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Ethanolic Fermentation of Pentoses in Lignocellulose Hydrolysates

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ABSTRACT

In the fermentation of lignocellulose hydrolysates to ethanol, two major problems are encountered: the fermentation of the pentose sugar xylose, and the presence of microbial inhibitors. Xylose can be directly fermented with yeasts, such as *Pachysolen tannophilus, Candida shehatae,* and *Pichia stipis,* or by isomerization of xylose to xylulose with the enzyme glucose (xylose) isomerase (XI; EC 5.3.1.5), and subsequent fermentation with bakers' yeast, *Saccharomyces cerevisiae.* The direct fermentation requires low, carefully controlled oxygenation, as well as the removal of inhibitors. Also, the xylose-fermenting yeasts have a limited ethanol tolerance. The combined isomerization and fermentation with XI and *S. cerevisiae* gives yields and productivities comparable to those obtained in hexose fermentations without oxygenation and removal of inhibitors. However, the enzyme is not very stable in a lignocellulose hydrolysate, and *S. cerevisiae* has a poorly developed pentose phosphate shunt. Different strategies involving strain adaptation, and protein and genetic engineering adopted to overcome these different obstacles, are discussed.

Index Entries: Lignocellulose; hydrolysate; fermentation; ethanol; yeast; xylose isomerase.

INTRODUCTION

There is presently great interest in the fermentation of lignocellulose hydrolysates to ethanol. One reason is that hydrolyzed lignocellulose represents a source of renewable energy that, by fermentation, can be

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converted to a liquid fuel or a chemical feedstock. Another reason is that the carbon dioxide produced by the combustion of a renewable feedstock does not represent a net addition to the atmospheric carbon dioxide as does the combustion of petrochemical feedstocks: it will not prevent the greenhouse effect, but will not make it worse. Whether used as an energy source or chemical feedstock, optimal utilization of lignocellulosic materials will demand a form of pretreatment that may consist of complete or partial fractionation, much the same as in the pulping industry today. Presently available processes, elevated temperatures, in the presence or absence of chemicals, produce byproducts that contain low concentrations of fermentable sugars and a variety of carbohydrate- and lignin-derived compounds. These compounds may be highly inhibitory to microbial life, and thus, to fermentation processes. One well-known example of such a byproduct is spent sulphite liquor from the pulping industry. When the polymeric carbohydrates, cellulose and hemicellulose, can not be used as polymers, they are hydrolyzed with acids or enzymes, producing lignocellulose hydrolysates with high concentrations of fermentable sugars, as well as the inhibitory carbohydrate- and lignin-derived compounds. With the presently available fermentation technology, ethanol is the most versatile conversion product, both as an energy source and a chemical feedstock.

The carbohydrate fraction of lignocellulosic feedstocks is composed of monosaccharides, such as glucose, xylose, mannose, galactose, and arabinose (*1*). The hexose sugars (glucose, mannose, and galactose) are relatively easily fermented to ethanol, whereas the pentose sugars (xylose and arabinose) are not. The amount of arabinose is so small that it hardly presents a real problem. The xylose fraction, on the other hand, constitutes 10 to 40% (*1*). Therefore, irrespective of whether the lignocellulosic hydrolysate is a byproduct with low carbohydrate content or the main product with high carbohydrate content, one is faced with a fermentation substrate that contains (i) a major carbohydrate fraction that is difficult to ferment to ethanol, and (ii) efficient microbial inhibitors. Both from environmental and economical points of view, the xylose fraction should be fermented to ethanol. All cost estimates of lignocellulose-derived ethanol show the significant impact of the price of the raw material (2).

