Xylitol Biosynthesis in the Yeast Candida

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Xylitol is an industrially important chemical due to its commercial applications. The use of xylitol as a sweetener as well as its utilization in biomedical applications has made it a high value specialty chemical.

xylitol agricultural residues grasses xylose reductase

1. Introduction

The industrially important specialty chemical xylitol, whose annual commercial production is now approaching 40,000 tons [1], has several commercial applications (**Figure 1**). The highest value commercial application for xylitol use is as an alternate sweetener in such products as chewing gum and various foods including ice cream and candy [2]. Beyond its utilization as a sweetener, biomedical applications exist for xylitol, including being used to prevent ear inflammations and its ability to stimulate murine hybridoma cell production [3][4][5]. Xylitol is also used as a sugar substitute for diabetics [5]. With respect to human health, it has been reported that xylitol promotes the growth of beneficial bacteria by promoting the synthesis of propionate and short-chain fatty acids in the human colon [6]. The chemical synthesis of xylitol from xylose still remains the dominant production method of xylitol production [5][7]. The industrial process to synthesize xylitol involves the chemical hydrolysis of xylan followed by the hydrogenation of the resultant hemicellulose hydrolysate by catalysts including palladium and nickel [5][7]. During the chemical synthesis of xylitol, both high temperatures and high pressure are usually required, with typical temperatures being 80–140 °C and 8–10 MPa hydrogen pressure being employed [5]. These processes are highly energy intensive, which makes the production costs of synthesizing xylitol very expensive considering the high temperature, pressure and metal catalyst used for a sustained period of time [5]. Another problem with the chemical production of xylitol is the synthesis of side products such as arabitol by the chemical catalyst, which necessitates the purification of the xylitol being produced [5]. Clearly, the cost of producing xylitol by a large-scale process is going to be high, which makes this possible production process not feasible economically in the long term [7][8][9]. A more realistic approach for large-scale xylitol production would be to use the pentose sugars present in hydrolysates of low-value plant biomass [10]. Low-value biomass residues are excellent candidates for the large-scale production of xylitol. Such low-cost residues are readily available as a raw material for xylitol synthesis compared to the process components used during the chemical bioconversion of xylose into xylitol. This could include utilizing hydrolysates of agricultural residues or hydrolysates derived from various species of grasses [11][12][13][14][15][16]. It has been well documented that a number of hydrolysates from agricultural residues contain a high level of pentoses that could be used for bioconversion into xylitol. Similarly, it has been shown that a variety of hydrolyzed grasses support biobased xylitol production. One advantage of grasses is that they generally require low fertilizer input while producing high yields. With the fiber content of the grasses being relatively high, it has

been demonstrated that both physical and enzymatic treatments of the grasses can result in a hydrolysate containing a high xylose concentration.

