APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 2043–2045 Vol. 61, No. 5
0099-2240/95/$04.00+0

Xylitol Formation and Reduction Equivalent Generation during Anaerobic Xylose Conversion with Glucose as Cosubstrate in Recombinant *Saccharomyces cerevisiae* Expressing the *xyl1* Gene

HELLE NØRGAARD THESTRUP† AND BÄRBEL HAHN-HÄGERDAL*

Applied Microbiology, Lund Institute of Technology, Lund University, S-221 00 Lund, Sweden

Received 21 January 1994/Accepted 2 March 1995

Glucose was used as a cosubstrate under anaerobic conditions in the conversion of xylose to xylitol by a recombinant *Saccharomyces cerevisiae* strain expressing the *xyl1* gene. Glucose was metabolized mainly through glycolysis, with carbon dioxide, acetate, and ethanol as end products and with reduction equivalents generated in the glyceraldehyde-3-phosphate dehydrogenase and acetaldehyde dehydrogenase reactions. At a high glucose supply rate, generation of surplus reduction equivalents resulted in simultaneous ethanol formation. On the other hand, at a low glucose supply rate, additional reduction equivalents were generated by simultaneous ethanol consumption. A significantly lower xylitol formation rate was observed.

Reduction of xylose to xylitol in *Saccharomyces cerevisiae* has been accomplished by transforming the yeast with a plasmid containing the *xyl1* gene encoding the enzyme xylose reductase (XR) from *Pichia stipitis* (5). Xylitol is not further metabolized in the yeast because of the low level of activity of xylitol dehydrogenase (7), and xylitol/xylose yields of close to 1 g/g have been achieved (4, 5, 8). The recombinant yeast requires a cosubstrate for the generation of reduction equivalents used in the reduction of xylose and for maintenance and growth. Use of different carbon sources as cosubstrates during xylitol formation has been studied (4, 8). If no cosubstrate was added, only insignificant amounts of xylitol were produced. Glycerol could support neither growth nor xylitol formation, and acetate was used only with a very low level of efficiency (0.3 g of xylitol produced per g of acetate consumed), whereas ethanol and glucose were used with xylitol/cosubstrate yields of 0.6 and >1 g/g, respectively (4). The highest xylitol/cosubstrate yield was obtained under low aeration conditions, which is also when the highest level of specific xylitol productivity was reached (4, 8). The reduction of xylose by XR and the generation of reduction equivalents by oxidation of the cosubstrate may be regarded as two coupled reactions, as shown in Fig. 1.

The aim of the present study was to determine which of the two reactions limits the xylitol formation in recombinant *S. cerevisiae* H475 (5). The study was performed under anaerobic conditions to prevent respiratory consumption of reduction equivalents, and glucose, supplied at two rates, was used as a cosubstrate. During fermentation, the rates of xylose and glucose consumption and product formation were monitored to elucidate the metabolic pathway used for glucose oxidation in relation to xylose reduction (Fig. 2 and Table 1).

The fermentations were performed in a 1.5-liter fermentor (Applicon Dependable Instruments, Schiedam, Netherlands) controlled by the EFC24 control system (Electrolux Fermentation, Malmö, Sweden). The temperature was set at 30°C and the pH was maintained at 6.5 by addition of 2.0 M NaOH. The agitation rate was set at 600 rpm, and the gas flow rate was 0.3 liters/min. The growth medium consisted of 6.7 g of yeast nitrogen base without amino acids per liter, supplemented with 0.05 g of L-tryptophan, L-histidine, and L-uracil per liter. Cells grown in shake flasks were used for inoculation of the fermentor. Ergosterol (Merck, Darmstadt, Germany) and Tween 80 (Sigma Chemical Co., St. Louis, Mo.), essential for anaerobic growth (1, 2), were added as previously described (15). Samples of 10 to 15 ml were taken every half an hour and analyzed for substrate and product concentrations by high-performance liquid chromatography (12) and for xylose reductase activity (7). The sample volume was chosen so that it balanced the glucose feed and the base addition, and a constant fermentation volume of 1.4 liters was maintained with a dilution rate of 0.005 h^{-1}. The optical density at 620 nm was used to estimate the cell dry weight, which was determined after drying the cells in a microwave oven. One A_{620} unit was equivalent to 0.3 g (dry weight) $liter^{-1}$. To achieve a high cell density, the cells were grown aerobically on glucose in the absence of xylose for the first 18 to 20 h. The air was replaced by nitrogen, 50 ml of a 200-g $liter^{-1}$ xylose solution was added, and a constant flow rate of glucose of 0.42 ml/min was initiated. The concentrations of the glucose solutions were 200 and 20 g $liter^{-1}$ during the high and low glucose feeds, respectively. The glucose concentration was below 10 mg/liter in both fermentations.

