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A Glycerol-3-Phosphate Dehydrogenase-Deficient Mutant of *Saccharomyces cerevisiae* Expressing the Heterologous *XYL1* Gene

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The gene *XYL1*, encoding a xylose reductase, from *Pichia stipitis* was transformed into a mutant of *Saccharomyces cerevisiae* incapable of glycerol production because of deletion of the genes *GPD1* and *GPD2*. The transformed strain was capable of anaerobic glucose conversion in the presence of added xylose, indicating that the xylose reductase reaction can fulfill the role of the glycerol-3-phosphate dehydrogenase reaction as a redox sink. The specific xylitol production rate obtained was 0.38 g g^{-1} h^{-1}.

The yeast *Saccharomyces cerevisiae* lacks the two enzymes xylose reductase (XR) and xylitol dehydrogenase and is not capable of aerobic growth on xylose or the fermentation of this pentose (6). The genes encoding XR and xylitol dehydrogenase have been identified in the xylose-fermenting yeast *Pichia stipitis*, and both genes have been transformed into *S. cerevisiae* (8, 10, 19). A strain possessing only the *XYL1* gene, encoding XR, is not able to grow on xylose but is able to convert xylose to xylitol in the presence of a cosubstrate (7, 12, 20). A highly reducing intracellular environment, accomplished by, e.g., the use of a highly reduced cosubstrate such as ethanol or anaerobic conditions, favors the reduction of xylose to xylitol. Under anaerobic conditions glycerol is formed by *S. cerevisiae* in order to regenerate NAD^+ (15, 16). NADH is consumed in the reduction of dihydroxyacetone to glycerol-3-phosphate catalyzed by glycerol-3-phosphate dehydrogenase. The xylose reductase from *P. stipitis* has a dual cofactor specificity, and the reduction may take place with either NADH or NADPH (23). For a mutant strain expressing *XYL1*, there will thus be a competition for NADH between the glycerol-3-phosphate dehydrogenase and the XR enzymes, giving parallel production of glycerol and xylitol (13). Two genes, *GPD1* and *GPD2*, encoding two isoforms of glycerol-3-phosphate dehydrogenase enzymes in *S. cerevisiae* have been identified and cloned (5, 11). Recently, a mutant in which both *GPD1* and *GPD2* were deleted was constructed (1). This double mutant was incapable of glycerol production and could be grown only aerobically. Under anaerobic conditions, the metabolism stopped and neither growth nor ethanol production was possible as a consequence of the inability of the cell to regenerate NAD^+ (2).

In the present study, the *gpd1*Δ*gpd2*Δ mutant was transformed with a *XYL1*-containing plasmid to examine (i) if a blocked pathway for glycerol production would enhance xylitol production because of decreased competition for NADH and (ii) if glycerol-free anaerobic fermentation could be sustained in *S. cerevisiae* by incorporating an alternative internal redox sink to glycerol.

The *S. cerevisiae* haploid strain used in this study was W303-1A (*MAT***a** *leu2-3,112 his3-11,15 ade2-1 trp1-1 ura3-1 can1-100 mal0 GAL SUC2*). The double null mutant (*gpd1*Δ::TRP1 *gpd2*Δ::URA3) for the two genes encoding glycerol-3-phosphate dehydrogenase was generated as previously described (1). The *XYL1*-containing plasmid pUA103 (8) and the control plasmid pMA91 (14) were transformed into *gpd1*Δ *gpd2*Δ mutants by the lithium acetate method (17). Transformants were selected on synthetic complete Leu medium plates (18). Expression levels of the *XYL1* gene with the pUA103 plasmid have previously been reported to be 0.3 to 0.6 U mg of $protein^{-1}$ in *S. cerevisiae* (8, 13, 20). The medium used was a synthetic medium, with glucose (20 g $liter^{-1}$) as the carbon and energy source. The medium was prepared according to the method of Verduyn et al. (22), with the following changes: the amount of $(NH_4)_2SO_4$ used was 7.5 g $liter^{-1}$, that of KH_2PO_4 was 3.5 g $liter^{-1}$, that of $MgSO_4 \cdot 7H_2O$ was 0.75 g $liter^{-1}$, and the concentrations of all trace metals were doubled. Furthermore, histidine and adenine (120 mg $liter^{-1}$) and unsaturated fatty acids, in the form of Tween 80 (420 mg $liter^{-1}$) and ergosterol (10 mg $liter^{-1}$), were added to the medium. Antifoam (Sigma 289) was added at 0.15 g $liter^{-1}$. The glucose was autoclaved separately from other medium components at 121°C for 25 min. Vitamins, histidine, and adenine were sterilized by filtration. Ergosterol and Tween 80 were sterilized according to the method of Verduyn et al. (21). Inoculum cultures were grown in 250-ml conical flasks in a shake water bath at 30°C for 48 h.

