Appl Microbiol Biotechnol (1986) 24:287–293

Applied and Microbiology Biotechnology

Improved ethanol production from xylose with glucose isomerase and *Saccharomyces cerevisiae* using the respiratory inhibitor azide

Bärbel Hahn-Hägerdal, Sissi Berner, and Kerstin Skoog

Applied Microbiology, Chemical Center, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

Summary. Ethanol was produced from xylose, using the enzyme glucose isomerase (xylose isomerase) and *Saccharomyces cerevisiae*. The influence of aeration, pH, enzyme concentration, cell mass and the concentration of the respiratory inhibitor sodium azide on the production of ethanol and the formation of by-products was investigated. Anaerobic conditions at pH 6.0, 10 g/l enzyme, 75 g/l dry weight cell mass and 4.6 mM sodium azide were found to be optimal. Under these conditions theoretical yields of ethanol were obtained from 42 g/l xylose within 24 hours.

In a fed-batch culture, 62 g/l ethanol was produced from 127 g/l xylose with a yield of 0.49 and a productivity of 1.35 g/l·h.

Introduction

The utilization of lignocellulosic feed-stocks involves the upgrading of the pentose fraction, mainly derived from hemicellulosics (Ladisch et al. 1983). If the feed-stock is to be converted to a liquid fuel, one attractive route that has been given great attention in recent years is the fermentation of pentoses to ethanol, thus giving rise to the same end-product as the hexose fermentation.

Xylose is fermented to ethanol under oxygen-limited conditions by certain yeasts (Lohmeier-Vogel and Hahn-Hägerdal 1985). However, the regular baker's yeast, *Saccharomyces cerevisiae*, is not one of those organisms. *S. cerevisiae* has the ability to ferment xylulose, an isomerization product of xylose. The reaction is catalyzed by the enzyme glucose isomerase (in reality a xylose isomerase), which is industrially used for the production of "high-fructose syrup".

The idea of using the enzyme glucose isomerase in conjunction with regular baker's yeast, *S. cerevisiae*, for the direct production of ethanol from xylose, was first presented by Schneider et al. (1980). It has since been the subject of much debate, above all for the economic implications that the utilization of yet another expensive enzyme would have on the market price of ethanol produced from a lignocellulosic feed-stock. However, the system has been studied and certain fermentation parameters have been defined. These studies were aimed at transferring the bacterial gene for the enzyme into the yeast (Chiang et al. 1981). In these studies one finds either a high productivity and a low yield or vice versa. They have mainly emphasized the fermentation parameters for yeast fermenting xylulose. None of the studies involve the simultaneous isomerization and fermentation of D-xylose to ethanol with the enzyme and baker's yeast. The present study was therefore undertaken in order to define parameters for the simultaneous isomerization and fermentation of D-xylose to ethanol, using glucose isomerase and baker's yeast with the aim of obtaining a theoretical yield and a high productivity. The influence of aeration and the respiratory inhibitor sodium azide on by-product formation were also investigated, as it had earlier been found that the addition of azide could repress by-product formation in *Candida tropicalis* by as much as 90% (Lohmeier-Vogel and Hahn-Hägerdal 1985).

Offprint requests to: B. Hahn-Hägerdal

Materials and methods

Experimental conditions

Baker's yeast, *S. cerevisiae,* was purchased from a local distributor. Glucose isomerase, Optisweet-P, was a generous gift from MKC, Hannover, FRG.

In all experiments 5% (w/v) xylose (Sigma Chemical Company, St. Louis, Mo., USA) in 0.1 M phosphate buffer (pH 6.0) was used as substrate. The growth medium was 0.25% (w/v) yeast extract (Difco, Detroit, Mi., USA), 0.025% (w/v) $(NH_4)_2HPO_4$, 0.20% (w/v) $MgSO_4 \cdot 7H_2O$ and 0.02% (w/v) $CoCl_2 \cdot 6H_2O$. Azide at different concentrations was included in the substrate solution.

Fermentations were carried out at 30° C, either in 500 ml baffled Erlenmeyer flasks containing 100 ml medium in a water bath at 160 rpm (corresponding to an oxygen transfer rate of 46 mM/h as determined by the sulphite method (Cooper et al. 1944) or in resting 150 ml flasks, filled and sealed with rubber stoppers supplied with tubs for sampling and gas outlet.