An initial specific xylitol formation rate as high as 0.12 g/g h^{-1} was obtained when a high glucose supply rate was applied. Previously, the specific xylitol productivities obtained with recombinant *S. cerevisiae* expressing the *xyl1* gene were reported to be between 0.05 and 0.09 g/g h^{-1} (4, 5, 8). The highest values were obtained under oxygen-limited conditions with ethanol as the cosubstrate (8). The specific XR activity (3, 7) in cell extracts of the recombinant yeast was about 10 nkat/mg of protein, independent of the fermentation time or the fermentation conditions. It was previously determined to be 8.49 nkat/mg of protein (5).

Markedly higher initial rates of xylose consumption and xylitol formation were observed during the high glucose supply rate than during the low glucose supply rate (Table 1). The xylose concentration and the cell density were only slightly different in the two fermentations. However, the rates of reduction equivalent generation differ. The formation of ethanol at the high glucose supply rate implies that surplus reduction

* Corresponding author. Mailing address: Applied Microbiology, Lund Institute of Technology, Lund University, P.O. Box 124, S-221 00 Lund, Sweden. Phone: 46 46 108428. Fax: 46 46 104203. Electronic mail address: Barbel.Hahn-Hagerdal@tmb.lth.se.

† Present address: Dansk Bioprotein A/S, DK-5230 Odense M, Denmark.

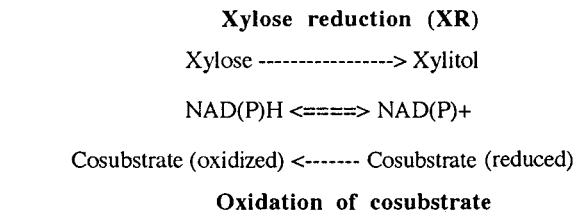

FIG. 1. Reduction of xylose by XR and generation of reduction equivalents by oxidation of the cosubstrate.

equivalents are consumed in the alcohol dehydrogenase reaction. At the low glucose supply rate, a significantly higher xylitol/glucose yield was found and anaerobic ethanol oxidation was observed. This was probably due to the consumption of reduction equivalents in the XR reaction. The results therefore indicate that the reduction equivalents were consumed more rapidly in the XR reaction than they were generated at the low glucose supply rate.

Ethanol has previously been reported to be unable to support xylitol formation in recombinant *S. cerevisiae* under anaerobic conditions (8). Anaerobic ethanol oxidation does not involve ATP formation, which is necessary for cell maintenance. In the present study, the anaerobic ethanol oxidation was accompanied by simultaneous glucose oxidation with concomitant ATP formation, which could explain why ethanol was anaerobically oxidized to generate reduction equivalents.

