CHAPTER 1

INTRODUCTION
Abstract
In this chapter, an overview about xylitol is presented. A literature survey on their physicochemical properties, functions, applications, methods of production, pathway and strategies used for enhanced biotechnological production is summarized.

1.1 Introduction
In today’s era people are becoming more and more calorie conscious as a safety measures for maintaining physical fitness. This trend has shifted the research focus towards low calorie or zero calorie sweetener. These sweeteners are of two type’s viz artificial and natural sweetener. These sweeteners find applications in food, pharmaceuticals and chemicals.

Artificial sweeteners are sucralose, saccharin, acesulfame K, aspartame, stevioside and neotame [1]. They are good sweetener, but their inability to provide bulk to food products limits their applicability. Moreover, non-nutritive sweeteners confer bitter, metallic aftertaste and incapable to give taste of sucrose.

Chemically, polyols are polyhydroxy alcohols. Unlike high-intensity artificial sweeteners, which are used in very small amounts, polyols are used in the same quantity as sucrose. Some of the commercially available polyols are erythritol, lactitol, maltitol, sorbitol and xylitol. These sweeteners provide fewer calories and result in a much slower and minor rise in blood sugar level. Hence, these are considered as safe for diabetic patients and therefore the products sweetened with these products may legally be labeled “sugar-free”. These health benefits increases importance of usage of polyols as sugar replacers in a variety of products.

One of such important low calorie sweetener with multiple applications is xylitol. Xylitol is widely consumed in the food, beverages, pharmaceuticals and confectionery. Xylitol serve as humectants, bulking agents, and freeze point depressants. In foods, xylitol widely used in chewing gums, baked food, jellies,
Biocatalytic process for production of xylitol from crude pentose stream of renewable biomass feedstock

In pharma, xylitol is used in toothpaste, mouthwashes, cough syrup and lozenges. Xylitol has received generally regarded as safe (GRAS) status from the US FDA [2].

1.2 History of Xylitol

In 1890, xylitol was discovered in the laboratories of Fisher and Stahe in Germany and Bertrand in France. In 1943, xylitol was found in nature in some plants. In 1962, xylitol metabolic pathway was found in mammalian tissues. Scientists had classified xylitol as polyol. Engineers and chemists started searching for alternative sweetener due to low sugar supply during Second World War. In 1975, The Finnish Sugar Company started the manufacturing of commercial production of xylitol in southern Finland with a capacity of over 3,000 tons/year [3]. At present, xylitol usage is legally approved for use in foods, pharmaceuticals and health products in more than 50 countries [4]. Xylitol market is growing with CAGR of 6% every year and is estimated to be over US$ 1 billion/year by 2020 and priced at US$ 5–6 per kg [5].

1.3 Natural occurrence of xylitol

Xylitol is naturally available in small quantities in many fruits, berries and vegetables [6]. Reineclaudes or yellow plums have the highest content with 1% on dry solids basis. Some of the xylitol containing plants and vegetables are bananas (Musa sapientum L.), raspberries, strawberries, reineclaudes, carrots, fresh cauliflower, white mushrooms etc. [6]. Xylitol is also a normal metabolic intermediate of carbohydrate metabolism in man and animals. The normal xylitol concentration in blood is 0.03-0.06 mg/100 ml of blood [7].
1.4 Physical and Chemical properties of xylitol

Since xylitol was discovered in laboratories, in natural sources and achieved high importance in various industries, the physical and chemical properties of xylitol have been characterized. Due to better properties over the other sugar alcohols, it is used as a preferred sweetener in foods, pharmaceuticals and cosmetics. Physical and chemical properties of xylitol are shown in Table 1.1. Chemical structure for xylose and xylitol are shown in figure 1.1.

Table 1.1 Physical and chemical properties of xylitol

<table>
<thead>
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<th>Sr no</th>
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<th>Values</th>
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<tbody>
<tr>
<td>1</td>
<td>Empirical formula</td>
<td>C₅H₁₂O₅</td>
</tr>
<tr>
<td>2</td>
<td>Molecular weight</td>
<td>152.1 g/mol</td>
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<tr>
<td>3</td>
<td>Appearance</td>
<td>White crystalline powder</td>
</tr>
<tr>
<td>4</td>
<td>Test</td>
<td>Sweet</td>
</tr>
<tr>
<td>5</td>
<td>Odour</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Relative Sweetness</td>
<td>Equal to sucrose</td>
</tr>
<tr>
<td>7</td>
<td>Caloric value</td>
<td>2.4 cal/g</td>
</tr>
<tr>
<td>8</td>
<td>Optical rotation</td>
<td>Optically inactive</td>
</tr>
<tr>
<td>9</td>
<td>Melting point</td>
<td>93.4-94.7 °C</td>
</tr>
<tr>
<td>10</td>
<td>Boiling point</td>
<td>216 °C</td>
</tr>
<tr>
<td>11</td>
<td>Solubility in water(20 °C)</td>
<td>64.2 g /100 ml</td>
</tr>
<tr>
<td>12</td>
<td>Solubility in ethanol</td>
<td>1.2 g / 100 ml</td>
</tr>
<tr>
<td>13</td>
<td>Solubility in methanol</td>
<td>6.0 g / 100 ml</td>
</tr>
</tbody>
</table>
Figure 1.1 Chemical structures of xylose and xylitol

1.5 Applications

US FDA has approved the use of xylitol for special dietary foods. In 1983, JECFA (a Joint Expert Committee of WHO and FAO) announced xylitol as a safe sweetener for foods [8]. It is used as natural sweetener alone or in combination with other sweeteners in the preparation of a wide variety of low calorie or zero calorie products. Addition of xylitol in yoghurts, jams and deserts improves color, texture and taste of product. Applications of xylitol include chewing gum, candies, toffees, ice cream, chocolates, caramels, mouth washes, tooth paste, syrup, chewable tablets, dietic and diabetic foods in pharmaceuticals [3, 5]. Xylitol is not metabolized by the Streptococci (especially Streptococcus mutans), normally present in the flora of the human mouth. Streptococci do not produce caries-promoting acids from xylitol. The clinical and field studies have demonstrated the caries-inhibiting effect of xylitol [7].
1.6 Process for xylitol production

Figure 1.2 shows the summary of various technologies used for xylitol production

1.6.1 Extraction

Xylitol is found naturally in fruits and vegetables (lettuce, cauliflower, yellow plums, raspberry, strawberry, grape, banana), as well as in yeast, lichens, seaweed and mushrooms. Very less xylitol concentration (< 900 mg/100g) from these sources obtained by solid-liquid extraction. Hence, extortion is not cost competitive [3, 5].