Analysis

Fermentation product concentrations and the consumption of xylose were determined using a Varian 5000 Liquid Chromatograph equipped with a Varian Auto Sampler series 8000 and a Varian refractive index detector.

The column used was a BioRad HPLC organic acid analysis column, 300·7.8 mm, Aminex HPX-87H. The liquid phase was 0.01 M H_2SO_4.

Enzyme activity

The activity of the enzyme glucose isomerase was determined according to Gong et al. (1980). A 2.5 ml 2 M fructose solution (consisting of 0.05 M phosphate (pH 7.0), 0.83 mM $MgCl_2$, and 0.83 mM $CoCl_2$) was used as a substrate. Then 0.1 ml enzyme solution was added to the substrate solution at 60° C. After 10 min of incubation the reaction was stopped by adding 0.3 ml 50% trichloroacetic acid. The glucose formed was determined by the glucose oxidase method (Trinder 1969).

Results and discussion

According to the manufacturer, the optimal pH for the glucose isomerase activity is 6.2 and the optimal temperature 75° C. The yeast *S. cerevisiae,* on the other hand, functions optimally at pH 4.5—5 and at around 30° C. In order to work in the same reaction, intermediate conditions for the enzyme and the yeast had to be found.

First we studied the activity of the enzyme as a function of pH (Fig. 1). Below pH 6 there is a dramatic loss of activity, indicating that the conversion should be carried out at a pH as close as possible to 6.

We then investigated the stability of the enzyme, both as a function of pH (Fig. 2) and in the presence of increasing concentrations of ethanol (Fig. 3). Again a pH lower than 6 had a dramati-

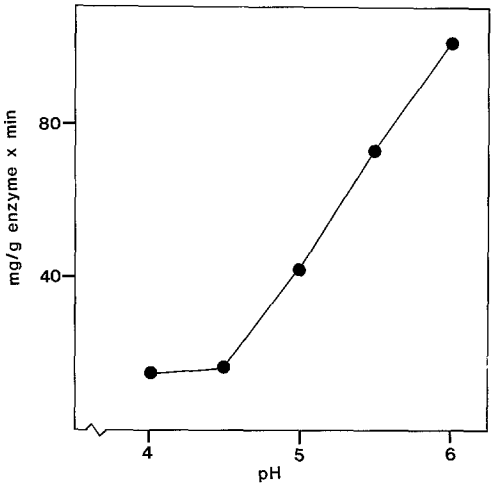

Fig. 1. Activity of glucose isomerase as a function of pH represented as glucose produced from fructose

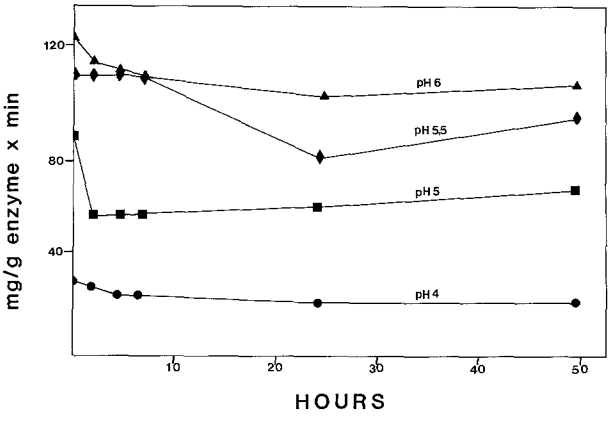

Fig. 2. Stability of glucose isomerase as a function of pH and time at 30° C. Remaining activity determined as glucose produced from fructose at pH 7.0 and 60° C