FERMENTATION OF XYLOSE TO ETHANOL

There has been intensive research in the fermentation of xylose to ethanol during the last decade (3). Xylose can be fermented to ethanol with bacteria, yeasts, and fungi. At present, yeasts give the highest product yields and the highest productivities. *Pachysolen tannophilus* (*4*), *Candida shehatae,* and *Pichia stipitis* (*5*) are the most thoroughly studied xylose-fermenting yeasts. The well-known bakers' yeast, *Saccharomyces*

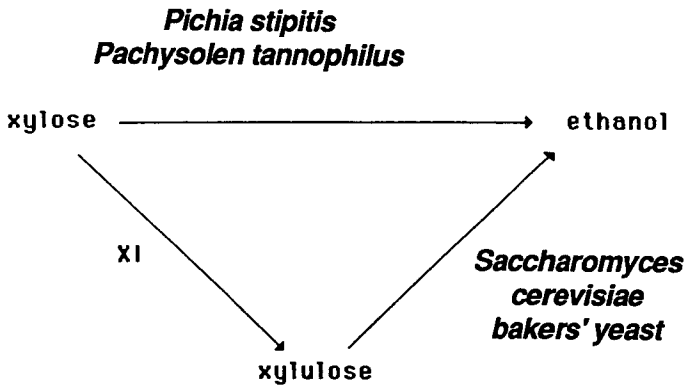

Fig. 1. The fermentation of xylose with directly fermenting yeasts and with a combination of isomerization and fermentation with bakers' yeast.

cerevisiae, generally used in industrial ethanol production, does not ferment xylose, but its isomerization product xylulose (*6–11*). Xylose can be converted to xylulose with the commercially available enzyme, glucose isomerase, which, in nature, is a bacterial xylose isomerase (Fig. 1). The equilibrium is less than 20% towards xylulose (*12–15*). However, simultaneous isomerization and fermentation with bakers' yeast can produce product yields and productivities comparable to those obtained in hexose fermentation (*16*). Similar results have since been obtained with the yeast *Schizosaccharomyces pombe* (*17*).

Oxygen Requirement

P. tannophilus, *C. shehatae*, and *P. stipitis*, which ferment xylose directly to ethanol, require a very low but extremely well-controlled level of oxygenation for optimal ethanol production, otherwise a considerable fraction of the xylose substrate is converted to the byproduct xylitol, or ethanol is reassimilated. Numerous attempts have been made to establish the optimal oxygenation level for maximum ethanol formation from xylose in yeasts. Oxygen limitation has been described in terms of "loosely screwed caps," the ratio of culture volume to vessel volume in relation to shaking and rotation rates (*18*), specific oxygen uptake rates (*19*), oxygen transfer rates (*20,21*), dissolved oxygen tension (*22*), and oxygen supply rate (Dellweg, personal communication). The only conclusion from these studies has been that oxygenation is necessary for maximum ethanol production, and that this level is very low, so low that it can not be directly measured with presently available techniques.

Xylose Reductase and Xylitol Dehydrogenase

It was initially thought that the oxygen requirement during xylose fermentation could be entirely explained by the differences in cofactor requirements for the two first enzymes in the xylose assimilating pathway,

xylose reductase (XR), and xylitol dehydrogenase (XDH). The first enzyme needs NADPH and the second NAD^+. In 1983, it was elegantly shown for *C. utilis* that anaerobic xylose utilization was prevented by the redox imbalance caused by the differences in cofactor requirements for XR and XDH (*23*). In the years to follow, XR (*24–27*) and XDH (*28–30*) from primarily *P. tannophilus* and *P. stipitis* strains were purified and characterized, both biochemically and kinetically.

The XR from both *P. stipitis* and *P. tannophilus* were found to be capable of using both NADPH and NADH, which seemed to explain why these yeasts can produce ethanol from xylose under anaerobic conditions (*31*). At least for *P. stipitis*, it would also explain why only trace amounts of xylitol have been observed to accumulate extracellularly. Later, it was found that xylitol accumulates intracellularly in this organism (*32*). In *P. stipitis*, it seems to be the same enzyme that has dual cofactor requirement, whereas for *P. tannophilus*, there seem to be two different enzymes requiring two different cofactors (*33*). The fact that the K_m of XR in *P. stipitis* is two times higher for NADH than for NADPH indicates that NADPH is the preferred cofactor (*25*).