1.6.2 Chemical process

Currently industrial production of xylitol from xylose is done by chemical process. Chemical process involves chemical hydrogenation or reduction of pure xylose (99.0 % purity) at high temperature and high pressure in specialized equipment using Raney nickel as catalyst. The potential sources of xylose are xylan containing hard woods or soft woods such as birch wood, sugar cane bagasse, straw and corn cobs. Xylose is extracted from lignocellulosic feedstock by acid hydrolysis, after color removal and purification, xylose treated for chemical hydrogenation at 80–140°C and hydrogen pressures up to 50 atmospheres in the presence of metal catalysts (Raney nickel).
Fig 1.2 Technologies available for xylitol production (Parajo et al, 1998)
The step wise description of industrial chemical process is as follows.

Step 1. Acidic hydrolysis of xylose containing lignocellulosic feedstock
Step 2. Xylose hydrolysate purification till desired purity achieved
Step 3. Pure xylose hydrogenation to xylitol using nickel catalyst
Step 4. Purification of xylitol from mixture of polyols
Step 5. Xylitol crystallization

Although, chemically produced xylitol is similar in structure and properties to the natural substance, this process has some serious issues. The chemical process produces xylitol with only 50 % yield from xylose, and xylitol produced is with other polyols due nonselectivity of the process. This xylitol solution requires further purification by chromatography and then concentration and crystallization of the product to obtain pure xylitol [9]. This chemical process requires specialized equipments to get required temperature and pressure, produces toxic chemicals, and creates environmental threats due to harsh conditions. These issues make current industrial process as costly and environment unfriendly [10, 11]

Currently, Danisco (industry of DuPont Company) is a major supplier of xylitol in world. It manufactures xylitol from xylose by chemical hydrogenation using hardwood sources. Danisco, also developed integrated process to produce xylitol from xylose wastes of the pulp and paper industry.

1.6.3 Biological process

Biological process for production of xylitol is a promising alternative for chemical production method. It involves two approaches, one is fermentation based using microorganism and other is enzyme based. Fermentation based approach involves use of fungi, yeast, bacteria and recombinants. Enzyme based approach involves use
of xylose reductase (XR). The microbiological process uses bacteria, fungi, yeast, and recombinant strains to produce xylitol from pure xylose or a hemicellulosic hydrolysate. Xylose can be used as pure xylose or xylose hydrolysate with or without detoxification. Enzymatic process is discussed in detail as below.

1.6.3.1 Enzymatic process
The enzymatic production of xylitol from pure xylose is a good alternative for chemical production due to complete stochiometric conversion to xylitol. Kitpreechavanich et al. studied the enzymatic conversion of d-xylose into xylitol using xylose reductase (XR) of Candida pelliculosa coupled with the oxidoreductase system of Methanobacterium sp. and achieved 90% conversion of xylose to xylitol at 35°C and pH 7.5 in only 24 h time period. This reaction completed with stochiometric conversion of xylose and equivalent consumption of NADPH, with successful regeneration of coenzyme using a membrane reactor [12]. Similarly, Neuhauser et al. also studied the fed batch enzymatic conversion of xylose to xylitol using NADH dependent C. tenuis XR coupled with formate dehydrogenase (FDH) from C. boidinii at pH-controlled enzyme reactor with recycling of enzyme and 2.8 g/L/h productivity [13].

1.6.3.2 Fermentation process
Biotechnological production of xylitol from lignocellulosic waste
Xylose and other sugar such as mannose, galactose, arabinose and rhamnose are obtained from lignocellulosic biomass and finds tremendous applications in both pharmaceutical and food industry. Xylose is second largest available cheap sugar on the earth. Xylose to xylitol conversion process plays significant role in bioprocess refinery concept. Thus, the process development from xylose to xylitol using biomass feedstock is gaining more attention of scientific community.
Figure 1.3 shows a simplistic flowchart for biological production of xylitol from lignocellulosic. Waste generated in agriculture is tested for xylitol production because all these waste generates xylose after acidic hydrolysis method. A pretreatment or hydrolysis step method on these lignocellulosic feedstock releases pentose sugar more susceptible to biotechnological usage. Examples of such pretreatment methods are phosphoric acid [14] and sulphuric acid [15]. The waste treatment with such acids at temperature of above 100 °C, leads to generation of other toxic inhibitor which inhibits the microorganisms, hence detoxification of xylose hydrolysate becomes top most priority before biological treatment.