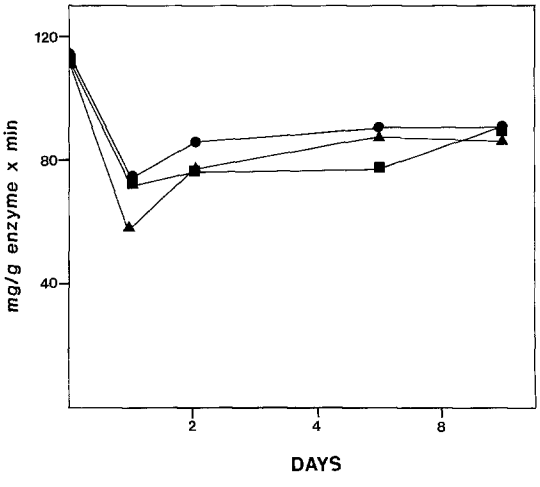

Fig. 3. Stability of glucose isomerase at pH 6.0 and 30° C, in the presence of ethanol (■) 100 g/l; (●) 50 g/l; (▲) no ethanol added. Remaining activity determined as in Fig. 2

cally depressing effect on the enzyme activity. Furthermore, the data indicated that even a very short exposure of the enzyme to a pH lower than 5.5 irreversibly reduced the enzyme. On the other hand, ethanol concentrations of up to 100 g/l hardly influence the enzyme activity as long as the pH remains at 6.0 (Fig. 3). Thus, the conversion of xylose to ethanol using xylose isomerase has to be carried out with pH strictly fixed at 6. The variations in enzyme activity between Figs. 2 and 3 is probably due to the enzyme not being completely soluble, which makes sampling difficult.

Given these conditions, it might be questionable to let the enzyme and the yeast work in the same reaction mixture. However, at equilibrium

$$\text{xylose} \overset{\text{XI}}{\rightleftharpoons} \text{xylulose}$$

less than 20% of the xylose is converted to xylulose by the enzyme (Mitsuhashi and Lampen 1953; Hochster and Watson 1954; Slein 1955). Thus, adding yeast able to ferment xylulose should "pull" the reaction and finally result in complete conversion of all xylose to xylulose.

Apart from a strict control of pH during a combined isomerization and fermentation, several other parameters might influence product formation in this process. Due to the slow fermentation

