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Influence of Cosubstrate Concentration on Xylose Conversion by Recombinant, *XYL1*-Expressing *Saccharomyces cerevisiae*: a Comparison of Different Sugars and Ethanol as Cosubstrates

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Conversion of xylose to xylitol by recombinant *Saccharomyces cerevisiae* expressing the *XYL1* gene, encoding xylose reductase, was investigated by using different cosubstrates as generators of reduced cofactors. The effect of a pulse addition of the cosubstrate on xylose conversion in cosubstrate-limited fed-batch cultivation was studied. Glucose, mannose, and fructose, which are transported with high affinity by the same transport system as is xylose, inhibited xylose conversion by 99, 77, and 78%, respectively, reflecting competitive inhibition of xylose transport. Pulse addition of maltose, which is transported by a specific transport system, did not inhibit xylose conversion. Pulse addition of galactose, which is also transported by a specific transporter, inhibited xylose conversion by 51%, in accordance with noncompetitive inhibition between the galactose and glucose/xylose transport systems. Pulse addition of ethanol inhibited xylose conversion by 15%, explained by inhibition of xylose transport through interference with the hydrophobic regions of the cell membrane. The xylitol yields on the different cosubstrates varied widely. Galactose gave the highest xylitol yield, 5.6 times higher than that for glucose. The difference in redox metabolism of glucose and galactose was suggested to enhance the availability of reduced cofactors for xylose reduction with galactose. The differences in xylitol yield observed between some of the other sugars may also reflect differences in redox metabolism. With all cosubstrates, the xylitol yield was higher under cosubstrate limitation than with cosubstrate excess.

The pentose sugar xylose occurs abundantly in lignocellulose (16, 26, 42) and constitutes a low-cost raw material for biotechnological production of xylitol (36, 37) or fuel ethanol. The former is used as an anticariogenic sweetener in food and related products (20, 29). *Saccharomyces cerevisiae* is a well-known organism, generally regarded as safe in food production, which withstands the inhibitory environment of lignocellulose hydrolysates better than other microorganisms (38, 39). Since *S. cerevisiae* cannot metabolize xylose due to the lack of the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH), metabolic engineering has been used to provide it with a xylose-metabolizing pathway (14, 21, 22, 49, 50, 57). Transport of xylose into the cell is the first metabolic step of xylose conversion, regardless of whether the desired end product is xylitol or ethanol. Xylose is taken up by the facilitated diffusion hexose transport system in *S. cerevisiae*, which also transports glucose, fructose, and mannose (4, 28, 54). Since lignocellulosic substrates constitute a mixture of xylose and other sugars, such as glucose, mannose, and galactose, these may interfere with the transport of xylose, leading to sequential utilization of the sugars.

Recombinant *S. cerevisiae* expressing the XR-encoding *XYL1* gene from *Pichia stipitis* converts xylose to xylitol with 1:1 yield, because xylitol cannot be metabolized due to the absence of XDH (14). Therefore, a cosubstrate such as glucose, ethanol, or acetate is needed for growth, for regeneration of the reduced cofactor [NAD(P)H] required in the reaction, and for supply of metabolic maintenance energy (13, 30). The rate of xylose conversion by *XYL1*-expressing *S. cerevisiae* may be controlled by the uptake of xylose, the XR activity, or the supply rate of reduced cofactors. Previous studies have shown that the XR activity is not rate controlling (33) and that the supply rate of reduced cofactors is rate controlling to some extent (31). The uptake of xylose has also been suggested to share in the control (31, 33). Glucose and xylose are consumed simultaneously only under glucose-limited conditions, which has been attributed to competition for the transport system (33). Fructose and mannose have lower affinities than glucose toward the common transporter (2, 10, 23, 35, 47) and should allow simultaneous xylose uptake to a greater extent than glucose. Cosubstrates transported by other transporters, such as maltose, which is taken up actively through proton symport (15, 45, 46), galactose, which is transported by a galactose-specific facilitated diffusion system (9, 25), and ethanol, which freely diffuses over the cell membrane, should permit simultaneous xylose uptake and may be more efficient cosubstrates. In this study, we compared xylose conversion, using these cosubstrates in different concentrations, by adding a cosubstrate pulse to a cosubstrate-limited fermentation. The results are discussed in relation to published data on transport kinetics and interactions and on redox metabolism. Implications for xylitol production as well as ethanolic fermentation of lignocellulosic substrates are envisioned.