The fermentative production of xylitol involves usage of microorganism as biocatalyst using yeast, bacteria and fungi. This process uses xylitol production from commercial pure xylose or hemicellulosic hydrolysate with or without detoxification. The production of xylitol using bacteria and fungi has been studied to a lesser extent compared to that using yeast strains. Bacteria studied are Enterobacter liquefaciens [16], Corynebacterium sp. [17] and Gluconobacter oxydans. Very few studies are available for filamentous fungi also [18]. Yeasts are studied extensively as compared to bacteria and fungi in last few decades by several researchers, as yeasts are good xylitol producers [11, 19, 20]. Barbosa et al. studied forty four yeast strains of five genera for xylose to xylitol production and found that Candida guillermondii and C. tropicalis were the best xylitol producers [21]. These yeasts produced xylitol titer of 77.2 g/L from 104 g /L of xylose in high cell density fermentation under aerobic conditions. da Silva and Afschar optimized fermentation conditions in continuous cultivation of Candida tropicalis for xylitol production using C. tropicalis. They also produced xylitol with 77-80 % yield from 100 g /L d-xylose [22].
This study of yeast for xylitol production by various researchers has confirmed *Candida* sp as best xylitol producers and best candidate for further research. In the fermentation process using yeast, the yield of xylitol obtainable from D-xylose is in a range of 65–85% of the theoretical value [11]. Fermentative xylitol production is dependent on certain factors, such as process parameters, expensive nutrients, process type and huge water consumption. Thus, Industrial application of fermentation process is challenging and time consuming. Overall process time increases due some process activities like sterilization of media, fermenter, seed culture development with substantial input of energy, labor, time leading to decreased process productivity. But, it confers advantages over chemical process due to its overall lower cost, selectivity, use of xylose without any purification, milder reaction condition and no any environmental threat [10].

Hence, the fermentative production for xylitol stands as good viable alternative for chemical process but its viability has some challenges such as optimization of process, culture variables, microbial stability, and nutritional factors related to carbon, nitrogen and micronutrients.
Fig 1.3 Stepwise presentation of biological production of xylitol from biomass
1.7 Biochemical pathway for xylitol

After xylose entry into the microbial cell, NADH or NADPH dependent xylose reductase (XR) reduces xylose to xylitol. Coenzyme specificity varies in different yeasts. Xylitol is either secreted from the cells or subsequently oxidized to d-xylulose by xylitol dehydrogenase. The xylulokinase converts xylulose to xylulose-5-phosphate by phosphorylation. Then, xylulose-5-phosphate enters the hexose monophosphate pathway. The xylose catabolic pathway is shown in figure 1.4.

Nonoxidative reactions of d-xylulose-5-phosphate by ribulosephosphate-3-epimerase, ribosephosphate isomerase, transaldolase and transketolase result in the formation of glycerol-3-phosphate and fructose-6-phosphate, which can then be converted to pyruvate in the Embden-Meyerhof pathway. Pyruvate either can be decarboxylated and reduced to ethanol or can enter the tricarboxylic acid cycle.

An oxidative pentose phosphate bypass might also be present in some d-xylose fermenting yeasts. In this pathway, a portion of the fructose-6-phosphate is oxidized via 6-phosphogluconate to ribulose-5-phosphate, thereby releasing CO$_2$ and generating NADPH need in biosynthesis pathways. Two NADP$^+$ molecules are reduced to NADPH with release of carbon dioxide. The energetics of the reactions is such that the equilibrium strongly favors NADPH formation.

In an alternative bypass, phosphoketolase can split d-xylulose-5-phosphate in to glyceraldehyde-3-phosphate and acetyl phosphate. The enzyme is present in *Pachysolen tannophilus*, where it may be involved in acetic acid formation under anoxic conditions.
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Figure 1.4 Pathway for xylose and glucose metabolism in yeasts.

Xylose → Xylitol → Xylulose → Xylulose-5-P → Hexose monophosphate Pathway

Non oxidative reactions:
- Glyceraldehydes-3-phosphate
- Fructose-6-phosphate

Oxidative cycle (NADPH regeneration):

EMP pathway:
- Ethanol + CO₂

TCA Cycle

NAD⁺ NADH

Pathway for xylose and glucose metabolism in yeasts.
Xylose metabolism in yeasts produces variety of products like xylitol, ethanol, CO$_2$, acetic acid and polysaccharides. Product yields depend on the regulation of carbon flow through available metabolic routes. As xylose to xylulose-5-phosphate is necessary for its utilization by the central metabolic pathways, it becomes difficult to isolate xylitol production from d-xylose in yeasts from pathway converting d-xylose to other products. Hence, a fair play of optimization required between xylose metabolic flux to xylitol, and xylose flux used for further metabolism. However, under oxygen-limiting conditions, the xylitol accumulates and excretes into the external medium. When xylitol is used as a carbon source, it has to be taken up from the external medium. Therefore, xylitol permeates the membrane in both directions. The uptake of xylitol was also found in *C. guillermondii* [23]. After total d-xylose consumption, the xylitol concentration starts to decrease, indicating that yeast takes up xylitol as a substrate for cell growth.
1.8 Literature Survey:

Compiled literature survey for various free or immobilized yeast cells used for xylitol production from various biomass, pure sugars is mentioned in Table 1.2.

Table 1.2 Compilation of various microorganism and feedstock used for xylitol production

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Microorganism</th>
<th>Feedstock</th>
<th>Fermentation performance</th>
<th>Ref</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td><em>C. guillermondii</em> FTI20037</td>
<td>Sugarcane bagasse</td>
<td>0.81 g/g and 0.60 g/L/h</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td><em>C. guillermondii</em> FTI20037</td>
<td>Rice straw</td>
<td>0.84 g/g and 0.14 g/L/h</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td><em>C. tropicalis</em> HDY -02</td>
<td>Corn cobs</td>
<td>0.73 g/g and 0.74 g/L/h</td>
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</tr>
<tr>
<td>4</td>
<td><em>C. tropicalis</em> JH030</td>
<td>Rice straw</td>
<td>0.71 g/g and 0.44 g/L/h</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td><em>Pichia stipitis</em> NRRL Y-30785</td>
<td>Corn stover</td>
<td>0.61 g/g and 0.18 g/L/h</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td><em>C. tropicalis</em> IFO 0618</td>
<td>D-xylose</td>
<td>0.64,2.67</td>
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<tr>
<td>7</td>
<td><em>C. tropicalis</em> DSM 7524</td>
<td>D-xylose</td>
<td>0.70,0.28</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td><em>Debaryomyces Hansenii</em> NRRL Y-7426</td>
<td>Sugarcane bagasse</td>
<td>0.71,0.22</td>
<td>30</td>
</tr>
<tr>
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<td><em>D. hansenii</em> UFV-170</td>
<td>D-xylose</td>
<td>0.54,0.24</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td><em>C. guillermondii</em> FTI 20037 (Ca alginate, STR)</td>
<td>Sugar cane hydrolysate</td>
<td>0.81,0.41</td>
<td>32</td>
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<tr>
<td>11</td>
<td><em>C. tropicalis</em></td>
<td>Corn cob hydrolysate</td>
<td>0.66,1.9</td>
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</table>
### Chapter 1