Both XR and XDH are induced by xylose and arabinose in *P. tannophilus* (*34*). In both *P. tannophilis* and *P. stipitis*, the enzymes appear to be repressed by hexoses (*35*). In *P. tannophilus*, the induction of XR is temperature-sensitive (*36*). Ligthelm et al. (*37*) found evidence that induction of the two different XR activities in *P. tannophilus* was related to the degree of oxygenation of the culture. On the other hand, for *C. shehatae*, the ratio of NADPH to NADH activity of XR and the ratio of XR to XDH were independent of the degree of oxygenation (*38*). The same has been found for *P. stipitis* (Skoog and Hahn-Hägerdal, Appl. Environ. Microbiol. 1990, in press).

Growth

Although *P. tannophilus* and *P. stipitis* produce ethanol from xylose under anaerobic conditions, they do not grow (*31*). Using immobilized cells, it was suggested that growth is necessary for maximum ethanol production with *P. tannophilus* (*39,40*). Using different respiratory inhibitors, such as azide, Lighthelm et al. (*41*) found experimental evidence to support the suggestion of Maleszka and Schneider (*42*) that mitochondrial function is necessary for optimal ethanol production from xylose with both *P. stipitis* and *P. tannophilus*. These and other observations then led Schneider (*43*) to suggest that ethanol production in xylose-fermenting yeasts should be considered as being the result of secondary, rather than primary, metabolism. For the xylose-fermenting yeast, *Candida tropicalis*, it was observed that the respiratory inhibitor azide improved the ethanol yield and reduced the xylitol byproduct formation (*44*). An analysis of the mass balances revealed that this was because of the uncoupling effect of the inhibitor, rather than its effect as an inhibitor of respiration (*21*). Even

though more ethanol was produced per gram of cell mass, a major part of the sugar carbon appeared to have been lost as carbon dioxide.

Ethanol Tolerance

P. stipitis exhibits a low ethanol tolerance (*45,46*). The ethanol tolerance has so far not been reported for *P. tannophilus*. It has only been found that cell growth was enhanced when ethanol was removed from the fermentation broth (*47*). In view of a recent review relating the effects of ethanol to the toxicity of an intracellular accumulation of acetaldehyde (*48*), one might speculate that the low ethanol tolerance of xylose-fermenting yeasts is related to the fact that oxygen is a prerequisite for ethanol formation. Both for *C. tropicalis* and *P. stipitis*, it has been observed that there is an equilibrium between ethanol formation and consumption under oxygen-limited conditions (*21*).

Transport of Xylose

The slow fermentation of xylose in *P. stipitis* has led several investigators to study transport systems, and so far, the reports are somewhat contradictory. High- and low-affinity transport systems with K_m values of 0.06 and 2.3 m*M*, respectively, have been identified (*49*). In another study, the corresponding values were found to be 0.9 and 380 m*M*, respectively (*50*). Glucose was found to inhibit the high-affinity system, and to compete with the low-affinity system (*49*). In another study, it was found that glucose at concentrations higher than 2% repressed the xylose uptake (*51*). Glucose and xylose also appear to have different transport systems (*50*). In view of the fact that *P. stipitis* has a rather limited tolerance to ethanol, one would like the pentose and hexose sugars in the lignocellulose hydrolysate to be fermented simultaneously so that the produced ethanol would not inhibit the efficient fermentation of xylose. Furthermore, preliminary investigations indicate that oxygen stimulates the uptake of ^{14}C-xylose in *P. stipitis* (Skoog and Hahn-Hägerdal, Appl. Environ. Microbiol. 1990, in press).

Inhibitors

The potential microbial inhibitors present in lignocellulose hydrolysates are numerous: acetic acid, furfurals, phenolics, aromatic acids and aldehydes, sulphite, lignosulphonates, as well as ions from hydrolysis and neutralization. Still unknown inhibitors remain to be identified, and it is expected that inhibitors act synergetically. In addition, the inhibitory effect is related to the amount of cells used in a fermentation. There are presently two ways to circumvent the inhibitory effects of a lignocellulose hydrolysate: (i) pretreatment to remove the inhibitors with, e.g., ion-exchange resins (*52*) and steam stripping (*53*), and (ii) strain adaptation (*54,55*).