In Table 2, the carbon balance results are summarized. The rates of sugar consumption and the rates of product formation were converted into the molar equivalent of carbon. In both fermentations, the product formation rates balanced the substrate consumption rates. Production of succinate and other dicarboxylic acids was not observed (9). The rate of glycerol formation was very low (2 mol/g h^{-1}) and was not taken into account. Glycerol is generally produced during anaerobic fermentations to oxidize excess NADH produced during cell mass formation (9). Reduced glycerol formation by a recombinant *S.*

TABLE 1. Rate of glucose consumption and xylitol formation and the xylitol conversion yield in relation to xylose and glucose consumed during high and low glucose supply rates

Rate of glucose consumption (mmol/g h^{-1})[a]	Cell density (g/liter)	Xylose concn (mM)	Rate of xylitol formation (mmol/g h^{-1})[b]	Xylitol conversion yield (mol/mol)[c]	
				$Y_{xylitol/xylose}$	$Y_{xylitol/glucose}$
0.33	10	400	0.78	1.1	2.2
0.06	7	500	0.39	0.9	7.8

[a] The specific glucose consumption rate was calculated directly from the feed rate of glucose, because the residual glucose concentration in the fermentation broth was very low (<10 mg/liter).

[b] The initial rate of xylitol formation was calculated from the progress curves in the period 20 to 30 h after the start of the fermentation. The rate of washing out of the substrates and products due to the dilution by the glucose feed and the sampling was taken into account.

[c] The xylitol yields in relation to consumed xylose and glucose, $Y_{xylitol/xylose}$ and $Y_{xylitol/glucose}$, were determined by dividing the rate of xylitol formation by the rates of xylose consumption and glucose consumption, respectively.