#### Process conditions affecting xylitol production

For the industrial production of xylitol, it is very important to increase the xylitol production rate and yield by optimising xylitol production conditions. Several factors play critical role in maintaining the growth of microorganism and xylitol production. The most extensively studied factors for microbial growth are temperature, pH, aeration rate, inoculum concentration and nutrients (carbon, nitrogen, and vitamin sources etc.). Proper control of these products shows paramount importance in obtaining good titer, yield and high efficiency. There are various and many studies performed related to the various process and media parameter optimization, which are discussed below.

#### 1.9.1 Effect of aeration and agitation

Aeration plays an important role in deciding the fate of pyruvate produced by glycolate metabolism. Under aeration, oxidative metabolism occurs and all pyruvate produced is converted via the TCA cycle. Under microaerophilic condition, oxy-reductive metabolism (fermentation) occurs and pyruvate is reduced to ethanol or other compounds. It is also found that the two pathways also able to occur simultaneously. Hence, amount of oxygen or aeration condition decides the xylitol production.

<p>| | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>12</td>
<td>D. hansenii (Ca alginate)</td>
<td>Corn cob hydrolysate</td>
</tr>
<tr>
<td>13</td>
<td>C. guillermondii (Polyvinyl alcohol hydrogel)</td>
<td>Sugar cane bagasse hydrolysate</td>
</tr>
<tr>
<td>14</td>
<td>C. mogii ATCC 18364 (Liquid emulsion membrane)</td>
<td>Xylose</td>
</tr>
</tbody>
</table>
production from xylose or only energy generation for cell growth and maintenance [37]. In bacteria, d-xylose metabolism involves first d-xylose to xylulose conversion by xylose isomerase (XI), followed by xylulose 5-phosphate production by xylulokinase (XK) and then further metabolism by the pentose phosphate pathway (PPP). In yeasts and filamentous fungi, d-xylose is reduced to xylitol by xylose reductase (XR) and further oxidized to d-xylulose by xylitol dehydrogenase (XDH). This reduction and oxidation process is regulated by the difference in the enzymes specificity for coenzymes, NADPH in the case of XR and NAD+ in the case of XDH. This redox imbalance plays an important role in accumulation of xylitol in yeast under controlled agitation and aeration [38]. Figure 1.4 shows the key reactions in d-xylose metabolism during xylitol production using xylose as substrate.

El-Baz et al. studied the effect of aeration on xylitol production using C. tropicalis in a medium with 20 g/L xylose. They found that xylitol production was increased when medium aeration was reduced by doubling the medium volume from 20 mL to 40 mL in 250 mL Erlenmeyer flask [37].

Mussatto and Roberto also studied effect of aeration and agitation on xylitol production by C. guillermondii FTI20037 from rice straw hemicellulosic hydrolysate at different agitation rates (200 rpm, 300 rpm and 500 rpm) in a fermenter. They concluded that xylitol production is affected by stirring speed, reaching a maximum yield of 0.84 g/g at 300 rpm [25].

Meyrial et al. evaluated the C. guillermondii in microaerophilic conditions for xylitol production form xylose and other nonxylose sugar. They concluded that xylitol produced with 0.63 g/g of xylitol with negligible ethanol but non xylose sugar was mainly converted to ethanol and biomass [39]. Horitsu et al concluded that xylitol accumulated by C. tropicalis at low dissolved oxygen concentrations due to high
levels of NADPH and NADH in the cells, leading to reduction of d-xylose and conversion into xylitol [29].

1.9.2 Effect of nitrogen sources on xylitol

Nitrogen sources are of interest in biological production of xylitol due to high cost of media. There are two types of nitrogen sources studied, one is organic nitrogen source viz yeast extract, peptone and casamino acids and other is inorganic nitrogen source viz urea, ammonium sulphate, ammonium phosphate and sodium nitrate. There are many studies using urea as nitrogen source for xylitol production [40]. Rodrigues et al evaluated effect of nitrogen sources like urea and ammonium sulphate on xylitol production by Pichia stipitis YS-30 using corn stover hydrolysate as xylose source. They concluded that when urea was used instead of (NH4)2SO4, xylose consumption and ethanol production rates increased by 25% and 34%, respectively [28]. Ko et al studied xylitol production using urea as a nitrogen source replacing yeast extract in waste wood fermentation with producing xylitol titer of 112.27 g/L. This strain favored urea as nitrogen source as 1.3 fold xylitol production increased as compared to yeast extract as nitrogen source. These results emphasizes that urea is the most promising nitrogen source among other nitrogen sources [41]. In contrast, Hongzhi et al. in his media optimization study with corn hydrolysate for xylitol production using different nitrogen sources like peptone, yeast extract, urea, ammonium nitrate and ammonium sulphate, concluded that only (NH4)2SO4 and yeast extract had a significant impact on xylitol production. Dahiya also evaluated the effect of eight inorganic nitrogen source and four organic nitrogen source on xylitol production and obtained maximum xylitol yields of 16.7 g/L and 30.6 g/L with ammonium acetate and yeast extract, respectively [18]. Onishii et al concluded that carbon/nitrogen ratio plays significant role in polyol formation in Pichia. Low
nitrogen medium favours xylitol production as compared to high nitrogen medium [42]. Rodrigues et al concluded that half cell yield was obtained with urea as nitrogen source compared with ammonium sulphate using corn stover hydrolysate for xylitol production [28].