MATERIALS AND METHODS

Strains, plasmid, and transformation. *S. cerevisiae* CEN.PK113-17A (*MAT*α *leu2-3,112 ura3-52 MAL2-8*^{c} *SUC2*), constructed by M. Ciriacy (Düsseldorf, Germany), K.-D. Entian, and P. Kötter (Frankfurt, Germany) and kindly provided by E. Boles (Darmstadt, Germany), was transformed with plasmid pUA103 (14) by the lithium acetate method (44), yielding strain YNQM1, expressing the *P. stipitis XYL1* gene. The strains were stored in a solution of 15% glycerol and 0.9% NaCl at −80°C.

Inoculum and media. For each fermentation, a new inoculum from the frozen stock was grown on plates (containing, per liter, 6.7 g of yeast nitrogen base without amino acids [Difco, Detroit, Mich.] supplemented for auxotrophic requirements, 20 g of glucose, and 20 g of agar) incubated at 30°C for 2 to 3 days. A colony from the plate was used to inoculate 50 ml of mineral medium (55) in

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a 250-ml baffled flask, containing 20 g of glucose per liter and supplemented with 0.05 g of uracil or uracil and leucine per liter as required, and the culture was incubated overnight at 30°C and 140 rpm in a Gallenkamp (Leicester, England) INR-200 orbital incubator. A 1,000-ml baffled shake flask containing 200 ml of mineral medium, with 20 g of the sugar used as the carbon source in the subsequent fermentation per liter, was inoculated with 25 ml of the overnight culture and incubated as described above. The cells were harvested by centrifugation in a Beckman (Geneva, Switzerland) J2-21 centrifuge at 4,440 × *g* for 15 min, resuspended in 0.9% NaCl, and used for inoculation of the fermentor.

All fermentations were conducted in mineral medium supplemented with amino acids and with a carbon source added as described below. In anaerobic batch and continuous cultivations, 0.42 g of polyoxyethylenesorbitan monooleate (Tween 80; Sigma, St. Louis, Mo.) per liter and 0.01 g of ergosterol per liter were added to the medium.

Fermentations. Fed-batch and continuous fermentations were conducted anaerobically in computer-controlled glass fermentors (Belach Bioteknik AB, Stockholm, Sweden). The fermentation conditions were 30°C, pH 5.5 controlled by addition of a solution of 100 g of KOH per liter, agitation at 200 rpm, and a N_2 flow rate of 0.2 liter min^{-1} except when ethanol was used as the cosubstrate, in which case an airflow rate of 0.3 liter min^{-1} was used. The gas outlet condenser was cooled to 2°C by means of water circulation from a cooling bath. Foaming was reduced by manual addition of silicone antifoam when required.

Glucose- and maltose-limited chemostat cultivations were conducted with a mixture of xylose and the cosubstrate as previously described (31), and a cosubstrate pulse was added after a steady state was reached. The fermentation volume was adjusted to 500 ml by means of a pump controlled by a level sensor.

To reach a high initial cell mass, fed-batch fermentations were started as aerobic batch cultivations on 30 g of the sugar used as the cosubstrate (glucose, when ethanol was used as the cosubstrate) per liter, with 300-rpm agitation and air flushing at 0.3 liter min^{-1}. The initial volume was 575 ml, and no xylose was present in the medium during the batch phase. When the initial sugar was completely consumed, anaerobic conditions were applied and 60 to 70 g of xylose per liter (100 ml of a 200-g $liter^{-1}$ solution) was added. A cosubstrate feed was initiated, and the feed rate was adjusted so that the cosubstrate concentration was near zero in the fermentor (i.e., cosubstrate-limited conditions). After a steady xylose conversion rate was reached, a pulse of 25 to 35 g of cosubstrate per liter (50 ml of a 400-g $liter^{-1}$ solution) was added.