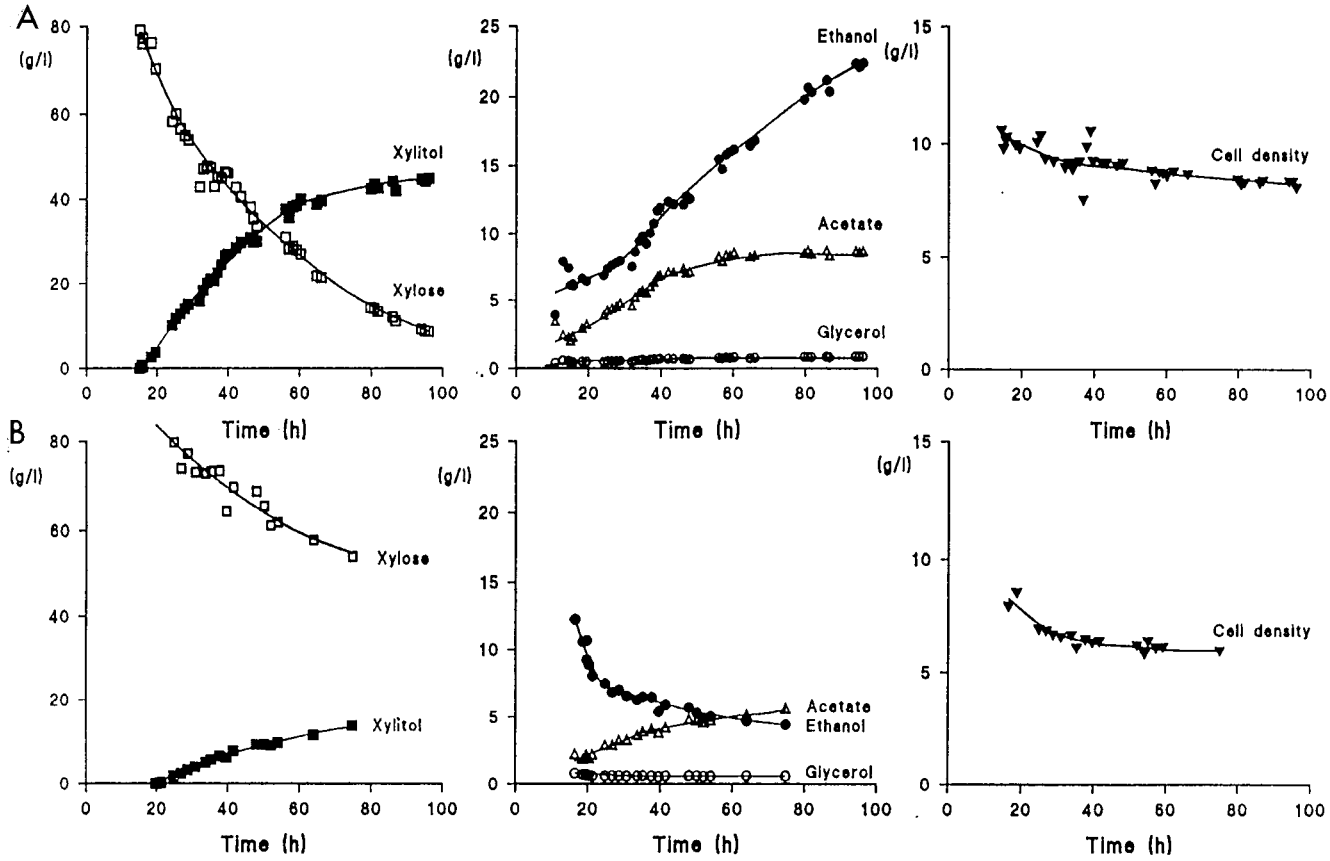

FIG. 2. Anaerobic conversion of xylose to xylitol, cosubstrate utilization, and by-product formation at high and low glucose supply rates by recombinant *S. cerevisiae* H475 harboring the *xyl1* gene for xylose reductase. (A) Glucose supply of 1 g/liter h^{-1}. (B) Glucose supply of 0.1 g/liter h^{-1}.

TABLE 2. Carbon balance calculated on the basis of the rates of substrate consumption and product formation

Carbon balance (mmol of C/g h^{-1})[a]								
Xylose	Xylitol	Balance	Glucose	Ethanol	Acetate	CO_2[b]	Cell mass[c]	Balance
−3.7	4.0	0.3	−1.98	0.82	0.46	0.64	0.10	0.04
−2.1	1.9	−0.2	−0.29	−0.24	0.35	0.13	0.11	0.06

[a] The rates of substrate consumption and product formation were calculated from the progress curves in the period 20 to 30 h after the start of the fermentation. The rate of washing out of the substrates and products due to the dilution by the glucose feed and the sampling was taken into account.

[b] Carbon dioxide in the off gas was measured by a Binos infrared gas analyzer (Leybold-Heraeus).

[c] Cell dry weight was converted into moles of carbon according to the elementary composition formula reported for *S. cerevisiae*, $CH_{1.83}O_{0.56}N_{0.17}$ (11). The decrease in cell mass seen in Fig. 1 is due to the fact that the rate of cell mass production was lower than the rate of washing out due to dilution by the glucose feed.

cerevisiae strain expressing the *xyl1* gene compared with that of a reference strain not harboring the *xyl1* gene was previously observed during xylitol formation with glucose as the cosubstrate (5). The decrease in glycerol formation indicates that excess NADH was oxidized in the XR reaction rather than in the glycerophosphate dehydrogenase reaction.

In both fermentations, the amount of carbon dioxide produced was equivalent to the amount of ethanol and acetate produced from glucose. This indicates that insignificant amounts of glucose were oxidized through the pentose phosphate pathway, which agrees with earlier reports on glucose flux through this pathway in *S. cerevisiae* (6). The formation of reduction equivalents therefore only involves the glyceraldehyde-3-phosphate dehydrogenase and acetaldehyde dehydrogenase reactions, in addition to the alcohol dehydrogenase reaction at a low glucose supply rate. An attempt to close the redox balance, similar to the carbon balance, failed. This is most likely due to uncertainty about the estimated NADPH consumption and NADH production rates during cell mass formation (13).

The rates of xylose consumption and xylitol formation decreased during fermentation, which partly may be ascribed to the decrease in xylose concentration. In extracts of the recombinant yeast cells, the K_m for xylose was determined to be 63 mM, in agreement with the K_m values of 42 and 95 mM for xylose for XR expressed in *P. stipitis* (10, 14). Thus, for 90% saturation of the XR enzyme, a xylose concentration of approximately 600 mM would have been required. However, the rates of xylose consumption and xylitol formation decreased more rapidly than the rate of decrease in xylose concentration (data not shown) and therefore cannot be fully explained by the decreased saturation of the XR enzyme. The decrease in xylose concentration may also affect the transport of xylose into the cells, and accumulation of by-products such as ethanol and acetate could adversely affect the xylitol formation rate.