1.9.3 Effect of xylose concentration

As renewable lignocellulosic materials are abundantly available in nature, it becomes efficient raw material for high value added biochemical. E.g. lignocellulosics are corn cobs, rice straw, sugarcane bagasse, wheat straw, sawdust and oat hulls. The hemicellulosic can be hydrolyzed to xylose and then fermented to xylitol. Several studies have used corn cob hydrolysate as carbon source [43, 44]. Kamat et al evaluated isolated *Cyberlindnera (Williopsis) saturnus*, from mangrove forests using detoxified corn cob hydrolysate for xylitol production. They observed that strain produced 29.1 g /L xylitol from 65 g/L xylose, 13/L glucose and 6.3 g/L arabinose with complete consumption of glucose in first 24 h [45]. Misra et al. obtained 20.92 g/L xylitol using *C. tropicalis* as yeast in corn cob hydrolysate using 1% (v/v) sulphuric acid and 15.19 g/L xylitol from 52.71 g/L xylose from concentrated hydrolysate [44]. Rocha et al obtained xylitol of 4.77 g/L titer while studying ethanol production from non-detoxified cashew bagasse hydrolysate (29.08 g/L glucose, 24.48 g/L xylose and 11.33 g/L arabinose), using *K. marxianus* CE025 [15]. Srivani and Setty optimized various process parameters using *C. parapsilosis* NCIM-3323 from synthetic xylose and obtained xylitol titer of 28.14 g /L at optimum initial xylose concentration, pH and temperature: 60 g/L, 3.5 and 30°C respectively [46]. Vajzovic et al. studied effect of inhibitors like furfural, 5-hydroxymethylfurfural and acetic acid on xylitol production using 30 g/L xylose and concluded that inhibitor concentration above 3 g /L has negative impact on xylitol production [47].
Osmophilic yeast produces higher concentration of xylitol at higher initial concentration of xylose. Microorganism tolerating higher concentration of sugar i.e. higher osmotic pressure produces xylitol with higher production rate and yield. Horitsu et al studied the effect of interaction of substrate concentration and aeration rate using yeast *C. tropicalis*. They concluded that cell concentration is lower with low substrate, low aeration rate and higher xylitol production and higher cell growth with higher initial xylose concentration and aeration rate [29]. Meyrial et al evaluated effect of initial xylose concentration (10 g/L-300g/L) using *Candida guillermondii* and found out that xylitol titer increased with increase in initial xylose. The high xylose yield of 0.75 g/g obtained with 300 g/L initial xylose and low yield obtained with low initial xylose due to xylose flux mainly towards biomass formation. Xylitol production rate was also higher and 2.4 fold specific productivity obtained with 200 g/L initial xylose as compared to that of 10 g/L initial xylose [39]. Xylitol production study with *Petromyces albertensis* with varying concentration of initial xylose resulted 36.8 g/L xylitol titer with 100 g/L xylose but declined at 150 g/L initial xylose. This might be due inhibition of metabolic enzymes by higher substrate concentration or high osmotic pressure [18].

**1.9.4 Effect of presence of other sugars in the medium**

Hsiao *et al* studied the effect of glucose on xylitol production and xylose consumption by yeast *Candida* and *Schizosaccharomyces*. They found out the inhibitory effect of glucose on xylose consumption by yeast for short period till glucose reached lower concentration. This shows that catabolite repression is not the regulatory mechanism and short time inhibition is due to intracellular concentration of glucose or its catabolite [48]. Meyrial *et al* selectively evaluated the *Candida guillermondii* to ferment other sugars like glucose, arabinose, mannose and galactose
instead of xylose. They concluded that rapid fermentation of these sugars with carbon flux mainly for growth and ethanol production [39]. *Candida guillermondii* is potential candidate for xylitol production as it selectively converts xylose from mixture of sugar of hemicellulosic hydrolysate.

### 1.9.5 Effect of presence of methanol

The addition of methanol stimulates xylitol production as 8.5 % xylitol titer increased as compared to control when d-xylose medium was added with 1.0 % v/v methanol. This might be due to excess of NADH produced by oxidation of methanol, which in turn increases xylose to xylitol reduction. This phenomenon also observed in sorbitol and iditol production by the methanol utilising yeast *Candida boidini* [49].

### 1.9.6 Effect of biotin

Lee *et al* studied the effect of biotin on xylitol production using *Pachysolen tannaphylus* and *Candida guillermondii*. In presence of biotin, *C. guillermondii* favours xylitol production and *P. tannophylus* boosted ethanol production over that of xylitol [50].

### 1.9.7 Effect of temperature and pH

Several studies in biological process for xylitol production were carried out at temperature range of 30–37°C. Thermotolerant yeast like *K.marxianus* allows less risk of contamination and increased productivity due to increased enzymatic activity [51]. Rodrussamee *et al* studied the potential of thermo tolerant *K. marxianus* DMKU3-1042 for xylitol and ethanol production from hemicellulosic hydrolysate at high temperatures (30° C, 40° C and 45° C). Cell growth and sugar conversion obtained at all temperatures [52]. Ethanol titer was 2.5 g/L at 72 h, and xylitol titer was 4.3 g /L after 48 h at 30 °C but xylitol titer was 7.0 g/L at 40 °C. Three isolated
strains of *K. marxianus* (IMB2, IMB3 and IMB4) from Indian distillery were studied for xylose consumption at high temperatures (40–45°C) at different pH (4.5, 5.0 and 5.5). It was concluded that lower temperature (40°C) and pH 5.5 were better for ethanol 2.08 g/L and xylitol titer 7.36 g/L using the IMB4 strain after 96 h of fermentation [53]. Srivani and Setty optimized environmental conditions like temperature (25–35°C) and initial pH (3–6) for xylitol production by *Candida parapsilosis* NCIM-3323. They noted maximum xylitol productivity at pH 3.5, but productivity decreased with further increase in pH, productivity decreased. In a temperature study, productivity increased up to temperature 30°C and afterwards, drop occurred in productivity [46]. Ramesh *et al.* obtained the optimum temperature (31.8°C) and pH (7.25) for xylitol production from corncob hemicellulosic hydrolysate by *Debaryomyces hansenii* var. hansenii (MTTC 3034) using a statistical optimization of response surface approach [54]. El-Baz *et al.* obtained maximum xylitol titer of 36.25 g/L in synthetic xylose medium using *C. tropicalis*.