Anaerobic batch fermentations were conducted at 30°C in magnetically stirred 120-ml stoppered flasks containing 100 ml of fermentation broth and equipped with cannulas for CO_2 removal.

Analyses. Glucose, mannose, maltose, galactose, ethanol, xylose, xylitol, glycerol, and acetate concentrations in the broth were determined by column liquid chromatography using a Gilson (Middletown, Wis.) CLC system. Glucose, maltose, ethanol, xylitol, glycerol, acetate, and xylose, when no mannose, galactose, or fructose was present, were separated on an HPX87-H column (Bio-Rad, Richmond, Calif.) at 45°C with 5 mM H_2SO_4 as the mobile phase at 0.6 ml min^{-1}. Mannose, galactose, and xylose were separated on an HPX87-P column (Bio-Rad) at 85°C with ultrapure water as the mobile phase at 0.6 ml min^{-1}. The compounds were detected by a Shimadzu (Kyoto, Japan) RID6A refractive index detector. Fructose was analyzed spectrophotometrically by using a D-glucose/D-fructose food analysis kit (catalog no. 139 106; Boehringer Mannheim, Mannheim, Germany).

Biomass concentrations were analyzed by optical density measurements at 620 nm and dry weight determinations (31). When added, antifoam disturbed the optical density measurements. Biomass concentration was also monitored online, using a capacitance probe connected to a model 214M biomass monitor (Aber Instruments, Aberystwyth, Wales). Two fermentors were monitored simultaneously through a model 624 biomass monitor multiplexer (Aber Instruments). The composition of the off-gas from the fermentors was monitored by a carbon dioxide and oxygen monitor (type 1308; Brüel & Kjær, Naerum, Denmark) involving photoacoustic and magnetoacoustic detection techniques for CO_2 and O_2, respectively. Two fermentors were monitored simultaneously through a multipoint sampler (type 1309; Brüel & Kjær). XR activity was measured as previously described (14) from cell lysates prepared by shaking with glass beads (31). Protein concentration was determined by the Bradford method (3), using a commercial Coomassie protein assay reagent (Pierce, Rockford, Ill.).

RESULTS

Xylose conversion in pulse experiments. Anaerobic batch fermentations of 20 g of glucose, mannose, maltose, and galactose per liter supplemented with 20 g of xylose per liter showed that strain YNQM1 was able to grow on the investigated cosubstrates and converted xylose to xylitol during the fermentations. Next, cosubstrate-limited chemostat cultivations were conducted to avoid the transient conditions inherent in batch cultivation and to make quantitative comparisons of xylose conversion rates under cosubstrate limitation and cosubstrate excess. A pulse of cosubstrate was added under steady-state conditions, and the effect on conversion of xylose to xylitol was determined. Due to poor strain stability in chemostat cultivation (see below), this approach had to be modified to pulse experiments in cosubstrate-limited fed-batch cultivations.

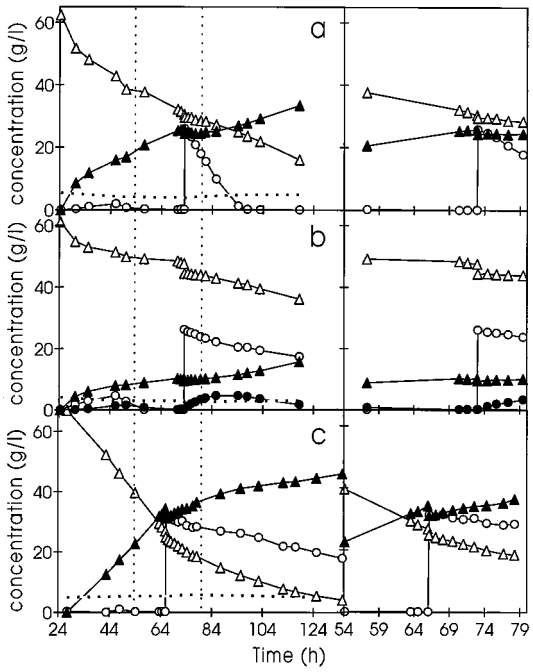

FIG. 1. Effects of pulse additions of cosubstrate on xylose conversion to xylitol in glucose (a)-, maltose (b)-, and galactose (c)-limited fed-batch fermentations. Concentrations of xylose (△), xylitol (▲), cosubstrate (○), biomass (·····), and glucose (●) in the fermentation broth are shown. The right-hand panel shows an enlargement of the time interval indicated by the dashed lines in the left-hand panel. Note that the addition of the sugar pulse caused a step dilution of all concentrations.

