Emerging technology for fermenting D-xylose

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In the past four years, numerous yeasts which convert D-xylose to ethanol have been reported. The conversion occurs most readily under aerobic conditions. Various aspects of this conversion have provided new insight into the mechanisms and metabolic regulation of ethanol fermentation in yeasts. Although specific fermentation rates, product yields and product concentrations are significantly lower with D-xylose than with D-glucose, technology is emerging which may prove to be feasible for commercial fermentation of D-xylose-containing waste streams.

D-xylose is the second most abundant sugar in nature, comprising up to 25% of the total dry weight of woody angiosperms and an even larger fraction of some agricultural residues where it exists as the polymer xylan. Even though it is not yet cheap nor commercially abundant, D-xylose, along with other hemicellulosic sugars, can be obtained in good yield (80-90% or more) through acid or enzymatic hydrolysis of the hemicellulosic fraction. Moreover, D-xylose (or oligomeric xylan) is present in many waste streams from sulfite and dissolving pulp mills, fiberboard and hardboard manufacturing plants. Combined use of D-xylose and D-glucose during production of chemicals or fuel (ethanol) from angiosperm feedstocks could improve the overall process economics and using D-xylose from waste streams could reduce disposal costs and provide alternative by-product credits for existing processes.

Xylose-fermenting organisms

The anaerobic production of ethanol from D-xylose was first demonstrated by Karczewka in 1951. This observation, however, went largely unrecognized - a major review in 1976 recorded that roughly half of all yeast species listed would assimilate D-xylose for aerobic growth but none would ferment it anaerobically. Moreover, in a recent taxonomic treatise on yeasts, 64% of the species listed are cited as capable of assimilating xylose aerobically, but none are cited as capable of fermenting this sugar. Another recent taxonomic synopsis, however, notes that a few yeast species - most conspicuously Pichia stipitis, Candida shehatae, Pachysolen tannophilus and Brettanomyces naardenensis - ferment D-xylose to ethanol at various rates. In the last four years at least 41 yeast species, including 23 of the genus Candida and eight of the genus Pichia, have been shown to produce some ethanol from D-xylose. In most instances, the conversion to ethanol occurs aerobically.

The discrepancy between results of classical taxonomic methods for determining fermentative activity and current findings stems largely from the fact that in many instances, production of ethanol from D-xylose by yeasts is either obligately aerobic or is greatly stimulated under aerobic conditions, and the classical methods do not provide for aeration during screens for fermentative activity.

Current efforts to ferment D-xylose largely began with the discovery by Wang, Shopsis and Schneider in 1980 that Schizosaccharomyces pombe and various other yeasts would ferment D-xylose, the keto-isomer of D-xylose, to ethanol. This finding was significant because D-xylose can be readily converted to D-xylulose by D-xylose isomerase (= glucose isomerase). Since this enzyme is so readily available and produced on such a large scale, technology for the conversion of D-xylose to ethanol by a two-stage isomerization and fermentation was rapidly developed. Interest in the two-stage process waned, however, when the direct fermentation of D-xylose to ethanol by Pa. tannophilus and other yeasts was discovered. Of these species only a few, most notably Pa. tannophilus and C. shehatae, will carry out the fermentation at rates of practical interest (Table 1).

Several conclusions are immediately evident from Table 1. First conventional D-glucose fermentations by S. cerevisiae are 6-35 times faster than even the best D-xylose fermentations by C. shehatae or Pa. tannophilus. In this same regard, the D-glucose specific fermentation rate (g ethanol (g cell dry weight)/h) of S. cerevisiae is 8-10 times faster than the D-glucose specific fermentation rate of these two other species. Second, reported ethanol yields (g product produced/g substrate consumed) with D-xylose are only about 56-82% of those reported for D-glucose. Third, maximum attainable ethanol concentrations from D-xylose are only about 23-46% of those attainable from D-glucose (Table 1).