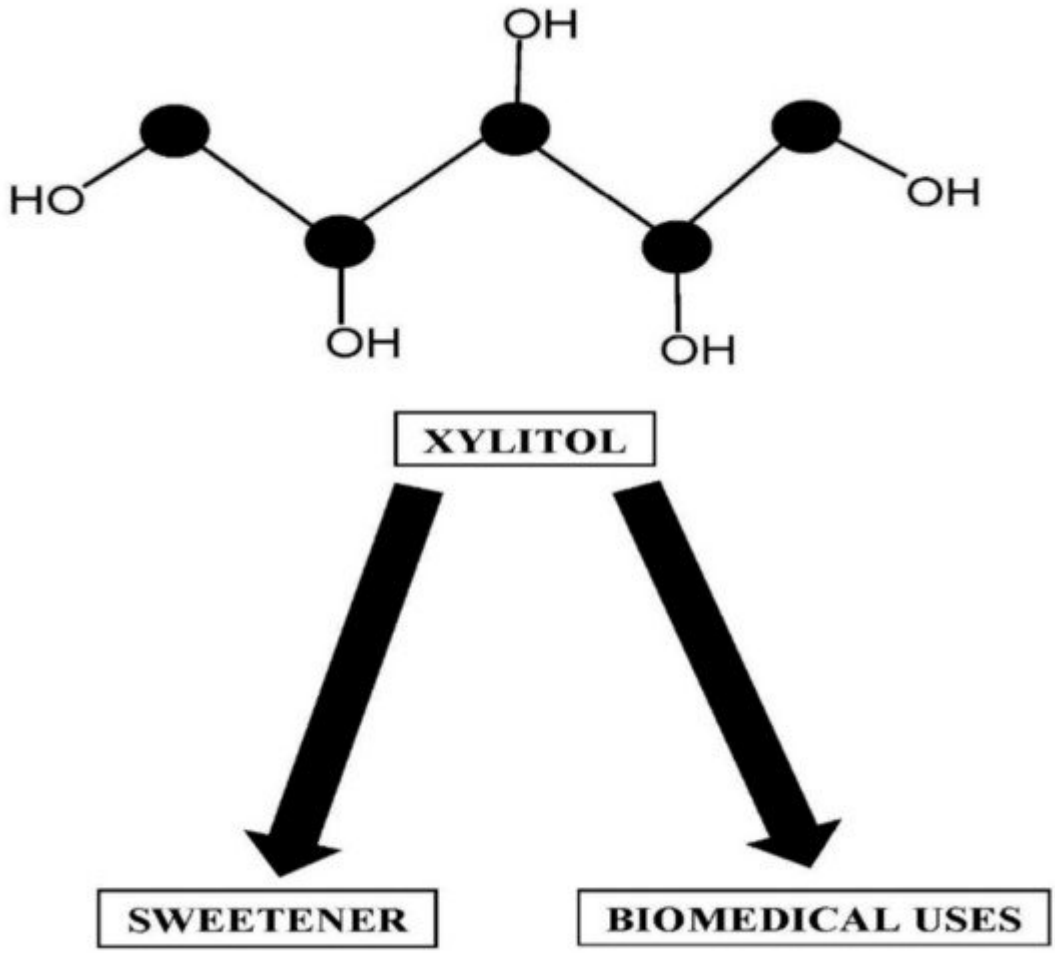

Figure 1. Structure of xylitol and its applications.

With hydrolysates containing high xylose levels, the bioconversion of the xylose present to xylitol can be accomplished by species of the yeast *Candida*. Prior work has identified a number of *Candida* species that are able to synthesize xylitol from the xylose present in hydrolyzed agricultural residues or grasses [17][18][19][20][21]. These yeast species contain the enzyme xylose reductase that primarily catalyzes the NADPH-dependent reduction of xylose to xylitol [1]. The resultant xylitol produced can be purified using activated carbon adsorption columns from the hydrolysate-containing medium [22].

2. Pathway of Xylitol Biosynthesis in the Yeast *Candida*

The ability of species of the yeast *Candida* to produce xylitol from xylose is due to the presence of the enzyme xylose reductase within their cells. Xylose reductase (EC 1.1.1.21) catalyzes the reduction of xylose by NADPH to the polyalcohol xylitol and $NADP^+$ (**Figure 2**). It should be mentioned that some isolated yeast xylitol reductases can also use NADH as a cofactor. There is much interest in better understanding the xylose reductase structure to

improve its efficiency in producing xylitol. Better understanding of yeast xylitol reductases could lead to the genetic engineering of this enzyme so that it has a greater affinity for its substrates, resulting in increased xylitol production. An engineered high activity xylitol reductase could efficiently synthesize xylitol from the high xylose-containing agricultural residues and grasses on an industrial scale. Xylose reductase has been investigated in a number of *Candida* species including *Candida tenuis*, *Candida guillermondii*, *Candida parapsilosis*, *Candida intermedia* and *Candida tropicalis* [23][24][25][26][27][28][29][30][31][32].

