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Physiological Response to Anaerobicity of Glycerol-3-Phosphate Dehydrogenase Mutants of *Saccharomyces cerevisiae*

S. BJÖRKQVIST,[1] R. ANSELL,[2] L. ADLER,[2] AND G. LIDÉN[1]*

Department of Chemical Reaction Engineering, Chalmers University of Technology, S-412 96 Göteborg,[1] and Department of General and Marine Microbiology, Medicinaregat 9C, University of Göteborg, S-413 90 Göteborg,[2] Sweden

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Mutants of *Saccharomyces cerevisiae*, in which one or both of the genes encoding the two isoforms of NAD-dependent glycerol-3-phosphate dehydrogenase had been deleted, were studied in aerobic batch cultures and in aerobic-anaerobic step change experiments. The respirofermentative growth rates under aerobic conditions with semisynthetic medium (20 g of glucose per liter) of two single mutants, *gpd1*Δ and *gpd2*Δ, and the parental strain ($\mu = 0.5$ h^{-1}) were almost identical, whereas the growth rate of a double mutant, *gpd1*Δ *gpd2*Δ, was approximately half that of the parental strain. Upon a step change from aerobic to anaerobic conditions in the exponential growth phase, the specific carbon dioxide evolution rates (CER) of the wild-type strain and the *gpd1*Δ strain were almost unchanged. The *gpd2*Δ mutant showed an immediate, large (>50%) decrease in CER upon a change to anaerobic conditions. However, after about 45 min the CER increased again, although not to the same level as under aerobic conditions. The *gpd1*Δ *gpd2*Δ mutant showed a drastic fermentation rate decrease upon a transition to anaerobic conditions. However, the CER values increased to and even exceeded the aerobic levels after the addition of acetoin. High-pressure liquid chromatographic analyses demonstrated that the added acetoin served as an acceptor of reducing equivalents by being reduced to butanediol. The results clearly show the necessity of glycerol formation as a redox sink for *S. cerevisiae* under anaerobic conditions.

Glycerol is a by-product of considerable importance during anaerobic production of ethanol by *Saccharomyces cerevisiae*. Glycerol formation under anaerobic conditions is normally explained by the necessity to consume the NADH formed in cell synthetic reactions, most notably, amino acid synthesis (10, 13). However, the formation of some organic acids, such as acetic acid and succinic acid, also leads to production of NADH, which is reoxidized in the glycerol formation pathway (14). Glycerol is also formed under aerobic conditions, but normally at much lower yields than under anaerobic conditions. A notable exception, however, is when a high-osmolarity medium is used, in which case the glycerol acts as an osmoregulator and substantial amounts may be produced (5). Depending on the strain, medium, and process conditions, 4 to 10% of the carbon source may be converted to glycerol (15). It is therefore highly relevant to study the physiological behavior of mutants of *Saccharomyces cerevisiae* with modified glycerol metabolism.

With glucose as the carbon source, glycerol is formed by conversion of dihydroxyacetone phosphate to glycerol-3-phosphate (G-3-P) and subsequent dephosphorylation of G-3-P to yield glycerol. The first reaction is catalyzed by glycerol-3-phosphate dehydrogenase (GPD). Two isogenes encoding different molecular forms of GPD have been identified. The first isoform of the GPD enzyme studied was found to be induced by osmotic stress (2) and was later demonstrated to be coded for by the *GPD1* gene (1, 12, 22). A second gene, *GPD2*, highly homologous to *GPD1* was cloned by Eriksson et al. (9). However, no obvious phenotype was observed for the *gpd2*Δ mutant. Recently, mutants with one or both isoforms of the *GPD* gene deleted were constructed (3). To physiologically characterize these mutants, we performed growth studies under well-controlled conditions in a bioreactor. In particular, we examined the importance of glycerol production in the intracellular redox control under anaerobic conditions.

MATERIALS AND METHODS

Yeast strains. The parent yeast strain used in this study was *S. cerevisiae* W303-1A (*Mat***a** *leu2 his3 ade2 trp1 ura3*). Mutants with *GPD1* (*gpd1*Δ::TRP1) and *GPD2* (*gpd2*Δ::URA3) deleted and one with both *GPD1* and *GPD2* (*gpd1*Δ::TRP1 *gpd2*Δ::URA3) deleted were generated in the W303-1A background as described by Ansell et al. (3).

Medium and growth conditions. The medium used was a semisynthetic medium, with 20 g of glucose per liter as the carbon and energy source. The medium was prepared as described by Verduyn et al. (21), except that we used $(NH_4)_2SO_4$ at 7.5 g/liter, KH_2PO_4 at 3.5 g/liter, and $MgSO_4 \cdot 7H_2O$ at 0.75 g/liter and doubled the concentrations of all trace metals. Furthermore, the medium was supplemented with 0.5 g of yeast extract per liter, 120 mg of the necessary amino acids (L-leucine, L-histidine, and L-tryptophan) per liter, and 120 mg of adenine and uracil per liter. Unsaturated fatty acids, in the form of Tween 80 (420 mg/liter) and ergosterol (10 mg/liter) were used in all cultivations, even the fully aerobic batch cultivations. Antifoam (Sigma 289) was added at 0.15 g/liter. The glucose was autoclaved separately from other medium components at 121°C for 25 min. Vitamins, yeast extract, amino acids, adenine, and uracil were sterilized by filtration. Ergosterol and Tween 80 were not autoclaved but merely steamed in a minimum volume of pure ethanol for 10 min as described in reference 20. Inoculum cultures were grown in 250-ml Erlenmeyer flasks in a shaking water bath at 30°C for 48 h.