Fed-batch fermentations started with biomass production in aerobic batch cultivation using the subsequently fed cosubstrate as the carbon source. After completion of the batch growth phase, xylose was added, anaerobic conditions were applied, and a feed of cosubstrate was initiated. The feed rate was adjusted so that cosubstrate-limited conditions were achieved, and a steady xylose conversion rate was established. A pulse of cosubstrate was added, and the effect on xylose conversion was observed. The concentration profiles of xylose, xylitol, cosubstrate, and biomass for the fermentations with glucose, maltose, and galactose are shown in Fig. 1.

After addition of the glucose pulse, the conversion of xylose was strongly retarded for 10 to 15 h (Fig. 1a). As the glucose was consumed, xylose conversion gradually increased, resuming the same rate as before the pulse when glucose limitation was regained. In contrast, the maltose pulse did not affect xylose conversion (Fig. 1b). The pulse addition of maltose caused a transient accumulation of glucose, consistent with similar observations found during maltose pulses added to maltose-limited chemostat cultivations (41). With galactose as the cosubstrate, rapid xylose conversion was achieved under galactose limitation, but the pulse addition of galactose markedly decreased the xylose conversion rate (Fig. 1c). Similar experiments were conducted with mannose and fructose, which both caused strong retardation of xylose conversion (data not shown). The rate of aerobic xylose conversion with ethanol as

TABLE 1. Specific xylitol production rates and xylitol yields on cosubstrate before and after a cosubstrate pulse of indicated magnitude

Cosubstrate	Sugar pulse (g liter^{-1})	$q_{xylitol}$ (g g^{-1} h^{-1})[a]		Change (%)	$Y_{Xol/Cosub}$ (mol mol^{-1})[b]	
		Before pulse	After pulse		Before pulse	After pulse
Glucose	25.9	0.086	0.0005	−99	1.13	0.026
Fructose	34.2	0.081	0.018	−77	1.21	0.18
Mannose	32.3	0.055	0.012	−78	0.84	0.13
Maltose	26.1	0.031	0.033	+6	1.24	0.63
Galactose	25.1	0.18	0.088	−51	7.23	1.51
Ethanol	35.7	0.062	0.053	−15	0.44	0.15

[a] Specific xylitol production rate.
[b] Molar yield of xylitol on consumed cosubstrate.

the cosubstrate decreased somewhat after addition of an ethanol pulse (data not shown).

Specific xylitol production rates were calculated under cosubstrate-limited conditions and under cosubstrate excess, using linear regression of the xylitol concentrations 12 to 17 h before and during the period of constant conversion rate after the pulse (4 to 12 h, depending on the cosubstrate) (Table 1). The biomass concentration did not change significantly during the fed-batch fermentations (Fig. 1), which made it possible to calculate specific xylitol production rates by using average biomass concentrations before and after the pulse. The glucose pulse caused a nearly total (99%) inhibition of xylose conversion, while the fructose and mannose pulses inhibited conversion of xylose to xylitol by 77 and 78%, respectively (Table 1). The maltose pulse had no effect on xylose conversion, while the galactose pulse inhibited xylose conversion by 51%, and the ethanol pulse caused a minor inhibition (15%) of xylose conversion (Table 1).

Molar yields of xylitol on consumed cosubstrate were calculated under cosubstrate-limited conditions (before the pulse) and during cosubstrate excess (the time period of elevated cosubstrate concentrations after the pulse). With all cosubstrates, the molar yield of xylitol on consumed cosubstrate was higher under cosubstrate limitation than with cosubstrate excess. Under cosubstrate limitation (before the pulse), the xylitol yields were similar on glucose and fructose and somewhat lower on mannose, while the molar yield on maltose was similar to that on glucose, which means that the yield per hexose unit was only half of that of glucose (Table 1). Galactose was the most efficient cosubstrate for xylose conversion, giving the highest specific xylitol production rate and a five- to sixfold-higher yield of xylitol compared with the other sugars, before the pulse (Table 1).

