0273-2289/98/70–72—0331$10.25

Factors that Affect the Biosynthesis of Xylitol by Xylose-Fermenting Yeasts

A Review

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ABSTRACT

Xylitol is a sweetener with important technological properties like anticariogenicity, low caloric value, and negative dissolution heat. Because it can be used successfully in food formulations and pharmaceutical industries, its production is in great demand.

Xylitol can be obtained by microbiological process, since many yeasts and filamentous fungi synthesize the xylose reductase enzyme, which catalyses the xylose reduction into xylitol as the first step in the xylose metabolism. The xylitol production by biotechnological means has several economic advantages in comparison with the conventional process based on the chemical reduction of xylose. The efficiency and the productivity of this fermentation chiefly depends upon the microorganism and the process conditions employed. In this mini-review, the most significant upstream parameters on xylitol production by biotechnological process are described.

Index Entries: Xylose fermentation; xylitol; xylose-fermenting yeasts; hemicellulosic hydrolysates.

XYLOSE-FERMENTING YEASTS: BIOCHEMICAL CONSIDERATIONS

Traditionally, yeasts were considered nonfermentors of xylose *(1,2)*. Nevertheless, in later years several yeasts able to ferment xylose have been identified, namely *Pachysolen tannophilus, Candida shehatae, Candida tropicalis, Pichia stipitis, Debaryomyces hansenii,* and *Candida mogii (3–7)*. Xylitol is the main byproduct of these yeasts during the xylose metabolism. How-

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ever, the factors that regulate the production and excretion of xylitol have not been clearly established *(3,8–10)*.

There are two possible metabolic routes for the utilization of xylose by microorganisms. In prokaryotic organisms, the first step of the metabolism involves enzyme induction followed by xylose isomerization into xylulose. Thereafter, the xylulose is phosphorylated to xylulose-5-phosphate *(11)*. In eukaryotic organisms such as yeasts, the oxido-reductive xylitol pathway to xylose prevails. In these reactions, the xylose is reduced to xylitol by the xylose reductase (E.C. 1.1.1.21) enzyme linked to nicotinamide-dinucleotide phosphate in the reduced form (NADPH). Next, the xylitol is oxidized to xylulose by xylitol dehydrogenase (E.C.1.1.1.9) linked to nicotinamide-dinucleotide in the oxidized form (NAD^+). The xylulose formed is phosphorylated to xylulose 5-phosphate that can be converted into pyruvate through the connection of the phosphopentose pathway with the Embden Meyerhof Parnas pathway *(12)*. The major enzymes for the metabolic pathway utilized by xylose-fermenting-yeasts are: xylose reductase, xylitol dehydrogenase, xylulokinase, phosphopentose epimerase, phosphopentose isomerase, transaldolase, transketolase, phosphohexoisomerase, glucose 6-P dehydrogenase, lactonase, and 6-P glucose dehydrogenase.

According to Gong *(11)*, the xylose reduction to xylitol and the activation of the phosphopentose pathway in yeasts is controlled by the $NADP^+$ and NADPH disposability. The $NADP^+$ generation during the xylose reduction can stimulate the activity of the 6-P-glucose dehydrogenase which, in turn, stimulates the activation of the phosphopentose pathway. Jeffries, 1982, cited by Gong *(11)*, suggests that the xylose metabolism in yeasts operates in a coordinated and closed cycle, assuring the NADPH regeneration necessary to reduce xylose to xylitol. The xylitol produced will be catabolized only to eventually produce glucose 6-phosphate for regeneration of NADPH and to maintain the cycle. This NADPH regeneration is essential for a economic xylitol production starting from xylose. Through this mechanism, the xylose metabolism in yeasts by the phosphopentose pathway and subsequent glucose 6-phosphate oxidation generates 2 moles of NADPH per each mole of CO_2 liberated.