The experiments were carried out in a 2.5-liter bioreactor (BioFlo III; New Brunswick Scientific) with a working volume of 2.0 liters at a temperature of 30°C and pH controlled at 5.0 by the addition of 2 M NaOH. The gas flow rate was controlled at 0.6 liter min^{-1} (at 0°C, 1 atm [1 atm = 101.29 kPa]) by a mass flow controller. A three-way valve made it possible to switch between air and nitrogen (certified less than 5 ppm of oxygen) in the inlet gas stream. The carbon dioxide and oxygen content of the exit gas was measured with a Bruel & Kjaer 1308 gas monitor as described by Christensen et al. (4). The carbon dioxide evolution rate (CER) could be calculated from the exit gas concentration and the measured gas flow rate, with account taken for withdrawn sample volume. The optical density was monitored on-line with a flow-injector-analysis manifold (2). Samples were also taken for the determination of cell concentration by dry weight. Duplicate samples were centrifuged,

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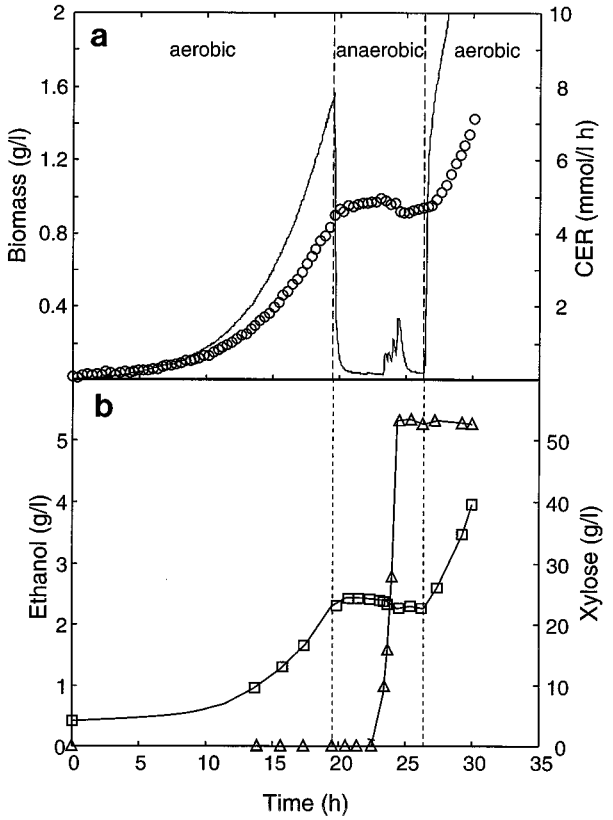

FIG. 1. (a) Biomass concentration, determined by flow injector analysis (○), and CER (solid line); (b) concentrations of xylose (△) and ethanol (□) for a batch cultivation of the *gpd1*Δ*gpd2*Δ mutant carrying pMA91.

washed with distilled water, and dried for 24 h at 105°C. Cell-free samples for high-pressure liquid chromatography (HPLC) analyses were regularly withdrawn from the reactor through a membrane filter module (PP19; ABC Corp.). Glucose, xylose, xylitol, ethanol, glycerol, and organic acids were determined by HPLC. A Shodex SH1011 column was used at a temperature of 56°C, with 5 mM H_2SO_4 as the eluent. All components were determined by a refractive index detector (Waters 410; Waters, Milford, Mass.). The first part of the cultivations was carried out under aerobic conditions. When the cell density of the cultures had reached approximately 0.8 g liter^{-1}, the inlet gas was changed to nitrogen.