The optimum temperature for xylitol production by *Candida* and *Saccharomyces* yeasts has been reported to be 30°C [37]. Sampaio *et al.* thoroughly studied and reported the xylose to xylitol conversion by *Debaryomyces hansenii* UFV-170 at different temperature and pH. They reported the optimum pH range as 4-8 with the maximum xylitol titer of 37.5-41.8 g/L, volumetric productivity, 0.70-1.0 g/L/h, specific productivity, 0.19-0.30 g/g/h and xylitol yield on consumed xylose, 0.70-0.76 g/g. The optimum temperature range for xylitol production ($T_{opt}$) was 30-35 °C, with xylitol yield, 0.74-0.77 g/g and volumetric productivity of 0.96-1.1 g/L/h. At low temperature, (15 °C), most of the xylose flux was towards TCA cycle and biomass generation, xylitol production was very low [31].
1.10 Bioreactor operation modes

Maximum reported studies in the literature for xylitol production are batch mode. However, some reports are available in other operation mode like fed batch mode. More xylitol titer reported is with fed batch mode [55,56]. Silva et al. studied semi continuous mode for xylose to xylitol conversion by porous glass immobilized C. guilliermondii in fluidized bed reactor (FBR). This reactor study was conducted for seven cycles (672 h) with fresh medium for every new cycle; first and second cycle gave xylitol yield of 0.79 and 0.57 g/g respectively and decreased in next cycles [55].

Santos et al also studied FBR with the immobilized-cell using sugarcane bagasse hydrolysate and evaluated the effect of aeration rate (AR) and carrier concentration (Cs) on xylitol production. They concluded that Cs and AR had negative effect on xylitol yield and only AR had positive influence on volumetric productivity. Productivity was 0.44 g/L/h and yield was 0.25 g/g at AR of 0.093 /min and Cs of 62.5 g/L, this might be due to faster cell metabolism at higher oxygen level. Xylose mass was diverted towards biomass production and less towards xylitol at highest levels of AR and Cs [56]. Salgado et al. evaluated series of two CSTR type bioreactors (2 L and 10 L) in series for lactic acid and xylitol production in sequential mode from hemicellulosic hydrolysate of vine shoot trimmings. They achieved xylitol titer of 5.1 g/L with product yield of 0.55 g/g at a dilution rate of 0.043 h⁻¹ [57].

1.11 Biomass hydrolysis and detoxification

Lignocellulosic biomass is mainly composed of cellulose (34-50% w/w), hemicellulose (19-34% w/w), lignin (11-30% w/w) and fewer amounts of pectin,
protein, extractives and ash. Composition of biomass varies with the plant species, age and growth conditions [68]. Cellulose is a major homopolysaccharide part forming skeleton and consisting of polymerized 10000 or more d-glucose units linked by a 1,4-glucosidic bonds. Hemicellulose is second major heteropolysaccharide consisting of 200 polymerised xylose, glucose, galactose, mannose, arabinose, glucoronic acid with acetyl side chains [59]. Hemicellulose and Lignin interlinks with cellulose to form structural matrix. Lignin, a third major part is a nonpolysachharidic consists of sinapyl alcohol, coumaryl- and coniferyl- units bonded by alkyl-, aryl, and combination of both ether bonds. Biomass is a composite material as cellulose, hemicellulose, and lignin are closely associated and lignin serves as a protective layer. Table 1.3 shows feedstock used for xylitol production and their xylose content.