Taken together, these facts present a dismal picture for the D-xylose fer-
mentation. Ultimately, D-xylose must compete economically with D-glucose as a feedstock. In view of the relative ages of the two technologies, however, there is room for some optimism. Moreover, it is possible that by using D-xylose from waste streams, a plant operator could avoid disposal charges and thereby offset some of the higher process costs incurred in the D-xylose fermentation.

Selection of improved strains

Improvements in the D-xylose specific fermentation rate and final ethanol concentration have been obtained principally by isolating better strains from nature, and by mutating and selecting strains in the laboratory. Genetics and strain selection are just beginning with D-xylose-fermenting yeasts. More progress has been made with *Pa. tannophilus* than with *C. shehatae* or *Pi. stipitis*. Methods for crossing strains have been developed for *Pa. tannophilus*; various aneuploid and polyploid strains have been constructed and their capacities for ethanol production have been assessed. Increasing the chromosome number from the haploid to the diploid level resulted in a significant increase in the ethanol yield. Further increases in ploidy enhanced the ethanol yield and D-xylose specific fermentation rate to a lesser extent. Selection of *Pa. tannophilus* strains capable of rapid growth on xylitol-plus-nitrate medium also results in stable isolates of *Pa. tannophilus* exhibiting up to a two-fold increase in the volumetric rates (g ethanol l\(^{-1}\) h\(^{-1}\)) of D-xylose (and D-glucose) fermentations under aerobic conditions, and a 1.5-fold increase in the specific fermentation rate under anerobic conditions. Anaerobically, the cells do not grow; hence the apparent increase in the rate is lower than when observed aerobically.

There is general agreement that, at least under aerobic conditions, *C. shehatae* and *Pi. stipitis* strains are superior to all other known yeast species in their rates of D-xylose fermentation. Similarities observed between *Pi. stipitis* and *C. shehatae* have led to the suggestion that these might be the teleomorphic and anamorphic forms (the sexually perfect and asexual stages) of the same organism. However, significant differences exist among the various named strains and some strains exhibit considerable instability in both their morphological and fermentative activities. Strains of *C. shehatae* exhibiting high respiratory- and low fermentative-activities have been isolated on xylitol agar.

A recent quantitative screening of 56 yeast isolates identified as Candida species, *C. tenuis*, *C. shehatae* and *Pi. stipitis* showed *Pi. stipitis* CSIR Y633 to give the greatest yield of ethanol (0.45 g ethanol (g xylene)\(^{-1}\)) at the highest volumetric rate (0.92 g ethanol (g cells)\(^{-1}\) h\(^{-1}\)).

Respiration deficiency

Selection for respiration deficiency, as described for endothia (determined by the tetrazolium overlay method) generally show greater fermentative activity than those exhibiting higher respiratory capacity. It is worth noting that on yeast malt agar, the strain exhibiting most conspicuous petite/Grande transitions, *C. shehatae* ATCC 22984, reverts to a heterogeneous mixture of phenotypes with petite-like cells predominating. The relationships between respiration capacity and ability to grow on a particular carbon source are complex and incompletely understood, and because these strains of *C. shehatae* are unstable, analysis is difficult.

If a yeast strain does not possess the biochemical machinery enabling it to carry out a balanced fermentation of

Table I. Comparison of xylose- and glucose-specific fermentation rates of various yeast and bacterial species