Under fully aerobic conditions with glucose as the carbon and energy source, the *gpd1*Δ*gpd2*Δ mutant transformed with a *XYL1*-containing plasmid (pUA103) had a specific growth rate of 0.15 h^{-1}, which was slightly lower than that of the *gpd1*Δ *gpd2*Δ mutant transformed with the control plasmid (pMA91), 0.21 h^{-1}. For the *gpd1*Δ*gpd2*Δ mutant carrying pMA91, growth as well as carbon dioxide evolution abruptly stopped when the mutant was subjected to anaerobic conditions (Fig. 1). This is in accordance with previous observations for the parental *gpd1*Δ*gpd2*Δ strain (2). However, neither growth nor carbon dioxide evolution stopped completely for the *gpd1*Δ*gpd2*Δ mutant carrying pUA103 under anaerobic conditions (Fig. 2), and ethanol was formed to some extent. Whereas no glycerol could be detected for the *gpd1*Δ*gpd2*Δ mutant carrying pMA91, traces of glycerol were formed by the *gpd1*Δ*gpd2*Δ mutant carrying pUA103. In the strain lacking XR, a small immediate effect of short duration on the CER was observed when pulses of xylose were added during anaerobic conditions (Fig. 1). The burst in CER was not caused by dissolved oxygen in the xylose solution added, since the effect was seen regardless of whether the xylose solution had been deaerated. Xylitol formation was below the detection limit, and no measurable consumption of xylose occurred. When the culture was again exposed to aerobic conditions, carbon dioxide evolution and growth quickly resumed, but neither xylitol formation nor xylose consumption was observed (Fig. 1). The effects of xylose addition on the strain carrying the *XYL1* gene were much larger, although a substantial amount of xylose had to be added before any clear effects could be seen (Fig. 2). Addition of up to 4 g of xylose liter^{-1} produced barely detectable changes (Fig. 3). However, after addition of 50 g of xylose liter^{-1}, the anaerobically measured CER and ethanol formation rate were again comparable to the aerobically measured ones. The specific xylitol formation rate under anaerobic conditions was calculated to be 0.38 g g^{-1} h^{-1}.

Apparently, the alternative redox sink provided by the reduction of xylose to xylitol can compensate, at least partly, for the lost capability of glycerol formation and make anaerobic growth possible in the *gpd1*Δ*gpd2*Δ mutant. Other redox sinks, such as acetoin, may also serve this purpose. Acetoin is, in fact, more effective than xylose in restoring metabolic activity (Fig. 3). Several explanations may account for this observation. The enzyme XR has a low affinity for xylose, with a reported K_m value of 63 mM (20). The *XYL1* gene was obtained from the yeast *P. stipitis*, which exhibits an active transport system for xylose (9) and is therefore able to accumulate xylose against a concentration gradient. *S. cerevisiae* lacks an active uptake system for xylose, and xylose is instead taken up via the glucose