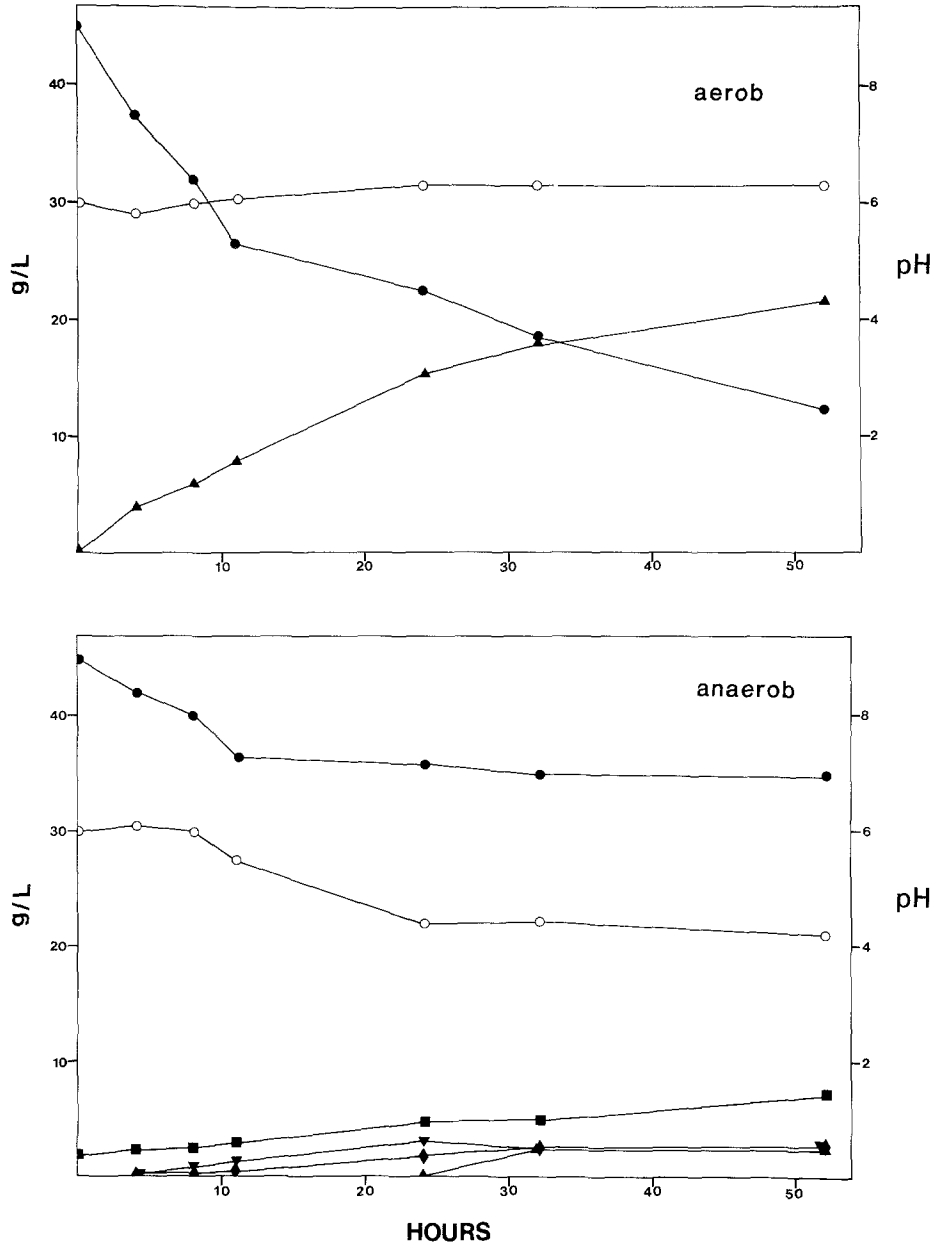

Fig. 4. Batch fermentation of xylose with baker's yeast in the absence of glucose isomerase pH 6.0, 30° C. (**a**) aerobic, 100 ml medium in a 500 ml shake flask equivalent to 48 mM/h; (**b**) anaerobic, 150 ml medium in 150 ml sealed bottle. (●) xylose; (■) ethanol; (▲) xylitol; (▼) acetic acid; (◆) glycerol; (○) pH

of xylulose with *S. cerevisiae* (Gong et al. 1981), high cell densities have been used to achieve high production rates (Chiang et al. 1981). Fermentation of xylulose was found to be accompanied by formation of by-products such as xylitol, arabitol, glycerol and acetic acid (Suihko and Poutanen 1984).

We have previously shown that by-product formation in xylose fermentation with *Candida tropicalis* can be repressed by up to 90% by the addition of the respiratory inhibitor azide (Lohmeier-Vogel and Hahn-Hägerdal 1985). For *C. tropicalis* as well as for *S. cerevisiae* (Hahn-Hägerdal and Mattiasson 1982), it was also found that a certain degree of aeration was important when fermentations were carried out in the presence of azide.

Thus, apart from optimal concentrations of enzyme and yeast cells, optimal aeration levels and azide concentrations were also investigated. The studies were carried out with commercial active baker's yeast cake *(S. cerevisiae)* rather than a pure culture, in order to be able to obtain high cell densities easily.

First a comparison was made between aerobic (100 ml in a baffled 500 ml shake flask) and anaerobic (a filled and sealed resting bottle) assimilation of xylose by *S. cerevisiae* in the absence of the enzyme xylose isomerase (Figs. 4a and b). Under aerobic conditions the major part of the xylose substrate was assimilated with the formation of xylitol. Under anaerobic conditions only 10—15% of the substrate was assimilated and low concentrations of ethanol, xylitol, glycerol and acetic acid accumulated in the medium.

In the next step, xylose isomerase and baker's yeast were used together for the conversion of xylose to ethanol under anaerobic conditions, without azide and with two different concentrations of azide, 2.3 mM and 4.6 mM (Figs. 5a, b and c). The presence of azide represses the formation of xylitol and acetic acid. As a consequence, the pH dropped by only 0.5 units, whereas without the presence of azide, pH dropped to 4. This resulted in stable enzyme activity in the presence of azide and a total loss of enzyme activity in its absence. Finally, the presence of azide resulted in theoretical yields of ethanol and inhibition of by-product formation.