It has been observed by several investigators that *P. stipitis* performs poorly in untreated lignocellulose hydrolysates, such as spent sulphite liquor (*56,57*). In particular, the acetic acid seems to be strongly inhibiting. Steam stripping to reduce the acetic acid to concentrations below 0.6 g/L (*58*) or pH adjustment to 5.7 (*56*) improved the ethanol yield with *P. stipitis*. The latter treatment converts acetic acid to its charged form, which prevents it from passing through the cell membrane, dissociating intracellularly, and lowering the intracellular pH. *P. stipitis* is also strongly inhibited by high ionic strength. The performance of *P. stipitis* has been found to be greatly improved through adaptation (*55*).

P. tannophilus appears to be a more resistant organism for fermentation of untreated lignocellulose hydrolysates in that it does not seem to be inhibited (*57,59*). This organism was originally isolated from tanneries, which may be considered as hostile an environment as even an untreated lignocellulose hydrolysate. However, the strategy for the survival of this organism is to produce sugar alcohols, primarily xylitol and glycerol, rather than ethanol.

The performance of *S. cerevisiae* in combination with commercial glucose isomerase was found to be superior to the direct xylose-fermenting yeasts in both spent sulphite liquor and hydrogen fluoride-pretreated and acid-hydrolyzed wheat straw (*57*). In the presence of glucose isomerase, the yield of ethanol became comparable to that obtained in hexose fermentations. The additional cost of the enzyme to achieve this enhanced yield has to be evaluated against the cost of the mandatory pretreatment when *P. stipitis* is the fermenting organism. However, this approach also suffers from several problems: the pH and temperature optima for the enzyme and the yeast differ greatly: 4.5 and 7.5, 30°C and 65°C, respectively. Moreover, the enzyme is irreversibly inhibited at pH below 4.5 (*16*). Even if the pH is controlled during the fermentation of an untreated lignocellulose hydrolysate, there is an irreversible loss of enzyme activity (Fig. 2, *57*). Also, bakers' yeast may not be optimal for the production of ethanol from lignocellulose hydrolysates. There is, therefore, great potential for improving both xylose isomerase and *S. cerevisiae*.

Xylose Isomerase

Glucose isomerase, commercially used in the immobilized form for the production of "high fructose syrup," is a bacterial enzyme that, in nature, functions as a xylose isomerase (EC 5.3.1.5). The commercial enzymes were selected with respect to their glucose isomerase activity, as well as their high temperature optima (*60,61*). The enzyme obtained from *Lactobacillus brevis* falls outside these critieria, as the pH and temperature optima are lower, and the K_m is much more favorable for xylose than for glucose (*62*).

An immobilized whole cell preparation of *L. brevis* xylose isomerase was made by modifying a method described previously (*63*). Compared

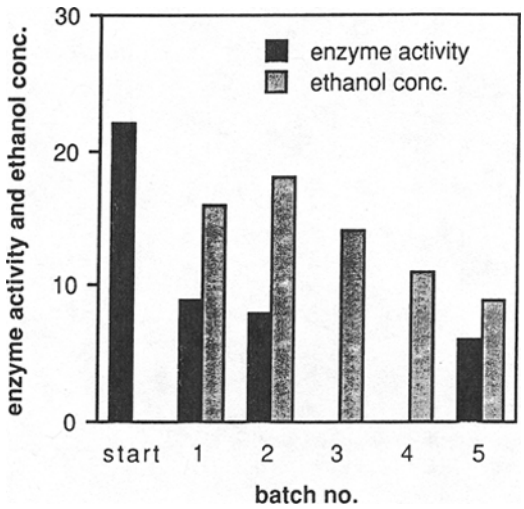

Fig. 2. Activity of immobilized xylose isomerase (XI) in repeated batch fermentations of untreated spent sulphite liquor with bakers' yeast. Activity measured either as glucose formed during the isomerization of fructose with the glucose oxidase method, or as ethanol formed during simultaneous isomerization and fermentation.