The levels of redox metabolism of the different cosubstrate sugars before and after the sugar pulse were compared by calculating the yields of acetate and glycerol on consumed cosubstrate. Acetate production causes net generation of reduced cofactors, required in the XR reaction, whereas glycerol production consumes reducing power (Fig. 2). The yields of glycerol and acetate per consumed hexose unit were similar for glucose, fructose, mannose, and maltose before and after the pulse (Table 2). During sugar limitation, before the sugar pulse, the yield of glycerol was low and the yield of acetate was high, while the opposite was true after the pulse, during sugar excess. With galactose, the yield of glycerol remained low throughout the fermentation, and the yield of acetate was significantly higher than that of the other sugars, both before and after the pulse.

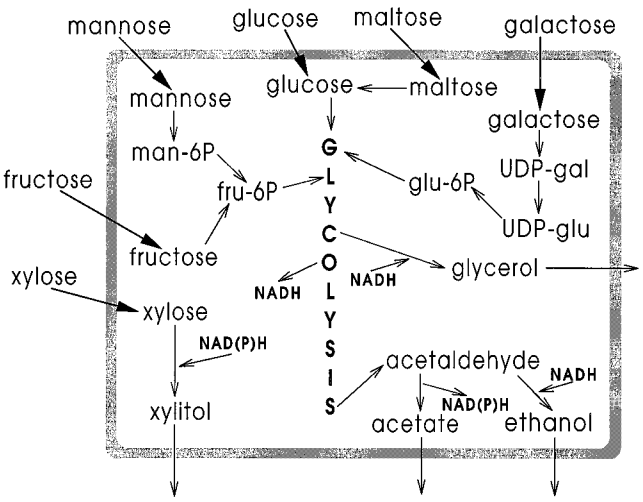

FIG. 2. Simplified scheme of the metabolism of glucose, fructose, mannose, maltose, and galactose and of xylose conversion in *XYL1*-expressing *S. cerevisiae.*

Activity and stability of XR. Previously constructed *XYL1*-expressing strains (14, 56) were not able to metabolize maltose and metabolized galactose only slowly and incompletely. In the present study, strain YNQM1 expressing *XYL1* was constructed by using the host strain CEN.PK113-17A, which grows on maltose, galactose, and all other sugars investigated both aerobically and anaerobically. Analysis of XR and XDH activities in the cell lysate showed that strain CEN.PK113-17A had no XDH activity and a low XR activity of 0.02 U/mg, which is due to an aldo-keto reductase with broad substrate specificity found in *S. cerevisiae* (24). After transformation with plasmid pUA103, the XR activity in shake flask cultures of strain YNQM1 was 5.5 U/mg.

In the fed-batch fermentations, XR activity was analyzed from samples taken (i) after the batch cultivation phase before the cosubstrate feed was started, (ii) just before the pulse addition of cosubstrate (cosubstrate-limited conditions), and (iii) 22 to 46 h after the pulse (conditions of cosubstrate excess). The XR activity at the first sampling point was between 4.9 and 8.5 U/mg in all fermentations. In the fermentations with glucose, fructose, and maltose, the activity clearly decreased during the period of cosubstrate limitation prior to the pulse and increased again under conditions of sugar excess (Table 3). Similarly, in the fed-batch fermentation with mannose, XR activity decreased under mannose limitation, but a subsequent increase during mannose excess could not be distinguished from the experimental error of the measurements

TABLE 2. Overall yields of glycerol and acetate per consumed hexose mole of cosubstrate sugar

Cosubstrate	$Y_{Gly/Cosub}$ (mol/mol of hexose)[a]		$Y_{Ac/Cosub}$ (mol/mol of hexose)[b]	
	Before pulse	After pulse	Before pulse	After pulse
Glucose	0.04	0.12	0.15	0.04
Fructose	0.05	0.13	0.17	0.02
Mannose	0.05	0.16	0.11	0.04
Maltose	0.05	0.11	0.12	0.01
Galactose	0.03	0.04	0.84	0.18

[a] Molar yield of glycerol on consumed mole of hexose.
[b] Molar yield of acetate on consumed mole of hexose.