These results are contradictory to previous findings which have shown that fermentation in the presence of azide could only be carried out under aerated conditions (Hahn-Hägerdal and Mattiasson 1982; Lohmeier-Vogel and Hahn-Hägerdal 1985). Therefore, we also investigated the effect of azide under oxygenated conditions in shake flasks corresponding to an oxygen transfer rate (OTR) of 46 mM/h (Figs. 6a—c). Under aerated conditions without azide present in the medium, ethanol is only produced in the beginning of the fermentation. It is then reassimilated and the metabolism is switched over to xylitol produc-

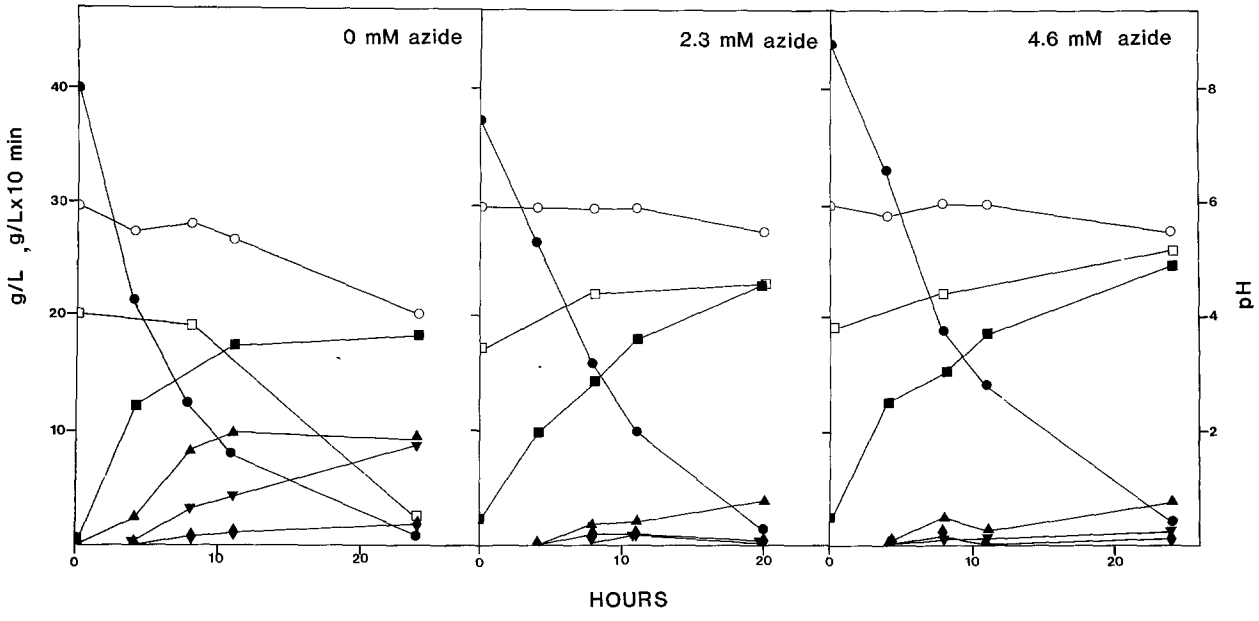

Fig. 5. Anaerobic fermentation of xylose with baker's yeast, 75 g/l, and glucose isomerase, 30 g/l. (**a**) no azide; (**b**) 2.3 mM azide; (**c**) 4.6 mM azide. (□) enzyme activity in g/l × 10 min. All other symbols as in Fig. 4

tion. At low concentrations of azide (0.2 mM), higher levels of ethanol were produced before reassimilation and, in addition to xylitol, acetic acid was also produced. At an azide concentration of 4.6 mM, by-product formation was almost totally repressed and theoretical yields of ethanol were produced when the culture was aerated. Since a similar result was obtained under anaerobic conditions, there appears to be no advantage in aerating the culture.

Anaerobic conditions and 2.3—4.6 mM azide were chosen to be the best conditions with respect to ethanol yield and repression of by-product formation.

Next, we studied the influence of different enzyme concentrations on product formation (Fig.