Taylor et al. *(13)* observed that the xylitol production is favored by an excess production of NADPH generated during the xylose catabolism. However, few papers report the quantitative aspects of the production and consumption of NADPH by yeasts. So, the xylitol production by yeasts seems to be essentially related with the NADPH pool and the xylose bioconversion into xylitol occurs primarily for coenzyme regeneration and probably as a mechanism of cellular detoxification.

The xylose reductase and xylitol dehydrogenase enzymes play a fundamental role in the xylose metabolism by yeasts *(14–16)*. The continued understanding of mechanisms regulating the activities of these enzymes may allow the establishment of conditions that favor the production of the desired product. According to Skoog and Hahn-Hagerdal *(17)*, the xylose reductase and xylitol dehydrogenase enzymes differ in specificity by the

coenzymes NADPH, NADH, NAD^+, and $NADP^+$. In *Candida utilis*, xylose reductase requires only NADPH as a cofactor, whereas xylitol dehydrogenase requires only NAD *(17,18)*. In other yeasts able to ferment xylose, like *Candida shehatae*, *Pichia stipitis*, and *Candida tenius*, xylose reductase requires both NADPH and NADH as cofactors *(18,19)*. The initial reactions of the xylose metabolism by yeasts are limited in different degrees because of a double specificity of xylose reductase by NADPH and NADH *(20)*. The accumulation of xylitol by yeasts in a medium containing xylose is associated with the complete absence of xylose reductase activity dependent on NADH *(19)*. The xylose reductase and xylitol dehydrogenase enzymes are inducible by cell growth in a medium containing xylose *(17)*. Bicho et al. *(21)* noticed that both in *Phachysolen tannophilus* and in *Pichia stipitis* the induction of these enzymes by the xylose was inhibited by glucose. These authors concluded that the repression of these enzymes was the principal regulating system in yeasts that metabolize xylose and that this repression can inhibit the yeasts' potential to ferment pentoses in lignocellulosic substrates.

XYLITOL-PRODUCING YEASTS

The development of an economic fermentative process for xylitol production involves the selection of microbial yeast strains with high productivity, the establishment of conditions that maximize the conversion of xylose into xylitol, and the optimization of these parameters for process scale-up. From a study of 58 species of yeasts that utilize xylose aerobically, Onishi and Suzuki *(22)* found that the best xylitol producers were *Candida polymorpha*, *Candida tropicalis*, *Candida guilliermondii*, *Pichia miso*, and *Hansenula anomala*. However, fermentation efficiency was only 40% between 7 and 10 d of cultivation. Gong et al. *(23)* also found that xylitol was the main metabolite formed during the xylose fermentation by yeasts. These authors concluded that a mutant strain of *Candida tropicalis* HXP2 is a promising xylitol producer, since its fermentation efficiency was approx 90% after 4 d cultivation. *Candida tropicalis* and *Candida guilliermondii* yeasts also proved to be suitable for xylitol production, presenting a high fermentation yield (81%) after 48 h cultivation, and an insignificant formation of byproducts *(9)*. The yeast *Debaryomyces hansenii* was able to produce xylitol with a conversion efficiency of approx 70% in only 28 h cultivation *(19)*. Although this microorganism is very promising, *Candida guilliermondii* has been considered as an outstanding xylitol producer. However, there are few studies on the xylose metabolism of this yeast, as well as on the factors regulating the xylitol production by this microorganism.

BIOTECHNOLOGICAL PARAMETERS IN XYLITOL FORMATION BY YEASTS

The xylose bioconversion into xylitol is regulated by different factors such as initial xylose concentration *(20,23–26)*, pH *(27,28)*, presence of glucose *(21,28,29)*, and aeration *(30–32)*.