TABLE 3. XR activities in lysates of cell samples taken at the indicated sampling times from fed-batch and chemostat fermentations[a]

Fermentation mode	Carbon source	Sampling time (h)	XR activity ± SD[b] (U mg^{-1})
Fed-batch	Glucose	23.5	5.8 ± 0.3
		72.9	3.8 ± 0.5
		97.9	6.1 ± 0.5
	Fructose	19.2	8.5 ± 0.5
		90.0	2.3 ± 0.5
		112.2	3.7 ± 0.2
	Mannose	23.2	4.9 ± 0.4
		95.9	1.7 ± 0.1
		132.2	1.9 ± 0.3
	Maltose	23.5	7.5 ± 0.5
		73.0	1.7 ± 0.3
		98.0	2.8 ± 0.4
	Galactose	27.5	5.7 ± 0.5
		66.1	7.9 ± 2.1
		112.3	5.1 ± 0.2
	Ethanol	23.1	8.0 ± 0.7
		95.9	1.2 ± 0.1
		132.1	0.7 ± 0.05
Chemostat	Glucose	17.9 (after batch)	7.4 ± 0.9
		136.0 (glucose limitation)	0.09 ± 0.005
	Maltose	20.0 (after batch)	8.9 ± 0.2
		89.0 (maltose excess)	8.8 ± 0.6
		218.9 (maltose limitation)	0.04 ± 0.003
		260.0 (maltose limitation)	0.02 ± 0.001

[a] In fed-batch fermentation, the first sample was taken after the initial batch cultivation, the second was taken before the cosubstrate pulse, and the third was taken after the pulse.
[b] Includes deviations for activity measurements and protein determinations.

(Table 3). In the fed-batch fermentation with galactose, the changes in XR activity were smaller than the experimental error of the measurements throughout the fermentation; with ethanol, the XR activity decreased throughout the fermentation, regardless of cosubstrate limitation or excess (Table 3). In chemostat cultivations on glucose and maltose, the strain lost all XR activity (Table 3). In the maltose-limited chemostat cultivation, the maltose concentration increased after initiation of the feed, and maltose-limited conditions were not reached until after 110 h. As found for the fed-batch cultivations mentioned above, the XR activity was stable as long as maltose was present in excess but decreased under maltose-limited conditions (Table 3). The onset of maltose limitation also correlated with a decrease in xylitol production.

DISCUSSION

The rate of conversion of xylose to xylitol by *XYL1*-expressing *S. cerevisiae* may be controlled at the level of (i) transport of xylose into the cell, (ii) XR activity, or (iii) supply of reduced cofactors. It has been suggested that the supply of reduced cofactors determines the rate of conversion of xylose to xylitol (31), and it has been shown that the XR activity does not exert significant control (33). The current and previously presented (33) results demonstrate that many cosubstrates inhibit xylose conversion when present in elevated concentrations. Published data on transport interactions between xylose and the different cosubstrates suggest that the cosubstrates inhibit xylose conversion by inhibiting transport of xylose. Thus, when these cosubstrates are present in high concentrations, the transport of xylose probably controls the rate of xylose conversion.

The facilitated glucose transport system of *S. cerevisiae* transports glucose, fructose, and mannose. The transport system has an affinity constant (K_m) in the range of 1 to 28 mM for glucose, depending on the magnitude of glucose repression (58). Depending on growth conditions and strain, others have reported affinity constants ranging from 0.5 to 125 mM for glucose (2, 4, 10, 11, 23, 35, 47). For fructose and mannose, affinity constants of 6 to 40 mM (2, 10, 47) and 12 to 27 mM (23, 35, 47), respectively, have been reported. The facilitated glucose transport system also transports xylose (4, 53), for which it has a comparatively low affinity constant of 49 to 300 mM (4, 18, 23, 28, 47, 54). Therefore, glucose, fructose, and mannose, when present in high concentrations, are expected to saturate the transport system, inhibiting the transport of xylose and thus xylose conversion. The present results show that glucose inhibited xylose conversion almost completely, whereas fructose and mannose had a somewhat lower effect, in agreement with the lower affinity constants of the transport system for these sugars. Since competitive inhibition is mutual, high xylose concentrations inhibit the uptake of glucose, fructose, and mannose. In glucose-limited chemostat cultivation, this has been noted as an increase in the residual glucose concentration after addition of xylose to the medium (31). A xylose concentration of 100 g liter^{-1} has been reported to inhibit the uptake of 5 g of glucose per liter by 83% (23).