<table>
<thead>
<tr>
<th>Cells</th>
<th>Fermentation rate</th>
<th>Yield</th>
<th>Max. conc. (g ethanol/l)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volumetric</td>
<td>Specific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pa. tannophilus</em></td>
<td>0.22</td>
<td>0.05-0.12</td>
<td>0.26-0.34</td>
<td>38.0</td>
</tr>
<tr>
<td><em>Pa. tannophilus</em></td>
<td>0.12</td>
<td>0.05</td>
<td>0.28-0.41</td>
<td>20.5</td>
</tr>
<tr>
<td><em>C. shehatae</em></td>
<td>1.31</td>
<td>0.28</td>
<td>0.29</td>
<td>26.0</td>
</tr>
<tr>
<td><em>C. shehatae</em></td>
<td>0.47</td>
<td>0.14</td>
<td>0.24-0.31</td>
<td>40.0</td>
</tr>
<tr>
<td>Glucose:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. shehatae</em></td>
<td>0.5</td>
<td>0.18</td>
<td>0.33</td>
<td>51.3</td>
</tr>
<tr>
<td><em>Pa. tannophilus</em></td>
<td>1.0</td>
<td>0.22</td>
<td>0.47</td>
<td>56.0</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>11.8</td>
<td>1.78</td>
<td>0.50</td>
<td>87.5</td>
</tr>
<tr>
<td><em>Z. mobilis</em></td>
<td>8.0</td>
<td>2.5</td>
<td>0.50</td>
<td>102.0</td>
</tr>
</tbody>
</table>

*grams ethanol produced/gram xylose consumed
grams ethanol (g cells dry wt)\(^{-1}\) h\(^{-1}\)
higher value obtained under aerobic conditions with 0.5% glucose additions
ferments D-xylose but not D-glucose
does not ferment D-xylose or D-glucose
xylose (see later), then the net accumulation of NAD(P)H under anaerobic conditions will stop metabolism. This effect is particularly apparent when a reduced carbon source such as xylitol is used.

Respiration deficiency has been previously reported to affect the use of other sugars by yeasts. For example, when respiratory competent strains of *S. cerevisiae* able to use D-galactose, maltose, sucrose, a-methyl glucoside and raffinose were converted into their petite forms, half of them lost the ability to ferment D-galactose and two-thirds of the petite strains would not use raffinose. An earlier report by Mahler and Wilkie had shown that conversion of wild-type *S. cerevisiae* to the petite form was accompanied by loss of the ability to grow on D-galactose, maltose or a-methyl glucoside. This phenomenon seems in some ways similar to effects observed on D-xylene metabolism, and it is likely that NAD(P) and NAD(P)H concentrations play important generalized roles in regulating sugar use by yeasts.

**Process considerations**

Not all process variables have been fully evaluated, but the effects of oxygen, D-glucose, nitrogen and pH are discussed in the following sections.

**Effect of oxygen**

Oxygen (aeration) has a profound effect on ethanol fermentations by yeasts; as has long been recognized - Pasteur described the inhibition of ethanol production by aeration in 188142. One hundred years later researchers realized that the Pasteur effect is only observed in washed (starved) cell suspensions. Less familiar but of equal or greater importance are the Custers, Kluyver and Crabtree effects. The Custers effect (also known as the negative Pasteur effect) is the transient inhibition of D-glucose fermentation following transfer of some yeasts (particularly *Brettanomyces*) to anaerobic conditions. The Kluyver effect is similar except that certain yeasts which will ferment D-glucose anaerobically, require oxygen to use other sugars. The Crabtree effect is the inhibition of respiration by low concentrations of D-glucose.

With the possible exception of the Pasteur effect, each of these regulatory mechanisms seems to have relevance to the D-xylene fermentation.

The aerobic fermentation of D-xylene by *C. tropicalis* is similar in some ways to the Custers or Kluyver effects. As mentioned previously, this organism (along with several less well documented *Candida* species) will produce small amounts of ethanol from xyllose under aerobic (or microaerobic) conditions, but it will not when completely deprived of oxygen. Unlike the Custers effect in which the cells adapt to anaerobic fermentation of D-glucose after 7-8 h, complete anaerobic inhibition of the D-xylene fermentation has been observed for at least 20 days.