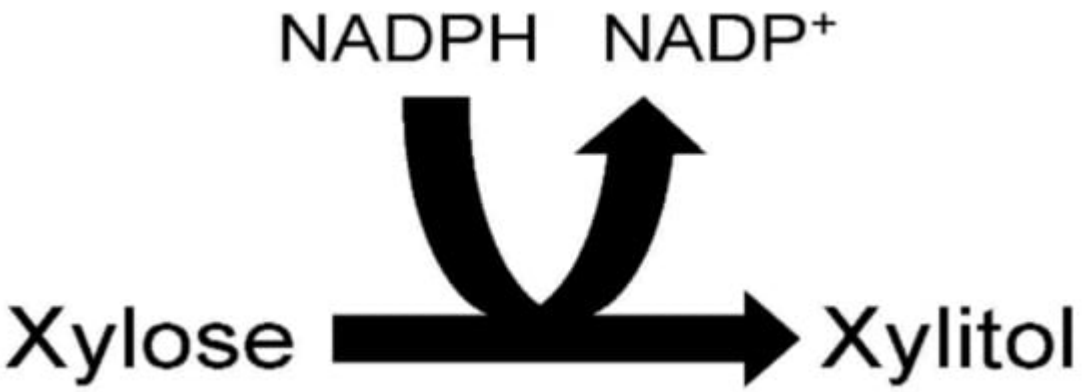

Figure 2. Xylose reductase reaction.

The xylose reductase from *C. tenuis* has been purified by dye ligand affinity chromatography and ion exchange chromatography to homogeneity [23]. Structurally, the purified enzyme was found to be a monomer or homodimer with each subunit having a molecular weight of 36,000–43,000 daltons [23][26]. This reductase from *C. tenuis* appears to differ from other family members of the aldo-keto reductase group, which exist purely as monomers. The isoelectric point of the reductase was determined to be 4.7 [23]. The reductase prefers NADPH compared to NADH as a cofactor based on binding specificity and it has been shown that $NADP^+$ is a strong competitive inhibitor of the reaction [23]. The reductase was shown to contain a catalytic tetrad consisting of tyrosine, lysine, aspartic acid and histidine residues essential to its activity [26]. The lysine residue has been shown to be located near the coenzyme-binding site. Further, the presence of the epsilon amino group of the lysine residue in the reductase is thought to be a key element in its mode of catalysis [26]. In a pH 7.0-buffered assay incubated at 25 °C, the *C. tenuis* reductase had a K_m and k_{cat} of 87 mM and 18.2/s, respectively, for xylose. The reductase had a K_m of 4.8 µM and 21.9/s, respectively, for NADPH, while it had a K_m of 25.4 µM and k_{cat} of 18.1/s, respectively, for NADH [23].

The xylose reductase gene fragment isolated from *C. guilliermondii* ATCC 20118 was cloned into *Pichia pastoris* GS115 [24]. This resulted in two xylose reductase activities being synthesized [24]. The xylose reductase activity, secreted extracellularly, was found to utilize only NADPH as its cofactor [24]. The molecular weight of this xylose reductase was shown to be 36,000 daltons [24]. The reductase was composed of 317 amino acids with the pI of the reductase shown to be 5.7 [24]. It was noted that the reductase was highly hydrophobic relative to amino acid composition including many leucine residues. It was also shown that three cysteine residues and seven histidine residues were present within the reductase structure similar to the *P. pastoris* xylose reductase [24]. In *C.*

guilliermondii FTI 20037, a prior report characterized xylitol reductase in a crude extract prepared from cells cultured in a medium containing sugarcane bagasse hydrolysate [25]. The kinetics of the crude reductase were analyzed and it was observed that the xylose reductase had a K_m of 64 mM for xylose and a K_m of 9.5 µM for NADPH [25]. The optimal pH for the reductase was 5.5 and the enzyme was most stable when buffered at a pH between 5.0 and 5.5 [25]. The optimal temperature for the reductase was 65 °C and its enzyme activity was still stable after being heated to 60 °C for 10 min [25].