Maltose did not inhibit xylose conversion, since maltose is transported by a specific transporter and therefore does not interfere with xylose transport by the glucose transport system. The effect of the maltose pulse may have been somewhat disturbed by the minor excretion of glucose after the pulse. However, the glucose concentration remained below 2.4 g liter^{-1} in the time interval used for calculation of the xylitol production rate after the pulse, and the glucose/xylose ratio was 0.06, compared with 1.1 in the pulse experiment with glucose. The ratio of 0.06 was not high enough to significantly affect the xylose uptake. In contrast to maltose, galactose, which is also transported by a separate transporter, inhibited xylose conversion severely. The glucose transport system has been found to be inhibited by galactose in galactose-grown cells, due to a mutual cross-inhibition between the galactose transporter and the glucose transport system (34). The inhibition was found to be of a noncompetitive nature, and the authors suggested that it may be mediated by a regulatory protein which, when bound to galactose or a substrate of the glucose transport system (glucose, fructose, or mannose), inhibits the other transport system (34). Ethanol, which can freely diffuse over the cell membrane, inhibited xylose conversion somewhat after pulse addition. Ethanol has been found to inhibit the glucose transport system noncompetitively, because of interference with the hydrophobic regions of the cell membrane (28).

The yield of xylitol on the cosubstrate was reduced in the presence of high cosubstrate concentrations during the pulse (Table 1). In the cases of glucose, fructose, mannose, galactose, and ethanol, the reduced yield under cosubstrate excess can at least partly be explained by the inhibition of xylose transport, but even with maltose, which does not affect xylose transport, the yield was halved in the presence of excess maltose compared with maltose-limited conditions. The changes in xylitol, glycerol, and acetate yields effected by the switch from cosubstrate limitation to excess show that the redox metabolism of the cell changed as the rate of cosubstrate consumption increased, leading to an increase in glycerol yield and a decrease in xylitol and acetate yields (Tables 1 and 2). This could be due to changes in the intracellular concentrations of NAD(P)H/NAD(P)$^+$ influencing the kinetics of the redox reactions, which may affect the xylitol yield on the cosubstrate.

The xylitol yield on glucose obtained under cosubstrate-

limited conditions (1.13 mol/mol) was in the same range as previously reported for similar fermentations with another *XYL1*-expressing *S. cerevisiae* strain (33, 51). The metabolism of glucose, fructose, mannose, maltose, and galactose is similar and mainly directed through the glycolytic pathway, after initial conversion of the sugar to glucose-6-phosphate or fructose-6-phosphate (Fig. 2). Therefore, the potential of these sugars to produce the reducing power required in the reduction of xylose to xylitol should be equal, and thus the yield of xylitol on these sugars should be similar. However, differences in glycerol and acetate yields on the different sugars (Table 2) indicated that the redox metabolism may differ. A lower glycerol yield on galactose than on glucose, observed after the pulse (Table 2), has previously been found in aerobic batch fermentations (27). It was attributed to a higher respiratory activity during growth on galactose, leading to a reduced need for glycerol production for maintenance of the redox balance (27). This interpretation, however, cannot be applied to the current observations under anaerobic conditions. Comparing the yields of glycerol and acetate on glucose and galactose before the pulse (Table 2), we estimated that galactose metabolism produced 12 times more reduced cofactors (two reduced cofactors are formed per molecule of acetate produced, and one is consumed per molecule of glycerol produced) than glucose. This can qualitatively explain the approximately six-times-higher xylitol yield obtained with galactose under cosubstrate-limited conditions (Table 1). High acetate yield was also found to be coupled to a high xylitol yield when glucose, fructose, and mannose were compared, but the poor xylitol yield on maltose was not accompanied by a low acetate yield. Acetate and glycerol yields alone do not give a complete picture of the redox metabolism, since additional reduced cofactors are produced in the pentose phosphate pathway. The flux through the pentose phosphate pathway was not quantified and may have varied between different cosubstrates.