The initial concentration of xylose in the fermentation medium has great influence on the xylitol production by yeasts. High concentrations of xylose in the medium further the xylose consumption by the yeasts and consequently, enhances the xylitol production (*26*). Gong et al. (*23*) noticed that *Candida tropicalis* HXP2 produced more xylitol when the xylose concentration was increased from 5 to 20%. This behavior was also exhibited by *Candida shehatae* Y-12856, *Pachysolen tannophilus* NRRL Y-2460, and *Pichia stipitis* NRRL Y-7124 (*33*). The correlation between xylitol accumulation and xylose concentration may be a consequence of an oxygen reduction resulting from high cell densities of highly concentrated substrates. The osmotic pressure exerted by xylose concentrations over 30% can interfere with the xylitol production (*26,34*). This interference can be reduced or even eliminated by altering the process to fed batch (*34*).

The extracellular pH has great influence on the metabolic process and on the product formation as well. This is evident from the behavior of the xylose-fermenting yeasts in relation to the pH of the medium. In general, the yeasts grow better in acid media at pH between 3.5 and 3.8. The tolerance limits are between 2.5 and 8.0, for several species. The optimum pH range for xylitol production by *Candida shehatae* is 3.5 to 4.0 (*35*). For *Pachysolen tannophilus* the xylitol production is maximum and constant at pH ranging from 3.0 to 5.8 (Watson, 1983, cited by Du Preez et al., (*cf. 36*). Silva and Afschar (*26*) detected significant xylitol production at pH 2.5 in cultures of *Candida tropicalis*.

Temperature has a significant effect on growth, metabolism, viability, and fermentative capacity of the yeasts (*11*). The optimum temperatures for yeast growth are between 20 to 30°C, although some species grow within the range of 0 to 47°C. The xylose-to-xylitol conversion into xylitol seems to be stimulated by the temperature increase (*9*). Indeed, Du Preez et al. (*36*) found that the xylitol production by *Candida shehatae* enhanced when the temperature was raised from 22 to 36°C. This was also observed for *Candida guilliermondii*, the maximum accumulated xylitol concentration (23 g/L) and the highest specific growth velocity (0.78/h) occurring at temperatures of 30 or 35°C (*9*).

The presence of hexoses, such as glucose, in the fermentation medium is also a critical factor that regulates the xylitol production by yeasts. The presence of glucose may repress the activity of the key xylose reductase enzyme involved in the xylose conversion into xylitol resulting in low yields of the product (*21*). In a fermentation medium without glucose an accentuated increase in the xylitol production by *Candida guilliermondii* was detected (*28,29*). In fermentations performed with *Candida shehatae* and *Pichia stipitis* containing a mixture of sugars (xylose and glucose), glucose was preferably consumed (*37*). These authors observed that a lag period was necessary to synthesize the enzymes of the xylose metabolism before this sugar was metabolized. However, this period can be significantly reduced with the inoculum growth in a medium containing xylose as a car-

bon source. In these conditions, the enzymes necessary for the xylose metabolism are induced and a simultaneous utilization of sugars is observed *(37)*. The simultaneous utilization of sugars, especially by yeasts, is a phenomenon still little studied and literature reports with conflicting opinions about the effect of hexoses on the utilization of xylose.

The constituents of the fermentation medium determine whether the fermentative processes are feasible. They should meet the elementary requirements for producing metabolites and forming biomass. It is known that the yeasts demand several inorganic ions such as: Ca^{+2}, Co^{+2}, Cu^{+2}, K^{+}, Mg^{+2}, and Mn^{+2} in micro and millimolar quantities for optimum culture growth in synthetic media *(38)*. Many of these ions activate the enzymatic reactions or participate in various biosynthetic reactions. Normally, it is necessary to supplement the fermentation medium with nutritional factors such as yeast extract, peptone, and meat extract. The yeast extract stimulates the yeast growth, mainly because it is rich in vitamins and amino acids. Increasing the concentration of this nutrient from 1 to 30 g/L resulted in the decrease of the xylitol production *(9)*, since the accumulation of xylitol by yeasts seems to be associated with growth limitation and acts as a secondary metabolite. A simple and inexpensive fermentation medium has been pursued by our research group *(31,39,40)*.