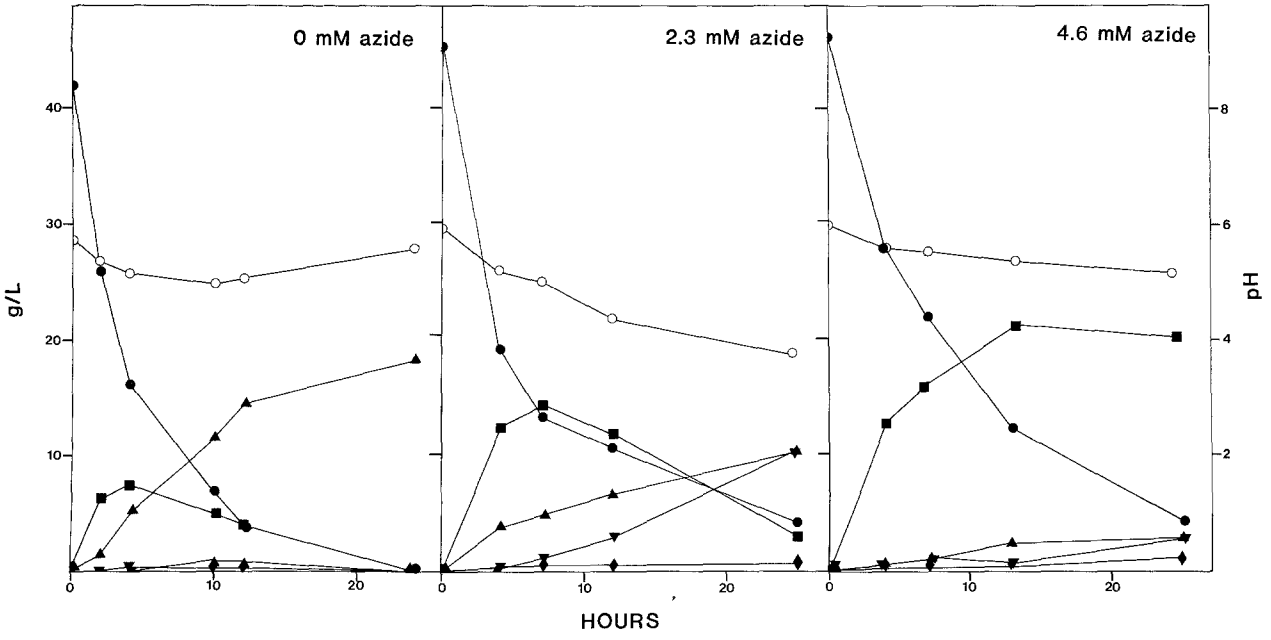

Fig. 6. Fermentation of xylose under aerobic conditions, with baker's yeast, 75 g/l, and glucose isomerase, 30 g/l. (**a**) no azide; (**b**) 0.2 mM azide; (**c**) 4.6 mM azide. Symbols as in Fig. 4