Production of ethanol from D-xylene by *P. tannophilus* and by *C. shehatae* in the complete absence of oxygen has been demonstrated in several laboratories. The specific rates of ethanol production by *P. tannophilus* under aerobic and anaerobic conditions are not very different but anaerobically, significant amounts of xylitol accumulate in the medium. With both organisms, aeration greatly stimulates the volumetric rate of ethanol production. Some researchers have reported that *P. tannophilus* exhibits only minimal ability to ferment anaerobically. Others report that the fermentation proceeds well anaerobically. The procedural basis for discrepancies among the various reports is not completely apparent, but it might result from the medium or the pH used for cell growth. As an example, cells grown on nitrate as a nitrogen source did not show anaerobic fermentation in the presence of nitrate, whereas cells grown on ammonium did. Our current studies show that shifting *P. tannophilus* cells from aerobic growth at pH 3.5 to anaerobiosis at pH 6.5 greatly inhibits fermentative activity. Taken together, these findings suggest similar but separate roles for nitrate and pH in regulating the anaerobic fermentative mechanism. It is possible that two different xylene reductases are present or active under the different conditions. Other factors may function as well.

Recently, Bruinenberg et al. presented a biochemical explanation for the inhibition of fermentation under anaerobic conditions. It is based on the necessity of maintaining a balance between intracellular NAD and NADH. Assimilation of D-xylene by yeasts and fungi commonly proceeds first through reduction by NADPH to form xylitol, then through oxidation by NAD to form D-xylulose. The oxidative portion of the pentose phosphate pathway provides the NADPH, resulting in the oxidation of D-xylene and transfer of reducing power from the NADPH pool to the NADH pool. *C. utilis*, a yeast which can only produce ethanol from D-xylene under aerobic conditions, possesses the common D-xylene reductase which uses only NADH as a cofactor, hence its assimilation of D-xylene is dependent on the oxidative pentose phosphate pathway. The D-xylene reduc-
tase(s) of yeasts which ferment D-xylose anaerobically (such as *Pa. tannophilus* or *Pi. stipitis*) accept either NADPH or NADH, allowing the cells to recycle anaerobically the NADH generated during the oxidation of xylitol to D-xylose, and thus to bypass the oxidative phase of the pentose phosphate pathway (Fig. 1). The explanation presented by Bruinenberg et al., has not been consistently supported by subsequent biochemical studies. For example, Ditzelmüller et al. purified a xylose reductase of *Pa. tannophilus* to 95% homogeneity and found that it was specific for NADP(H). By comparison, Verduyn et al. purified the xylose reductase from *Pi. stipitis* and found that the activity with NADH was 70% that with NADPH. Both organisms are capable of xylose fermentations.

Other enzymes enter into the regulation of xylitol metabolism under aerobic and anaerobic conditions. For example, in examining purified xylitol dehydrogenase from *Pa. tannophilus*, Ditzelmüller et al. found that at physiological pH (7), the equilibrium of the reaction catalysed by this enzyme favored the accumulation of xylitol and that the oxidation of xylitol was strongly inhibited by NADH and ATP. Given these findings, it is easier to understand why *Pa. tannophilus* accumulates xylitol under anaerobic conditions.

The stimulation of D-xylose fermentation by *Pa. tannophilus* by aeration can be attributed largely to the fact that *Pa. tannophilus* requires oxygen for growth. Mahmourides et al. have identified a transition from oxidative growth to fermentation during the cultivation of *Pa. tannophilus*. During the fermentative phase, ethanol accumulates and dissolved oxygen concentration in the medium increases. Schvester, Robinson and Moo-Young examined the oxygen requirement of *Pa. tannophilus* in a quantitative manner, and found that a significant level of aerobiosis was necessary to stimulate growth and enhance the rate of ethanol production. These authors also observed that ethanol production lagged behind growth and did not start until the dissolved oxygen concentration was near zero. As noted earlier, several researchers found that the specific rate of D-xylose fermentation by *Pa. tannophilus* is little changed under aerobic and anaerobic conditions, even though the product yields may shift dramatically.