**Table 1.3 Feedstock and their xylose content**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Feedstock</th>
<th>Xylose content(%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Corn stover</td>
<td>22.4</td>
</tr>
<tr>
<td>2</td>
<td>Corn fiber</td>
<td>16.8</td>
</tr>
<tr>
<td>3</td>
<td>Wheat straw</td>
<td>21.2</td>
</tr>
<tr>
<td>4</td>
<td>Switch grass</td>
<td>20.4</td>
</tr>
<tr>
<td>5</td>
<td>Office paper</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Hemicellulose is second major sugar in plant cell walls (cellulose being the first) abundantly available and a potential substrate for ethanol and xylitol production. Hemicellulose degradation by acidic hydrolysis yields xylose as main component with arabinose, mannose, galactose and glucose as other sugars. But with these sugar products, hydrolysis also produces 35 other byproducts or microbial inhibitors [59].
Inhibitors are furan derivatives (furfural and 5-hydroxymethylfurfural (HMF)), phenolic compounds, weak acids (acetic, levulinic, formic acid) and ions of heavy metals (nickel, aluminium, chromium, etc.) [60]. Furfural and HMF are potential inhibitor due to their high concentration and these are formed by hydrolysis of hexoses and pentoses, respectively. Fermentability of hydrolysates obtained by acidification for xylitol or ethanol production is inversely proportional to the concentration of furan compounds. Second types of inhibitors are phenolic compounds which cause biological membrane to lose its integrity destroying its ability to serve as selective barrier [61]. Acetic acid also acts as inhibitor after certain concentration level and is released from acetyl hemicellulose groups and formic acid is a product of HMF degradation. Hence before fermentation of hydrolysate several methods are employed to detoxify the hydrolysate such as physical (Evaporation of volatile inhibitors), chemical (addition of calcium hydroxide or calcium oxide), adsorption (ion-exchange resins or activated carbons) and biological (microbial and enzymatic) to remove inhibitors of xylitol production [62]. Mateo et al. studied effect of different detoxification methods like liming with calcium oxide, calcium hydroxide or sodium hydroxide and adsorption with activated carbon on hydrolysate of olive pruning residues. They concluded that minimal (30 %) decrease in phenolic compounds and slight decrease in acetic acid (<7%) was achieved without any sugar loss with sodium hydroxide treatment. The calcium based treatments removed more lignin derived compounds (56–71%), acetic acid (about 50%) and total furan content (approximately 70%) with higher sugar loss, making method unsuitable for detoxification of olive pruning hydrolysate. They found that activated charcoal can be best alternative as it reduces inhibitors (46% of acetic acid, 81% of phenolic compounds and 98% of total furans), with the possibility of regeneration [63].
Activated carbon [64] and ion-exchange resins [24] have shown some promising results for completely removing the major fermentation inhibitors. Also, nowadays microbial treatments for detoxification of lignocellulosic hydrolysate are gaining significant attention. Fonseca et al. studied biological detoxification of hemicellulosic hydrolysate obtained from corn fiber, rice straw and sugarcane bagasse using yeast *Issatchenkia occidentalis* CCTCC M 206097. They achieved maximum removal of inhibitors with less sugar loss when biological treatment applied after five fold concentration of hydrolysate. Inhibitor like syringaldehyde, ferulic acid, furfural and 5-HMF were reduced by 66.67%, 73.33%, 62%, and 82% respectively in 24 h of their biological treatment [65].

### 1.12 Microorganisms

Biotechnological methods for xylitol production have evaluated various microorganism and feedstocks. Most of these studies are focused on *Candida sp.* Biotechnological xylitol production is less expensive as compared to chemical process and has lot of advantages. Bacteria and fungi also evaluated for xylitol production. Rangaswamy and Agblevor studied 17 bacterial cultures of the genera *Serratia*, *Cellulomonas* and *Corynebacterium* for potential xylitol production and concluded that *Corynebacterium* sp. B-4247 produces maximum xylitol of 10.05 g/L [17]. Cirino et al. used recombinant *Escherichia coli* W3110 and achieved xylitol titer of 38 g/L [66]. Yeasts are potential producer of xylitol from hemicellulosic hydrolysates and occupies major portion of literature survey as shown in Table 1.0. According to literature reports, Microorganisms with high NADPH dependent XR activity are best candidate for xylitol production. Misra et al. also found that *C. tropicalis* as best xylitol producing yeast among 18 studied strains of yeasts with xylitol titer of 12.11 from 50 g/L initial xylose concentration [44]. Studies have
shown variable values for xylitol production by biotechnological processes using yeasts from industrial residues as shown in Table 1. It can be readily observed that the results are very discrepant and are related to the different microbial species and growth conditions involved (carbon and nitrogen sources, pH, aeration, and so forth). Controlling these conditions is of significant importance for optimizing the xylitol production process.

1.13 Xylitol production by immobilized yeasts

The most effective way to improve the process efficiency and productivity is the use of immobilized cells as it allows higher cell concentration in the reactor and reuse of cells in repeated batch. A good performance of immobilization system is related to support/matrix properties, procedures used reactor configuration and biocatalytic conditions. There are numerous matrices evaluated for immobilization of cells and for xylitol production. The use of Polyvinyl alcohol (PVA) for cells immobilization resulted into 48.5 g/L xylitol yield with 5 weeks of repeated nine consecutive batch mode of fermentation [66]. The immobilized microbial cell in polyacrylic hydro gel produces 0.73 g/g of xylitol yield from rice straw hemicellulosic hydrolysate [67]. The conversion of d-xylose to xylitol is more than 95 % by the NADH-dependent XR from yeast. Carvalho et al [68] and Branco et al [69] reported average productivity of 0.43 g/L/h and 0.21 g/L/h, respectively with repeated usage of alginate immobilized C. guillermondii in stirred tank reactor. In another study, Santos et al. obtained 70% xylose to xylitol conversion using sugarcane bagasse fiber immobilised C. guillermondii cells [70]. Cunha et al. reported with repeated use of immobilised C.guilliermondii cells, productivity increases [71]. Silva and Afschar studied the fluidised bead reactor with the porous glass immobilized the cells of C. tropicalis DSM 7524 without any success in recycling of cells [72].
Lohmeier-Vogel et al studied agars-immobilized *C. tropicalis* ATCC 32113 system with the glucose and d-xylose by using nuclear magnetic resonance. This immobilisation process not increased xylose or glucose metabolism and concluded that increased oxygen supply to immobilised matrix also not increases xylose metabolism [73].

### 1.14 Gaps of Literature study –

- Chemical process is scaled up in industry due to lack of competitive microbiological method
- Chemical process can work only when 99.9 % pure xylose is used as substrate. Otherwise, catalyst deactivation occurs
- Production of xylitol through the chemical process is expensive due to difficult separation and purification step
- Fermentation process on an industrial scale is not yet practiced due to reduced productivity
- Enzymatic production involves experiments with commercial xylose containing medium, no work is done using C5 stream
- Leaching of Co-enzyme results into loss of cell activity
- Reactivation of reducing potential (i.e. NADH) is required with XR enzyme immobilization system
Chapter 1

1.15 Problem formulation and focus of study

Despite a wide range of xylitol applications, the use of xylitol as sweetener is limited due to high price via chemical method. The biological process approach to xylitol production from xylose present in the lignocellulosic biomass may provide an alternative for the chemical process. Several microorganisms are evaluated to assess their potential for biochemical production using lignocellulosic hydrolysate stream. There are very few reports for efficient biotechnological production of xylitol from lignocellulosic waste without any detoxification. Hence, this creates one of the gaps for further study. Screening and development of hydrolysate inhibitor tolerant microbial strain plays a significant role in xylitol production at the industrial scale. A focus is required to thoroughly understand type of feedstock, hydrolysis method, microbial potential, biocatalysis conditions for development of integrated technology for biological production of xylitol at an economic industrial scale under biorefinery concept.