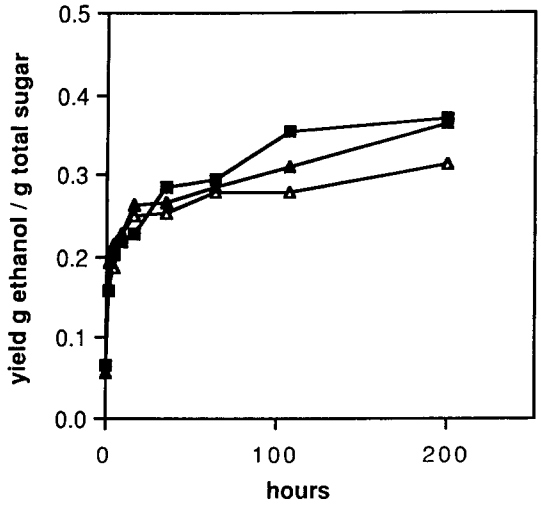

Fig. 3. Yield of ethanol in fermentations of untreated spent sulphite liquor with (△) *S. cerevisiae,* (▲) commercial immobilized XI and *S. cerevisiae,* and (■) immobilized XI from *L. brevis* and *S. cerevisiae.*

with the best commercial immobilized glucose isomerase, this preparation had a significantly higher activity at pH 5 in spent sulphite liquor when the direct isomerization of xylose was measured (*64*). When the best commercial glucose isomerase was substituted by the immobilized *L. brevis* enzyme in the fermentation of spent sulphite liquor with bakers' yeast, the yield was slightly better (Fig. 3). Because of its more favorable

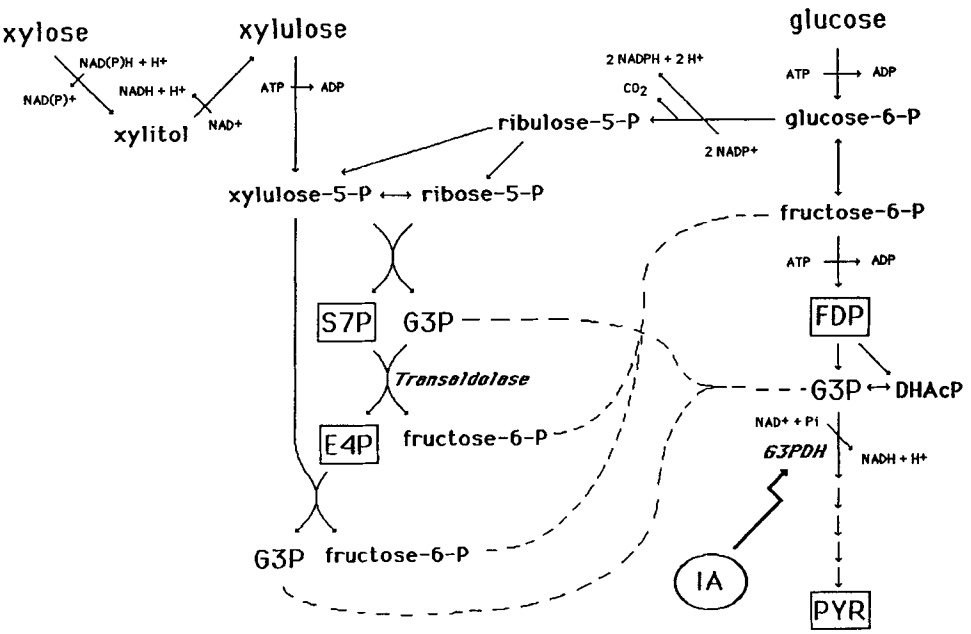

Fig. 4. Schematic representation of the interactions and the common intermediates in the pentose phosphate pathway (PPP) and glycolysis in yeasts. S7P = sedoheptulose-7-phosphate, G3P = glyceraldehyde-3-phosphate, E4P = erythrose-4-phosphate, FDP = fructose-1,6-diphosphate, DHAcP = dihydroxyacetone phosphate, G3PDH = glyceraldehyde-3-phosphate dehydrogenase, IA = iodoacetate, PYR = pyruvate. (From ref. 11, with permission)

pH optimum, it is expected that the *L. brevis* enzyme preparation will retain its activity on recirculation.