In *C. intermedia*, it was noted that the yeast contained two different forms of xylose reductase [27]. The isoforms of the enzyme were purified to homogeneity by a combination of affinity chromatography and ion exchange chromatography with relatively high yields. One form of the reductase was strictly specific for NADPH as a cofactor with a K_m of 61 µM and a k_{cat} of 14.6/s, while its K_m and k_{cat} for xylose were 82 mM and 14.6/s, respectively. The second form of the reductase was able to utilize either NADPH or NADH as a cofactor [27]. The K_m of this reductase isoform for NADPH was 56 µM and its k_{cat} was 11/s, while its K_m for NADH was 28 µM and its k_{cat} was 11.2/s. The K_m of this reductase for xylose was about 50 mM and its k_{cat} was about 10/s [27]. Structurally, both isoforms exist as homodimers with individual subunits having a molecular weight of 36,000 daltons. The isoelectric point of each isoform was found to differ with the NADPH-specific isoform having a pI of 4.38, while the second form had a pI of 4.59 [27]. It is interesting to note that the number of titratable cysteines in each isoform also differed. The NADPH-specific isoform had five titratable cysteines, while the second form had only two [27]. The inactivation of the cysteines in the NADPH-specific isoform resulted in a total loss of its activity [27]. It was thought that the ratio of available NADPH to NADH was a determining factor as to which isoform of the reductase was most active in the *C. intermedia* cells [27].

The xylitol reductase gene in *C. parapsilosis* was cloned in *Escherichia coli* and the reductase was purified to homogeneity using ion exchange chromatography, affinity chromatography and preparative electrophoresis [28]. The molecular weight of the *C. parapsilosis* reductase was 36,629 daltons and was composed of 324 amino acids. The reductase was shown to have a high catalytic efficiency for xylose as a substrate. The K_m for xylose was calculated to be 31.5 mM, with its k_{cat} being 46/s. Interestingly, this reductase preferred NADH as its cofactor instead of NADPH [28]. The K_m of the reductase for NADH was 3.1 µM compared to the K_m for NADPH being 36.5 µM [28]. The k_{cat} of the reductase for NADH was 45.9/s relative to the k_{cat} for NADPH being 4.6/s [28]. The optimal pH for the reductase was 6.0, while its temperature stability was greatest if stored at 4 °C. The reductase was subject to non-competitive inhibition by its product xylitol. The reaction mechanism for the *C. parapsilosis* reductase was thought to be an ordered sequential bi bi mechanism as has been proposed for other xylitol reductases [28].

In *C. tropicalis*, the properties of xylose reductase have been investigated [30][31][32]. The xylose reductase from *C. tropicalis* has been purified by cloning the xylitol reductase gene into *Escherichia coli* [30]. The purified reductase was crystalized and analyzed using X-ray diffraction [30]. The properties of a crude xylitol reductase activity isolated from a strain of *C. tropicalis* adapted to a hydrolysate of tree sawdust were studied [31]. The reductase was shown to be specific for $NADP^+$ as its cofactor [31]. The reductase was still 95% active following 120 days at −80 °C. Further, the reductase was observed to be stable at a pH range between pH 5 and 7. The crude enzyme was

stable for 24 h when incubated between 25 and 40 °C. The K_m of the reductase was calculated to be 81.78 mM for xylose and 7.29 µM for NADPH [31]. The V_{max} of the enzyme for xylose and NADPH was 178.57 µM/min and 12.5 µM/min, respectively [31]. The K_m and V_{max} values for the reductase were thought to be associated with the high rate of xylitol production noted by the strain. The molecular weight of the reductase was 36,600 daltons [30]. Another study using recombinant versions of the *C. tropicalis* reductase found that a serine residue at position 279 of its structure allowed increased catalysis compared to the presence of a leucine or an asparagine residue at position 279 [32]. The importance of a serine residue at position 279 of the reductase was thought to involve the binding of NADPH [32].

In summary, when comparing the previously investigated xylose reductases isolated from various species of *Candida*, there appear to be some similarities. First, the xylose reductases isolated from species of *Candida* appear to exhibit similar molecular weights. In general, the pH optimum of many of the characterized reductases from *Candida* species was similar. In addition, some of the reductases synthesized by the *Candida* species had a strict cofactor requirement for NADPH, while some synthesized a reductase activity that preferred NADPH as a cofactor but could still use NADH as a cofactor to catalyze the reaction. With respect to the kinetic properties of the *Candida* xylose reductases, the affinity of the enzymes for the nicotinamide cofactor was usually much greater than their affinity for xylose as a substrate.

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