Biomass utilisation as feedstock requires its acid hydrolysis which generates lot of inhibitor toxic to microorganism in biological process. Detoxification of hydrolysate adds further cost to the process and creates major challenge for process viability. So there is need for adaptation of microorganism to inhibitor or screening of microbial strain which will work in hydrolysate stream without any detoxification.

Xylitol production is influenced by nutritional, fermentation and physiological growth factors associated with micro-aerophillic conditions. Several studies have evaluated free or immobilised cells for optimising xylitol production in batch or continuous mode using different reactor
configurations. Cell immobilization by using various hydrogel is one of the progressive approaches for the creation of the immobilized biocatalysts.

1.16 Rationale and Significance of the Study

Rationale of the Study

✓ To enhance profitability of second generation lignocellulosic biorefinery by value added bioprocessing of crude C5 stream to xylitol
✓ Develop a green alternative, high performance bioprocess for cost competitive xylitol production
✓ Chemical process is costly and involves use of catalyst which is not environment friendly

Significance of the Study

✓ Crude xylose conversion to xylitol is seldom pursued both in academic and industrial manufacturing due to process challenges
✓ Overcoming these challenges via bioprocessing using industrial biotech as the driver will produce bioxylitol of superior quality and cost competitiveness
✓ This study opens up new frontier in bioxylitol production specific to Indian feedstocks and operating condition
✓ Biological process will produce xylitol at an economical cost from renewable feedstock
1.17 Aims and Objectives

**Statement of aim:** To enhance performance of bioxylitol production using high efficiency biological process

1. Use of immobilization as means to increase xylose to xylitol yield by reducing xylose flux towards biomass
   A. Evaluate various immobilization supports to achieve high efficiency immobilization and cell recycling
   B. Optimize media composition and bioprocess parameter in immobilized system
   C. Comparative study with free cell system
2. Develop optimal reactor design to overcome oxygen transfer limitation in xylose to xylitol bioconversion

1.18 Expected Outcome and Significance

1. Comparative evaluation and selection of reactor type
2. High yield
3. High productivity and titer
4. Lower capex and opex
5. Fundamental studies related to OTR and ICB kinetics
6. First time study of scale up relation associated with bioxylitol production in ICB.

**Significance**

Bioxylitol production using fermentative mode is not commercially practiced. Our study will overcome some of challenges related to the xylitol productivity, titer, yield, recyclability of cells, costly fermentation media, use of pure xylose as substrate to produce bioxylitol at competitive price as
compared to chemically derived xylitol. Our study will provide better insight into immobilization based xylitol production, effects of inhibition, OTR, reusability of biocatalyst and will help to accelerate commercial adaptation of bioprocessing of xylitol.

1.19 Organization of the thesis

Chapter 1 provides a general introduction to bio-based chemical, xylitol, its physicochemical properties, various methods of production, biochemical pathway, applications and advantages over other polyols and increasing market demand. The existing methods of production and biocatalyst are summarized. The research problem and challenges associated with fermentative production of xylitol are discussed. The research problem statement, aims and objectives, organization of the thesis are provided in this chapter.

Chapter 2 provides data about screening of supports and optimization of immobilization conditions for xylitol production from nondetoxified corn cob stream. Potential microorganism was immobilized on suitable matrix like sodium alginate, poly vinyl alcohol, polyacrylamide gel, agarose gel, gelatin and k-carrgeenan. Immobilization conditions were optimized using full factorial design methods to select the best significant variable and their concentration. Beads prepared with optimized conditions were tested in non-detoxified stream for fermentation performance and cell reusability.

Chapter 3 covers statistical optimization of xylitol fermentation media components like carbon source xylose, inorganic nutrients like ammonium sulphate, urea, buffering components like potassium dihydrogen phosphate
and process parameter like pH, temperature, agitation and weight of biocatalyst. Plakett Burman method and response surface method were used for statistical media optimization. Anova analysis was used to select the statistical significance of variable. Actual validation results were compared with predicted results.

Chapter 4 describes the comparative study for immobilized cell and free cells in optimized media. The various parameters were tested to check the advantages and effect of immobilization on xylitol production. The parameters tested and described are xylose concentration, xylose with and without inhibitors, pH, temperature, storage stability and recyclability study.

Chapter 5 describes study related with optimal reactor design with fluidised bed reactor and stirred tank reactor. The reactor design study involved height to diameter (H/D) ratio, change in sparger design, volumetric mass transfer coefficient study (K_L,a study). The immobilized cell with optimised media were treated in fluidized bed reactor with different H/D ratio. Further free cell study was performed in FBR with optimum H/D ratio to compare the performance with immobilized cell. Stirred tank reactor study involved with change in sparger design, volumetric mass transfer coefficient study with change in agitation, aeration. This study was performed with immobilized bead, optimized media in 5 L reactor with different K_L,a and results were compared with free cells. These immobilized beads were again tested in reactor with same fermentation condition to check the recyclability of beads.

Chapter 6 compiles summary and conclusion of the research work and author contribution to the body of knowledge.
Chapter 1

1.20 References:


Chapter 1


Chapter 1


55. Silva, S.S., Santos, J.C., Carvalho, W., Aracava, K.K., Vitolo, M. 2003. Use of a fluidized bed reactor operated in semi continuous mode for xylose-to-


Chapter 1