Saccharomyces cerevisiae

On reviewing the literature, it appeared that *S. cerevisiae* ATCC 24860 is one of the best xylulose-fermenting yeasts in terms of high ethanol yield and low xylitol byproduct formation (3). When the fermentation of glucose and xylulose with this organism was compared under anaerobic conditions, it was found that the rate of sugar consumption and ethanol production was approx 10 times lower with xylulose as the carbon source (*11*). One reason could be that the pentose phosphate pathway (PPP) enzyme transaldolase (TA) is low in *S. cerevisiae*, compared with xylose-assimilating yeasts, such as *Candida* and *Torula sp.* (*65,66*). The low TA activity might result in the PPP losing the competition with glycolysis for the common intermediate glyceraldehyde-3-phosphate (G3P), thereby resulting in a reduced metabolic flow through the PPP (Fig. 4). In the presence of iodoacetate, an inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase, it is expected that G3P would accumulate to

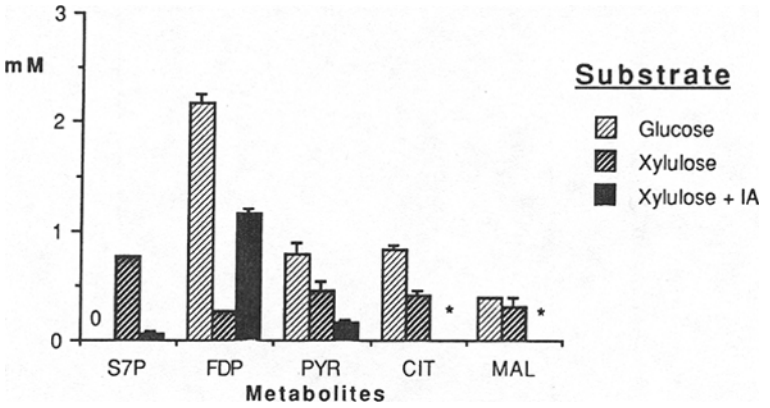

Fig. 5. Intracellular intermediary metabolite concentrations in cells of *S. cerevisiae* fermenting glucose, xylulose, and xylulose in the presence of iodoacetate. CIT = citrate, MAL = malate, other abbreviations as in Fig. 4.

be available for the PPP. This was indirectly observed in that the level of the intermediate of the PPP that reacts with G3P, sedoheptulose-7-phosphate, was lowered in *S. cerevisiae* when xylulose was fermented in the presence of iodoacetate (Fig. 5; *11*).

The sugar transport systems of *S. cerevisiae* may not be optimal for the simultaneous fermentation of both hexose and pentose sugars in a lignocellulose hydrolysate. To the best of our knowledge, there have so far not been any reports on the transport of xylulose in *S. cerevisiae*. Xylose, on the other hand, is transported by the low- and high-affinity glucose transport systems of *S. cerevisiae*, but with K_m values several orders of magnitude higher (*67,68*). Furthermore, it has been found that xylose inactivates the glucose-phosphorylating enzymes hexokinase PI, hexokinase PII, and hexokinase in *S. cerevisiae* (*69–71*).

The approach of adapting organisms to a hostile environment could also be used for *S. cerevisiae*. One place to look for adapted strains is a plant that has been continuously fermenting spent sulphite liquor for more than 40 yr (Lindén et al., to be published). Among 23 isolates from different places in the fermentation plant, seven strains were identified; two as *S. cerevisiae* and the others as *Pichia membranaefaciens*. The two *S. cerevisiae* fermented spent sulphite liquor better than bakers' yeast, and the yield of ethanol was increased from 0.37 to 0.41 (Fig. 6).