**Effect of D-glucose**

The relatively low yield of ethanol obtained during the fermentation of D-xylose seems to result, at least in part, from the concomitant fermentation of D-xylose and respiration of ethanol that occurs in *Pa. tannophilus*. It has been shown that the addition of small amounts (0.5%) of D-glucose to an active D-xylose fermentation has essentially no effect on the rate of D-xylose utilization, but markedly increases the aerobic yield of ethanol. The anaerobic yield of ethanol is not affected, leading to the conclusion that small amounts of D-glucose can suppress ethanol oxidation. *Pa. tannophilus*, therefore, appears to be glucose-sensitive and exhibits the Crabtree effect. *C. shehatae* differs in this regard in that its yield of ethanol, even when growing on pure D-glucose, does not generally exceed approximately two-thirds of the theoretical maximum. Moreover, its volumetric D-glucose fermentation rate is only about 50% greater than that observed with D-xylose.

**Effects of nitrogen and pH**

The nitrogen source is important for both *Pa. tannophilus* and *C. shehatae*. In the case of *Pa. tannophilus*, growth on nitrate can induce cells to higher specific fermentation rates when they are transferred to a less restrictive nitrogen source. *C. shehatae* does not use nitrate. Peptone, casein and especially yeast extract stimulate ethanol production in *Pa. tannophilus*, and an amino nitrogen source is very important for good ethanol production by *C. shehatae* (Fig. 2). Studies in our laboratory have shown that the maximum specific fermentation rate is attained with *C. shehatae* at pH 3.2-3.4, but that somewhat higher yields can be obtained at pH 2.6. The aeration provided by shaking 50 ml of medium in a 125 ml Erlenmeyer (equivalent to a sulfite-fermentation rate is attained with *C. shehatae*.)

**Other factors**

The fermentation of crude acid or enzymatic hydrolysates of wood presents problems over and above those encountered in the fermentation of pure D-xylose. Acid hydrolysis produces various incompletely characterized inhibitory compounds from the lignin and carbohydrate fractions. Enzymatic hydrolysates are easier to ferment but are presently more difficult to obtain: it is generally necessary to treat the lignocellulose with steam or mild solvent pulp to disrupt the lignified material before enzymatic depolymerization. Pretreatment releases the hemicellulose in a polymeric form along with some inhibitory compounds such as acetic acid or lignin degradation products.

One of the motives for considering the fermentation of D-xylose is to use hemicellulosic sugars generated in processes converting lignocellulose to ethanol. A few yeasts, most notably *C. lusitaniae* ATCC 34449, *C. blankii* ATCC 18735, *Pi. wickerhamii* ATCC 24215 and *C. tenuis* CBS 4435 (Ref. 10) can ferment both D-xylose and cellobiose. Cellobiose is produced during enzymatic hydrolysis and inhibits the activity of exocellobiohydrolase, an enzyme which is very important in the degradation of crystalline cellulose. An ability to ferment cellobiose therefore has obvious implications for simul-
taneous saccharification and fermentation. It should also be noted that *P. tannophilus* can ferment D-galactose and D-mannose to ethanol; it is, therefore, able to ferment most of the predominant plant mono- saccharides.

**Future directions**

The use of D-xylene as a feedstock for the production of ethanol has passed the initial flush of discovery and entered into the phase of process improvement and development. One should look for bench marks: (1) The production of at least 5-6% (w/v) ethanol with a yield of greater than 0.4 g/g in 36 h should be achieved before contemplating scale-up and commercialization. (2) Recovery and use of by-products such as acetic acid, glycerol and xylitol should be evaluated for their effects on process economics. (3) The possible production of novel chemicals from the pentose phosphate pathway along with traditional products such as citric acid, amino acids, vitamins and antibiotics should be considered. (4) An improved knowledge of the biochemical pathways, particularly of the regulatory and rate-limiting steps, is essential before one can expect progress through the application of contemporary genetic techniques. Finally, it is worth remembering that fermenting D-xylene is practicable only where it is a by-product of lignocellulose utilization, and that adapting yeasts and fermentation processes to particular waste streams or hydrolysates may present unique and difficult problems.

**Acknowledgement**

The author gratefully acknowledges Henry Schneider (NRC, Ottawa), Nancy Alexander (USDA, Peoria) and Kent Kirk (USDA, Madison) for useful discussions and critical readings of this manuscript.

**References**


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