Oxygen is important for the xylose metabolism by yeasts *(17,29–31)*. The biochemical and physiological aspects of xylose metabolism requiring oxygen are not entirely known yet. This metabolism appears to be related to sugar transport, coenzyme regeneration, and the ATP production during the oxidative phosphorylation. Some yeasts need oxygen for an optimum xylose fermentation. Under aerobic conditions, a high production of cell mass occurs, whereas under anerobic conditions a great part of the xylose is converted into xylitol and the ethanol production is small *(17,31,40,41)*. A likely explanation for the oxygen demand is based upon the necessity for recuperation of the coenzymes required in the initial steps of the xylose utilization *(18)*. According to these authors, the xylose metabolism through xylose reductase linked to NADPH and xylitol dehydrogenase linked to NAD^{+}, under anerobic conditions, brings about an overproduction of NADH, which paralyzes the subsequent metabolism reactions, and consequently stimulates the accumulation of xylitol in the culture medium. In fact, the xylitol production by *Pachysolen tannophilus* increases by decreasing the aeration rate *(41)*. The same happens to the xylitol production by *Candida guilliermondii (9,32)*, *Candida parapsilosis (30)*, *Debaryomyces hansenii (6)*, and *Candida mogii (7,42)*. The agitation of the medium is also an important parameter for the xylose fermentation into xylitol, since the oxygen transference rate is favored by higher agitation speeds. According to Ojamo et al. *(34)* for xylitol production by *Candida guilliermondii*, it is essential that the oxygen supply to the yeast be restricted. High xylitol yields are obtained under appropriate agitation and aeration conditions. Silva et al. *(29)* found that the xylitol production by *Candida guilliermondii* was favored by

increasing the agitation speed from 200 to 300/min, whereas increasing to 400/min promoted an increment in the xylose consumption to the disadvantage of the xylitol formation. The agitation role in this fermentative process is still little known. However, for xylitol production, it seems that a moderate agitation is necessary and that the maximum production is likely to be reached by means of an adequate agitation/aeration relationship.

The initial cell concentration also influences the xylose fermentation to xylitol by yeasts. Increasing the initial concentration of the inoculum from 0.25 to 3.0 g/L results in a greater xylose consumption *(43)*. Besides, the amount of xylitol produced by *Candida guilliermondii* increases by 20%. Similar results were obtained by Barbosa et al. *(9)* using the same yeast strain, but under distinct nutritional conditions. These authors obtained yields of approx 81% with media inoculated with high cell concentrations. This xylitol accumulation in the medium, with increased cell concentrations, is a consequence of the oxygen decrease, which favors the xylitol production by yeasts *(9,29,32)*.

SOME ASPECTS OF THE XYLITOL PRODUCTION FROM HEMICELLULOSIC HYDROLYSATES

In most studies on xylitol production by fermentative processes, xylose of analytical grade is commonly the main substrate. Despite the fact that agroforest residues are abundant, inexpensive, and contain a large proportion of xylose, few studies have reported the utilization of hemicellulosic hydrolysates coming from these materials for xylitol production. The main problem in the fermentation of these hydrolysates is the presence of toxic compounds released from the lignocellulosic structure during the hydrolytic process, as well as those originated from the sugar degradation *(44)*, which inhibit the microbial growth and the fermentative activity of the yeasts. In this way, several methods have been proposed with the purpose for minimizing this effect. According to Felipe et al. *(31)* for an effective xylitol production from sugarcane bagasse hydrolysate is very important a previous adaptation of the cells to the hydrolysate. Alves *(45)* verified that *Candida guilliermondii* produced more xylitol when the sugarcane bagasse hydrolysate is first treated with CaO to adjust the pH to 7.0 and subsequently treated with H_3PO_4 to lower the pH to 5.5, adding 2.4% of activated charcoal. Under these conditions, 90% of the initial xylose (48 g/L) contained in the hydrolysate was consumed after 40 h of batch fermentation, and the xylitol concentration was 24.2 g/L, which corresponds to a 67% conversion efficiency. As for eucalyptus hemmicellulosic hydrolysate, Silva et al. *(46)* observed that the maximum xylitol production (54 g/L) occurs when the hydrolysate is first treated with CaO until reaching pH 8.4 and then treated with H_3PO_4 until the pH decreases to 6.0. The pH of fermentation is another important factor in the fermentation of