XR and XDH activity is generally not considered to be present in bakers' yeast *S. cerevisiae*. In one study, *S. cerevisiae* was reported to have XR and XDH activities several orders of magnitude higher than those found in *P. stipitis* (*72*). Even when corrected for a possible printing error in the unit definition, one is left with about 10 times more XDH activity than in *P. stipitis*. It has so far not been possible to reproduce this remarkable result in other strains of *S. cerevisiae* (Lindén et al., to be published).

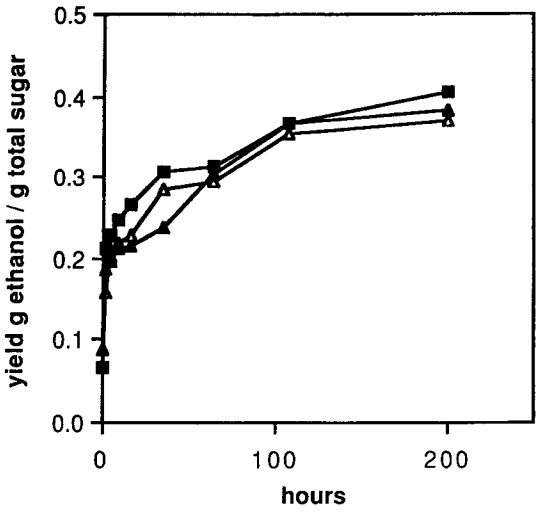

Fig. 6. Yield of ethanol in fermentations of untreated spent sulphite liquor in the presence of immobilized XI from *L. brevis* with (△) *S. cerevisiae* (bakers' yeast), (▲) *S. cerevisiae*, isolate no. 10, and (■) *S. cerevisiae*, isolate no. 3 from a fermentation plant for spent sulphite liquor.

Genetic and Protein Engineering

A great amount of effort has been made during the last 10 yr to transform *S. cerevisiae* with bacterial glucose (xylose) isomerase. The xylose isomerase gene from *Escherichia coli* has been purified and characterized (*73*). It was later expressed both in *S. cerevisiae* and *Sch. pombe*, however, with considerably reduced activity (*74,75*). Similar attempts were made with xylose isomerase genes from *Bacillus subtilis* and *Actinoplanes missouriensis* (*76*). In these cases, the xylose isomerase was not even catalytically active. The xylose uptake gene from *E. coli* (*77*) and the xylulokinase genes from *P. tannophilis* (*78*) and *S. cerevisiae* (*73*) have also been investigated in an attempt to improve ethanol production from xylose. Strain improvements have been attempted with hybridization (*79*), and by increasing the DNA content (*80*). All these efforts have so far met with surprisingly little success. A much more encouraging approach has been to transform *E. coli* with ethanologenic enzymes from *Zymomonas mobilis* (*81,82*). The yield of ethanol from xylose was reported to be 0.52.

Instead of transforming *S. cerevisiae*, one could stay with the combination of bakers' yeast and glucose isomerase, and improve the enzyme. Rather than looking in nature for an organism with a better xylose isomerase, the advent of genetic and protein engineering techniques will probably make it possible to tailor an enzyme suitable for the isomerization of xylose under conditions prevalent in lignocellulose hydrolysates (*83,84*).

An approach so far not described in the literature is the transformation of *S. cerevisiae* with the XR and XDH genes from *P. stipitis*. This ap-

proach is expected to be more successful than the transformation with bacterial genes, since genes from another yeast are used.

Once *S. cerevisiae* has been transformed with XR and XDH, the transformant could be further improved with a combination of genetic and protein engineering techniques. One would like to change the cofactor requirements of the enzyme XR with protein engineering techniques to obliterate the redox imbalance. The transaldolase activity could be enhanced through genetic engineering techniques to improve the fermentation rate of sugars that are assimilated through the PPP.

Finally, once the genetics of *P. stipitis* are better known, it would appear quite reasonable to "manipulate" the organism in such a way that the transport systems, the ethanol tolerance, and the oxygen dependence no longer constitute any problems in the fermentation of xylose.

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