD ratio decreased 2-fold with increasing oxygen availability from 10 - 30 mmol/h.

It is very difficult to compare data from different studies because oxygenation is measured and reported differently. Yet it is evident that yeasts producing xylitol require small amounts of oxygen that is specific for each yeast strain. It is observed that *D. hansenii* has the highest demand for oxygen compared to other yeasts (107).
Optimization studies

Nutritional, physiological, operational, genetical and metabolic parameters are important for the economic xylitol production by microbial strains at industrial scale. The scientific community has performed elaborate optimization studies using several statistical approaches (127). When optimizing the xylitol production rate of C. tropicalis ISO 0618 by employing the Box-Wilson method, Horitsu et al. (43) found that the interaction between D-xylose concentration and aeration rate is related to cell biomass concentration. Rodrigues et al. (100) used response-surface methodology for xylitol production optimization from sugarcane bagasse hydrolysate in a fed-batch process and reported the best experimental parameter for achieving a maximum of 0.78g of xylitol per g of xylose by using C. guilliermondii. Whereas, Carla et al. (11) used a fractional factorial design for selection of important variables on xylitol biosynthesis from rice straw hydrolysate by C. guilliermondii. The authors noticed that all four selected factors such as xylose concentration, inoculum level, agitation speed and nutrient supplementation have played a critical role in the xylitol fermentation and the most important factor is initial xylose concentration. Genetic algorithms coupling neural network was used for optimization of six medium components for xylitol production by C. mogii by Baishan et al. (2) and noticed 0.65g xylitol production per g of xylose utilized. In another study, Sreenivas Rao et al. (132) optimized incubation temperature, pH, agitation, inoculum size, corn steep liquor, xylose, yeast extract and KH₂PO₄ requirements for maximum xylitol production using Taguchi methodology and achieved 78.9% conversion at optimized environment with isolated C. tropicalis. Optimization studies are also reported for fed-batch fermentation based xylitol production by C. tropicalis ATCC 13803 by Kim et al. (53) and noticed 0.75% xylitol conversion rate per gram of xylose utilization.

Xylitol production by immobilized yeasts

Another way to improve the process parameters is the use of immobilized cells since it allows obtaining high cell concentration in the reactor, with the increase in the efficiency and productivity of the process. In addition, the use of immobilized cell systems make possible the recovery of cells for later use in repeated batch operations. A good performance of an immobilization system depends on immobilization matrix properties, procedures employed, reactor configuration and bioconversion conditions (93, 97). Reports on use of different matrices have been evaluated for immobilization of cells and for xylitol production. Carvalho et al. (13) and Branco et al. (7) working with alginate immobilized C. guilliermondii cells reported repeated use of these cells for bioconversion in stirred tank reactor with average productivity value of 0.43 g/l and 0.21 g/l/h, respectively. In another study, Santos et al. (111) observed more than 70% bioconversion of xylose to xylitol with C. guilliermondii cells immobilized on natural sugarcane bagasse fibers. The maximum yield was 0.73 g of xylitol per gram of xylose consumed was noticed by Liaw et al. (65) with Candida subtropicalis immobilized in polyacrylic hydrogel thin films whereas, Cunha et al., (18) reported increased productivity with increase in recycling of polyvinyl alcohol immobilized C. guilliermondii. Silva and Afschar (120) immobilized the cells of C. tropicalis DSM 7524 on a porous glass and used them in a fluidized bed reactor. The authors intended to reuse the immobilized cells several times by repeating the batch fermentation with substrate shift. However the yeast was degenerated after completion of the first cultivation and addition of fresh medium. Under continuous conditions, the immobilized cells of C. guilliermondii converted D-xylose into xylitol with a high productivity of 1.35 g.l⁻¹.h⁻¹.

Co-immobilization of different microbial strains and their use in xylitol bioconversion
revealed improved productivity values. The highest conversion rate was observed when benzene-treated cells were co-immobilized in the photo-crosslinkable resin prepolymer ENT 2000 and 4000 (85). Almost 100% of the D-xylose (4.5 g.l\(^{-1}\)) was converted into xylitol after 33 h of incubation when the volume ratio of immobilized methanogen to immobilized Candida pelliculosa was 1:2. In the co-immobilized cell system, the degree of conversion and the conversion rate of D-xylose were higher than those in the separately immobilized cell system. Co-immobilized cells were stable for about 2 weeks with approximately 35% conversion. Lohmeier-Vogel et al. (66) studied the glucose and D-xylose metabolism in agarose-immobilized C. tropicalis ATCC 32113 by nuclear magnetic resonance. NMR studies showed that neither glucose nor xylose metabolism was enhanced by use of an immobilization process. Attempts to improve the rate of D-xylose metabolism by increasing the oxygen delivery to the entrapped cells were not successful.