Even though the XR activities fluctuated during the fed-batch cultivations, this could not account for the observed instantaneous decrease in the xylose conversion rate after the pulse additions of cosubstrate. Despite the fluctuations, the XR activity was above 1 U/mg in all but one sample (Table 3). Previously, it was shown that a 20-fold difference in XR activity (0.51, compared with 10.8 U/mg) resulted in a <2-fold difference in xylitol production rate (33). The differences in XR activity before and after the pulse in the current fed-batch fermentations were less than twofold (Table 3) and could thus not have affected the xylose conversion rate significantly.

Strain YNQM1 totally lost its XR activity in chemostat cultivation (Table 3), showing that the strain was unstable under these conditions. The previously constructed strain GPY55-15B(pUA103), containing the same plasmid as YNQM1, had only about 0.5 U of XR activity per mg (14) and was stable during long chemostat cultivations (31). However, strain GPY55-15B containing plasmid pM2, mediating a 20-fold-higher XR activity of 10.8 U/mg (56), was unstable (33). Thus, the instability is not related to a particular plasmid construct or a particular host strain but is related to the level of XR activity, confirming the conclusion of a previous study comparing differences in stability of the two strains with the same genetic background but different XR activities (33).

The XR activity of strain YNQM1 was related to growth conditions, since the activity decreased under sugar-limited conditions in fed-batch and chemostat cultivation and increased again under conditions of sugar excess in some of the fed-batch fermentations (Table 3). A decrease in XR activity, which was not related to plasmid loss, was also noted in previous glucose-limited chemostat cultivations (33) and in glucose-limited immobilized cells (43). The *S. cerevisiae PGK* promoter, which regulates the expression of XR in plasmid pUA103 (14), is a strong glycolytic promoter, often considered to give constitutive, high-level expression of heterologous genes. However, the expression level is influenced by the carbon source and the growth phase in batch cultivation (5, 8, 19, 40, 52). Several authors found high expression in the presence of glucose and low expression in post-exponential growth phase (5) and with gluconeogenic carbon sources (8, 52), while others found low expression at high glucose concentrations, increased expression at low glucose levels, and repression by high ethanol concentrations (40). The *PGK* promoter contains several binding sites for regulatory proteins (6–8) which are responsible for regulation of transcription, but it is not clear exactly how the regulation is effected through these proteins. The fluctuations in XR activity noted in the present and previously investigated (32, 33, 43) strains may thus at least partly be due to regulatory responses of the *PGK* promoter to different nutritional conditions.

The inhibition of xylose conversion by other sugars has implications for the conversion of the xylose in lignocellulose to xylitol as well as for the production of fuel ethanol from lignocellulose with recombinant *S. cerevisiae*. Lignocellulosic substrates usually contain glucose, mannose, and galactose in addition to xylose (17, 26, 38, 48). Because of transport inhibition and catabolite repression in batch fermentation, glucose and mannose will be utilized sequentially, after which galactose and xylose will be utilized, possibly with some inhibition of xylose uptake by galactose. Especially in the case of xylitol production with *XYL1*-expressing *S. cerevisiae*, batch fermentation would be inefficient, since the cosubstrate would be consumed before xylose could be taken up. The cosubstrate-limited fed-batch fermentation technique offers possibilities to control the cosubstrate concentration so that maximal xylose conversion is achieved (30, 33). In natural xylose-fermenting yeasts, such as *P. stipitis* and *Pachysolen tannophilus*, hexoses and xylose are utilized sequentially, despite the presence of specific xylose transport systems in these yeasts. This is due to catabolite repression of XR and XDH (1). Introduction of a xylose permease into *S. cerevisiae* with constitutive expression of XR or of XR and XDH may enable simultaneous xylose and hexose conversion, increasing the xylose conversion efficiency in lignocellulosic substrates (12).

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