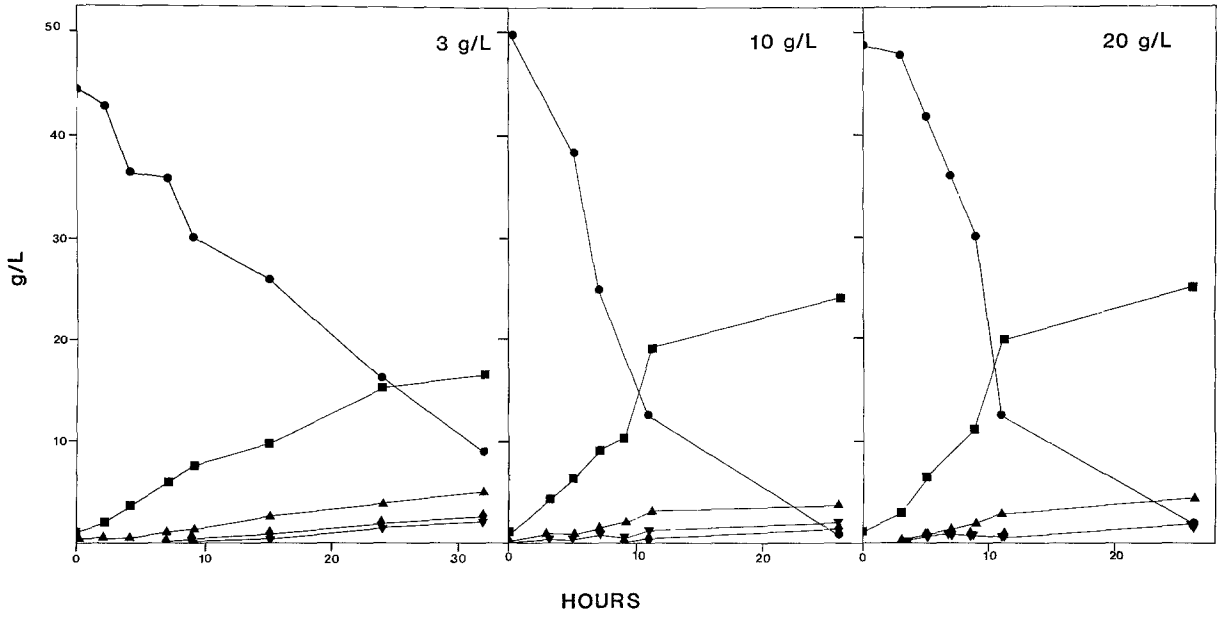

Fig. 7. Fermentation of xylose under anaerobic conditions, with baker's yeast, 40 g/l, pH at 6.0 and varying concentrations of glucose isomerase: (**a**) 3 g/l; (**b**) 10 g/l; (**c**) 20 g/l. Symbols as in Fig. 4

7a—c). Increased enzyme concentration resulted in increased ethanol production. A concentration of 10 g/l was found to be optimal since higher enzyme concentrations did not improve product formation.

Finally, we also investigated different levels of cell mass (Fig. 8). With increasing cell mass the rate of ethanol production increases. Because baker's yeast is easily obtainable, we chose 75 g/l for future experiments.

In conclusion, we have found that the conversion of xylose to ethanol with xylose isomerase and *S. cerevisiae* is best performed under anaerobic conditions with 10 g/l enzyme and 75 g/l dry weight of cell mass. A concentration of 4.6 mM azide increases the ethanol yield and represses the formation of by-products such as glycerol, acetic acid and xylitol.

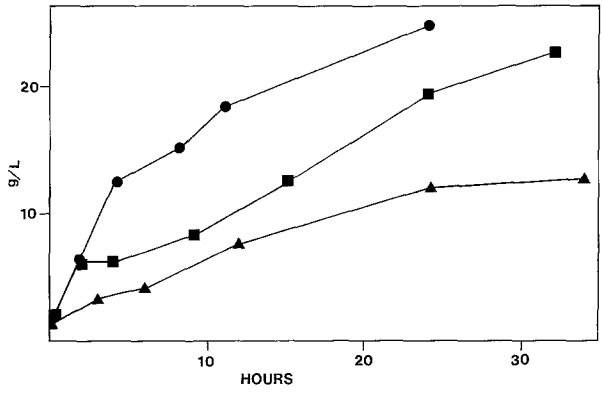

Fig. 8. Fermentation of xylose under anaerobic conditions with glucose isomerase, 30 g/l, pH 6.0, 4.6 mM azide and varying concentrations of baker's yeast: (▲) 30 g/l, (■) 40 g/l and (●) 75 g/l

Under these conditions a fed-batch fermentation was carried out aiming at producing a high ethanol concentration (Fig. 9).

Xylose was added twice to a concentration of 100 g/l. Ethanol was continuously produced up to 60 g/l. By-product formation was very low; at most 7.5 g/l xylitol, 4.5 g/l glycerol and 2.5 g/l acetic acid. The pH was carefully controlled to 6.0. Nevertheless, a steady decline in enzyme activity was observed. It was speculated that perhaps the acetate moiety might be inhibitory rather than the pH. However, a study of the enzyme stability in sodium acetate at pH 7 revealed that the enzyme activity was not influenced at all.

In conclusion, it is possible to produce ethanol from xylose with high yield, high product concentration and high productivity. In order for the process to be economical, ways to maintain the enzyme activity in the process and to recirculate the enzyme must be found.

Acknowledgements. This study was supported by the National Energy Administration and the Swedish Natural Research Council.