**Bioreactor process strategies**

Most of the xylitol bioconversions by employing the microbial strains are associated with batch culture methods either at flasks or lab batch stirred tank reactors with the use of free or immobilized cell systems and pure xylene or xylose containing hydrolysates (3, 31, 75, 79, 86, 87, 103, 126, 129, 132, 142, 150, 153) with productivity values ranging from 0.55 to 0.78 gram substrate per gram xylitol. Application potential of these batch processes at industrial scale is time consuming as batch processes are associated with preparatory activities such as regular inoculum development, sterilization of the reactor, etc involving considerable input of labour, energy and time leading to decreased productivity. Efforts have been made to improve the product volumetric productivity values using different reactor configurations and varying the process parameters. In this context, continuous culture techniques often provide better productivities and yields. Santos et al. (111) working on the development of a bioprocess for the continuous production of xylitol from hemicellulosic hydrolysate using C. guilliermondii immobilized cells reported 70% xylitol to xylitol bioconversion. Similar xylitol productivity values with C. guilliermondii FTI20037 under continuous fermentation using sugarcane bagasse hydrolysate have been reported by Martinez et al. (72) however, the authors noticed little impact of \(k_La\) on volumetric productivity which is an interesting phenomenon in xylitol production process. Faria et al. (23) evaluated the role of membrane bioreactor in a view to achieve the simultaneous separation of xylitol during continuous bioconversion process and noticed the best performance (86% conversion) with 0.2 \(\mu\)m pore diameter containing membrane at a dilution rate of 0.03 per hour. An improvement of 30% on xylitol production/conversion under continuous cultivation of D. hansenii was observed with the supplementation of small amounts of glucose and at lower aeration environments (137). In fact production of xylitol from hemicellulosic hydrolysate may be more effective with the use of mixed culture as in continuous process and process efficiency depends on removal of other monosaccharides from the hydrolysate by the co-microbial culture (21). In most of the continuous reactor configurations, a substantial improvement in productivity values can be achieved only by using low dilution rates of xylose with high residence time, which is very difficult to achieve in practice for bulk production.

Research has therefore focused on xylitol production by fed-batch mode where substrate concentration can be maintained at a suitable level throughout the course of fermentation, i.e., a level sufficient to induce xylitol formation but not to inhibit microbial growth. In addition, these processes generally operate with high initial cell density which normally leads to an increase in volu-
metric productivity. The yeast C. boidinii NRRL Y17213 gave better results when cultivated in a fed-batch fermentor compared to other ways of cultivation. The highest xylitol yield was 75% of the theoretical yield, compared to 53% in the batch culture. The productivity of 0.46 g.l⁻¹.h⁻¹ was twice as high as the highest obtained under batch conditions (140). Olofsson et al. (89) reported xylitol production yield of 0.67% under fed-batch condition using recombinant S. cerevisiae strain with wheat straw hydrolysate. Whereas, Oh et al. (87) working with glucose-limited fed-batch cultivation of recombinant yeast strain observed an 1.9 fold increase in specific xylitol productivity over a control strain containing only xylose reductase enzyme. In order to improve the volumetric productivity and to overcome loss of xylitol producing biocatalysts in repeated fed-batch reactors, cell recycle attachment with hollow fiber membrane was employed and 3.8-fold increases were observed compared with the corresponding values of batch-type xylitol production parameters (1). Xylitol productions by other reactor configurations are also reported in the literature. Branco et al. (7) reported only 41% conversion of xylose to xylitol in bubble column bioreactor using immobilized C. guilliermondii and sugarcane bagasse hydrolysate. More than 70% xylitol yield was reported with the use of semi-continuous process in stirred tank reactor by alginate immobilized yeast cells (13).

**Future prospects and conclusions**

Xylitol is gaining the commercial importance due to its application potential in health and pharmaceutical sectors. Xylose is the raw substrate used for xylitol production either by chemical hydrogenation or by bioconversion with certain microbial species. Chemical production of xylitol is cost-intensive, energy consuming process and production economics depend on purity of the xylose and the main source of xylose is xylan from hemicellulosic biomass. Hemicellulosic xylan can be converted to xylose either by chemical or enzymatic hydrolysis which is depend on the parameters related to biomass, hydrolysis and enzyme. Chemical hydrolysis of biomass produces microbial growth inhibitors and needs detoxification. Detoxification of hydrolysate can be performed by physical, chemical and biological methods. However, the major challenge is for economic pretreatment technology with energy efficiency, in addition to optimum convertibility associated with reduced formation of degradation products. Development of species specific hydrolyzing enzymes would offer selective hydrolysis of xylan from renewable biomass as well as eliminate or reduce the inhibitory effects of some hydrolysates and xylose utilization in presence of other monomeric sugars. However, combination of all these detoxification methods is most suitable and cost effective approach but adaptation is necessary according to the microbial metabolic pathways. Many scientific groups have screened for xylose-utilizers and noticed that Candida genus is the best for xylitol production. Molecular characterization of xylose utilizing yeast strains revealed the presence of a xylose utilizing clade in the phylogenetic tree. Xylitol production by any microbial strain is related to the balance of xylose reductase and xylitol dehydrogenase. Xylitol production depends on the nutritional, fermentation and physiological growth factors associated with micro-aerophillic conditions. Several studies have investigated the optimization of xylitol production using free or immobilized cells in batch or in continuous fermentation conditions using different reactor configurations. Considering the limitation of microbial conversion of xylose to xylitol, especially with the use of the necessary high dilution rates and residence time, it is important to focus on the development of xylose reductase dependent enzymatic bioconversion of xylose from hemicellulosic hydrolysate. The development of an independent microbial
metabolic cofactor regeneration system needs special attention. One of the other alternatives is to develop robust microbial systems by cloning the xylose reductase gene by recombination along with reduced cofactor generation system, however this has not been successful due to lack of continuous cofactor regeneration system. Use of co-substrates such as galactose for cofactor regeneration increased xylitol production indicating the need for further understanding and exploitation of this approach at the genetic level for successful development of recombinant strains. Screening and development of robust and novel microbial strains with hydrolysate inhibitor tolerance play a pivotal role in xylitol production at the industrial scale. A focus should be maintained on a common platform of understanding of the hydrolysate material, hydrolysis procedure, microbial performance, bioconversion environment and downstream processing is one of the most essential aspects for development of integrated technological solution for production of second generation bio refinery products like xylitol via biotechnological process at an economic industrial scale.

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References


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production by recombinant *Saccharomyces cerevisiae*. Biotechnol. 9: 1090-1095.


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