hemicellulosic hydrolysates. Its effect is related to the acetic acid concentration in the hydrolysate. Acetic acid concentration higher than 3.0 g/L inhibits the *C. guillermondii* capability to convert xylose into xylitol *(47)*. The nonionized acetic acid, which is found in the medium at pH < 7.0, probably acts as an inhibitor of the yeast metabolism. Therefore, the inhibition of xylose/xylitol bioconversion can be related to the coupled effect of low pH and undissociated acetic acid concentration over 5.0 g/L *(48)*. These results show that the hemmicellulosic hydrolysates from agroforest residues can be efficiently utilized in fermentative processes for xylitol production after an initial treatment designed to remove or reduce the compounds known to be toxic to cell metabolism.

LARGE-SCALE XYLITOL PRODUCTION: SCALE-UP CONSIDERATIONS

Knowing the factors that regulate the xylose metabolism to xylitol and determining the fermentative parameters that maximize this bioconversion is fundamental for establishing an economic process for xylitol production. The biotechnological process, whose efficiently has been demonstrated on a laboratory scale, demands optimization and scale-up studies before transfer to the productive sector. The scale-up of fermentative processes, particularly of aerobic fermentations involving non-newtonian fluids, is difficult to achieve. The methods employed are mostly empirical and based upon similitude concepts and nondimensional groups. The existing correlations are limited and, in general, the variables considered to be important to the process are the fermenter geometry, the agitation frequency, the potency transmitted to the system, the oxygen transfer, and the rheological properties of the fluid. For these empirical methods to be successfully utilized, it is necessary to identify the factor having the strongest influence on the process efficiency and then use it as a scale-up criterion. As mentioned before, the dissolved oxygen concentration in the medium is one of the factors that regulates the bioconversion of xylose into xylitol. The volumetric coefficient of oxygen transfer (K_La) should be used as a criterion to scale-up processes requiring oxygen. Thus, when the K_La is maintained constant, in both scales (laboratory and pilot plant), other variables (potency, agitation frequency, and diameter of the tank) can be determined for a larger scale, based upon empirical correlations already developed.

No literature was found on the scale-up of xylose fermentation to xylitol. Considering that the existing correlations were developed for particular cases under different operational conditions, it is necessary to determine suitable correlations for the development of new technologies for xylitol production on a large scale.

CONCLUDING REMARKS

Several studies have been published on xylitol production from xylose by yeasts. However, to determine the best fermentation conditions, most authors have used Erlenmeyer flasks in rotary shakers. Also, in most cases the discontinuous fermentation system has been employed. Few studies specify the conditions for xylitol production in large- or laboratory-scale bioreactors. Likewise, few studies exist on the utilization of hemicellulosic hydrolysates from agroforest residues as the raw material for this bioconversion.

The number of research groups interested in a new technology for xylitol production using the biotechnological processes has grown considerably.

Among the environmental factors that exert influence on the xylitol production by xylose-fermenting yeasts, the dissolved oxygen concentration is noteworthy and must be carefully controlled.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of "Fundação de Amparo à Pesquisa do Estado de São Paulo" FAPESP and "Conselho Nacional de Desenvolvimento Científico e Tecnológico" CNPq/Brazil. The authors are also grateful to Maria Eunice Machado Coelho for the revision of this paper.

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