We want to express our gratitude to Dr. Y. Takasaki, Fermentation Research Institute, AIST, Tsukuba, Japan, for giving valuable advice during the preparation of this manuscript.

References

Chiang LC, Gong CS, Chen LF, Tsao GT (1981) D-Xylulose fermentation to ethanol by *Saccharomyces cerevisiae*. Appl Environ Microbiol 42:284—289

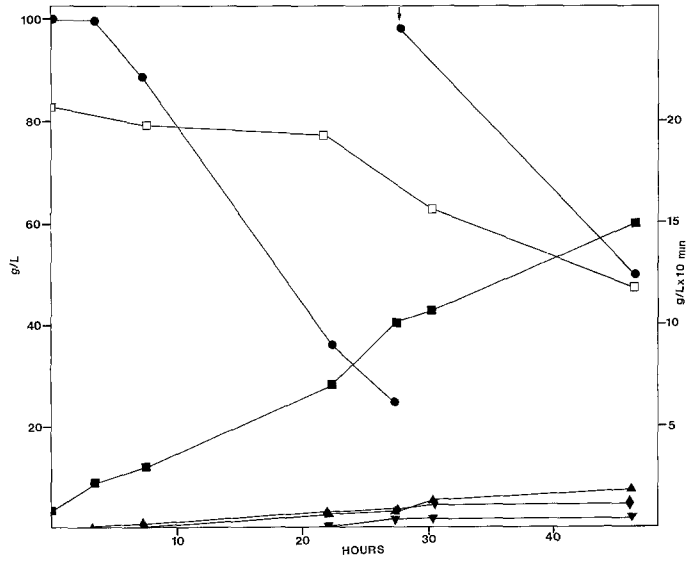

Fig. 9. Fermentation of xylose in a 1 l fermentor with a working volume of 800 ml at pH 6.0 with baker's yeast, 75 g/l, glucose isomerase, 30 g/l, and 4.6 mM azide under anaerobic conditions. Symbols as in Figs. 4 and 5

Cooper CM, Fernstrom GA, Miller SA (1944) Performance of agitated gas-liquid contactors. Ind Eng Chem 36:504—509

Gong CS, Chen LF, Flickinger MC, Chiang LC, Tsao GT (1981) Production of ethanol from D-Xylose by using D-Xylose isomerase and yeasts. Appl Environ Microbiol 41:430—436

Gong CS, Chen LF, Tsao GT (1980) Purification and properties of glucose isomerase of *Actinoplanes missouriensis*. Biotechnol Bioeng XXII:833—845

Hahn-Hägerdal B, Mattiasson B (1982) Azide sterilization of fermentation media. Ethanol production from glucose using immobilized *Saccharomyces cerevisiae*. Eur J Appl Microbiol Biotechnol 14:140—143

Hochster RM, Watson RW (1954) Enzymatic isomerization of D-xylose to D-xylulose. Arch Biochem Biophys 48:120—129

Ladisch MR, Lin KW, Volack M, Tsao GT (1983) Process considerations in the enzymatic hydrolysis of biomass. Enzyme Microb Technol 5:82—100

Lohmeier-Vogel E, Hahn-Hägerdal B (1985) The utilization of metabolic inhibitors for shifting product formation from xylitol to ethanol in pentose fermentations using *Candida tropicalis*. Appl Microbiol Biotechnol 21:167—172

Mitsuhashi S, Lampen JO (1953) Conversion of D-xylose to D-xylulose in extracts of *Lactobacillus pentosus*. J Biol Chem 204:1011—1018

Schneider H, Wang PY, Johnson BF, Shopsis C (1980) Pentose fermentation by yeasts. In: Stewart GG, Russel I (eds) Current developments in yeast research. Pergamon Press, pp 81—85

Slein MW (1955) Xylose isomerase from *Pasteurella pestis*, strain A-1122. J Am Chem Soc 77:1663—1667

Suihko ML, Poutanen K (1984) D-Xylose fermentation by free and immobilized *Saccharomyces cerevisiae* cells. Biotechnol Lett 6:(3) 189—194

Trinder P (1969) Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann Clin Biochem 6:24—27

Received December 30, 1985/Revised March 17, 1986