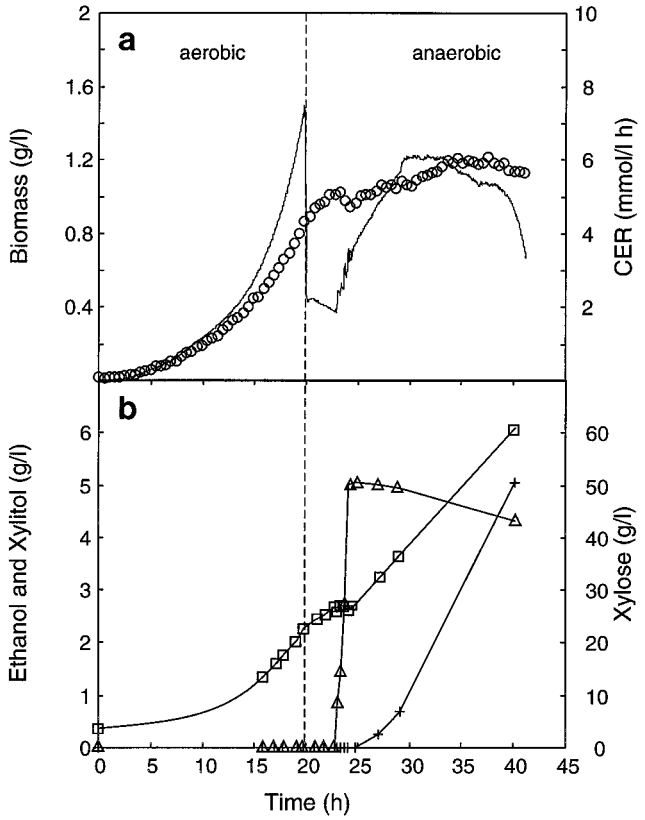

FIG. 2. (a) Biomass concentration, determined by flow injector analysis (○), and CER (solid line); (b) concentrations of xylose (△), xylitol (+), and ethanol (□) for a batch cultivation of the *gpd1*Δ*gpd2*Δ mutant carrying pUA103.

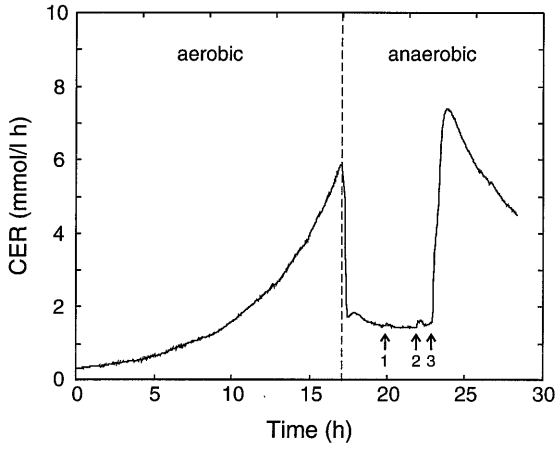

FIG. 3. CER for a batch cultivation of the *gpd1*Δ*gpd2*Δ mutant carrying pUA103. Arrows: 1, addition of xylose (0.5 g liter^{-1}); 2, addition of xylose (4 g liter^{-1}); 3, addition of acetoin (0.5 g liter^{-1}).

uptake system (3). This system is a facilitated system and does not allow accumulation against a gradient. Furthermore, there will be competition for the carriers between glucose and xylose. High concentrations of xylose are therefore needed to obtain sufficiently high intracellular concentrations for the XR to operate efficiently. The residual metabolic activity of the *XYL1*-carrying mutant under anaerobic conditions in the absence of added xylose is most likely caused by catalytic activity of the XR for substrates other than xylose. The XR is known to be capable of acting on various substrates, including, e.g., glyceraldehyde, arabinose, and galactose (23). An activity of 5% of that obtained with xylose has been reported with dihydroxyacetone as a substrate (23). This suggests that the traces of glycerol found in the present study are due to XR activity. The specific xylitol formation rate obtained with the mutant in the present study, 0.38 g g^{-1} h^{-1}, is several times higher than a previously reported value of 0.12 g g^{-1} h^{-1} obtained with a *XYL1*-carrying mutant, which was not mutated in its glycerol metabolism (20). However, in order to maintain a high conversion rate, a high xylose concentration in the medium is required. The ratio between glucose and xylose in the medium should also be favorable, which suggests that fed-batch or continuous operation should be used in order to maintain a high xylose conversion rate.

The experimental results presented here demonstrate that the reducing power of *S. cerevisiae* under anaerobic conditions, which is normally used to produce glycerol, may be successfully diverted in favor of another reduced product. This can be achieved by deleting the *GPD1* and *GPD2* genes and inserting another suitable reductase.

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