The research on xylose fermentation at the Department of Applied Microbiology was supported financially by the Swedish National Board for Industrial and Technical Development, the Nordic Fund for Technology and Industrial Development, the Swedish Natural Science Research Council, the Swedish Ethanol Development Foundation (SSEU), and the Knut and Alice Wallenberg Foundation. H. N. Thestrup acknowledges a scholarship from Nordisk Forskerutdannings Akademi.

REFERENCES

1. **Andreasen, A. A., and T. J. B. Stier.** 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J. Cell. Comp. Physiol. **41:**23–26.
2. **Andreasen, A. A., and T. J. B. Stier.** 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cell. Comp. Physiol. **43:**271–281.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Hallborn, J., M.-F. Gorwa, N. Meinander, M. Penttilä, S. Keränen, and B. Hahn-Hägerdal.** 1994. The influence of cosubstrate and aeration on xylitol formation by recombinant *Saccharomyces cerevisiae* expressing the *XYL1* gene. Appl. Microbiol. Biotechnol. **42:**326–333.
5. **Hallborn, J., M. Walfridsson, U. Airaksinen, H. Ojamo, B. Hahn-Hägerdal, M. Penttilä, and S. Keränen.** 1991. Xylitol production by recombinant *Saccharomyces cerevisiae*. Bio/Technology **9:**1090–1095.
6. **Lagunas, R., and J. M. Gancedo.** 1973. Reduced pyridine-nucleotides balance in glucose-growing *Saccharomyces cerevisiae*. Eur. J. Biochem. **37:**90–94.
7. **Lindén, T., J. Peetre, and B. Hahn-Hägerdal.** 1992. Isolation and characterization of acetic acid-tolerant galactose-fermenting strains of *Saccharomyces cerevisiae* from a spent sulfite liquor fermentation plant. Appl. Environ. Microbiol. **58:**1661–1669.
8. **Meinander, N., B. Hahn-Hägerdal, M. Linko, P. Linko, and H. Ojamo.** 1994. Fed-batch xylitol production with recombinant, *XYL1*-expressing *Saccharomyces cerevisiae* using ethanol as cosubstrate. Appl. Microbiol. Biotechnol. **42:**334–340.
9. **Oura, E.** 1977. Reaction products of yeast fermentations. Process Biochem. **1977:**19–35.
10. **Rizzi, M., P. Erlemann, N.-A. Bui-Tahn, and H. Dellweg.** 1988. Xylose fermentation by yeasts. 4. Purification and kinetic studies of xylose reductase from *Pichia stipitis*. Appl. Microbiol. Biotechnol. **29:**148–154.
11. **Roels, J. A.** 1983. Energetics and kinetics in biotechnology, p. 23–69. Elsevier Biomedical Press, Amsterdam.
12. **Skoog, K., B. Hahn-Hägerdal, H. Degn, J. P. Jacobsen, and H. S. Jacobsen.** 1992. Ethanol reassimilation and ethanol tolerance in *Pichia stipitis* CBS 6054 as studied by ^{13}C nuclear magnetic resonance spectroscopy. Appl. Environ. Microbiol. **58:**2552–2558.
13. **van Dijken, J. P., and W. A. Scheffers.** 1986. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Rev. **32:**199–224.
14. **Verduyn, C., R. van Kleef, J. Frank, H. Schreuder, J. P. van Dijken, and W. A. Scheffers.** 1985. Properties of NAD(P)H-dependent xylose reductase from xylose fermenting yeast *Pichia stipitis*. Biochem. J. **226:**669–677.
15. **Visser, W., W. A. Scheffers, W. H. Batenburg-van der Vegte, and J. P. van Dijken.** 1990. Oxygen requirements of yeasts. Appl. Environ. Microbiol. **56:**3785–3792.