Experimental procedures. The experiments consisted of aerobic batch cultivations and step change experiments in which responses to anaerobic conditions were studied. In the step change experiments, cells were initially grown aerobically, and during strictly exponential growth, a step change of the inlet gas from air to pure nitrogen was made. All aerobic batch experiments and step change experiments were performed in a BioFlo III bioreactor (New Brunswick Scientific, Edison, N.J.) with a working volume of 5 liters at a temperature of 30°C. An inoculum culture of 5 ml was added at the start of the experiment. The pH was maintained at 5.0 by addition of 2 M NaOH, and the stirrer rate was 500 rpm. Air or nitrogen sparging through the fermentor was controlled by a mass flow controller (Hi-Tec, Ruurlo, The Netherlands) at a flow rate of 1.5 liters/min. A three-port valve made it possible to change the inlet gas stream between air and nitrogen. The nitrogen gas used had a guaranteed oxygen content of less than 5 ppm [ADR class 2, 1(a); AGA]. The composition of the outlet gas was continuously measured with a Bruel & Kjaer 1308 gas monitor (7). Gas measurements

* Corresponding author. Phone: 4631-7723039. Fax: 4631-7723035. E-mail: Liden@cre.chalmers.se.

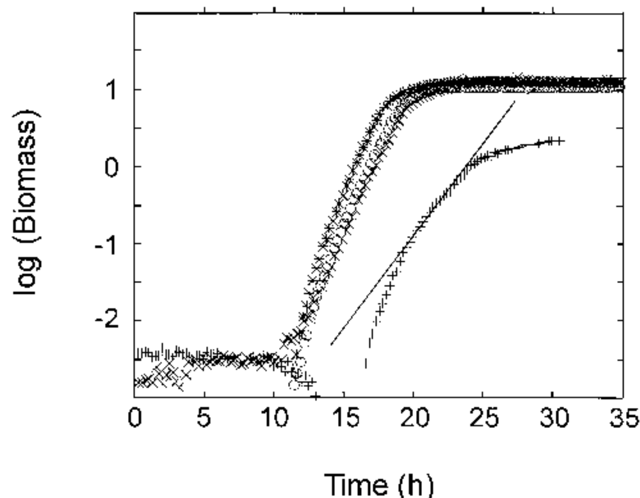

FIG. 1. Logarithm of biomass concentration, measured by on-line FIA, versus time in aerobic batch cultures of the W303-1A parental strain (*), the *gpd1*Δ mutant (×), the *gpd2*Δ mutant (○), and the *gpd1*Δ *gpd2*Δ mutant (+). Glucose (20 g/liter) was used as the carbon and energy source. The region for calculation of the specific growth rate of the *gpd1*Δ *gpd2*Δ mutant is indicated by the line.

were averaged for 2 min and stored in a data file. The instrument was calibrated with a gas mixture containing 5.00% CO_2, 20.00% O_2, and nitrogen as inert gas. The volumetric mass transfer coefficient value for the reactor was determined to be 0.05 s^{-1}, and from the measured outlet oxygen concentration it could be calculated that the dissolved oxygen concentration was above 80% of the air saturation concentration in all aerobic batch cultivations.

An on-line flow injection analysis (FIA) system was used for determination of optical density at 610 nm. Samples were automatically taken from the bioreactor every 15 min. Samples for high-pressure liquid chromatography (HPLC) analyses were withdrawn from the broth via a module membrane filtration unit (probe system PP19; ABC, Puchheim, Germany) every hour. At least 4 ml of the initial portion of each sample was discarded to ensure that a representative sample was taken. Whole culture samples were also withdrawn for dry-weight determinations and assays of GPD1 activity.

In a separate experiment with the *gpd1*Δ *gpd2*Δ mutant, culture fluorescence was measured with an optical probe tuned for NAD(P)H detection (FluoroMeasure System; BioChem Technology, Malvern, Pa.). In this experiment, a 2.5-liter Chemap SG fermentor and an on-line HPLC system were used as previously described (17). The medium and growth conditions were otherwise identical to those described above, except that no yeast extract was added to the medium, since this would substantially increase the background fluorescence.

Analyses. (i) Biomass. The FIA manifold for biomass optical density determination consisted of a six-port, pneumatically operated valve with a 50-μl sample loop, a magnetically stirred mixing chamber with a volume of 0.5 ml, and a spectrophotometer equipped with an 80-μl flow cuvette. To get a stable signal, it was important to remove gas bubbles, which was done in a small degassing chamber with a connection on one side and connections on the top and bottom. Each fresh sample was pumped through the side connection. Gas bubbles and some of the sample solution were pumped through the top connection, and the remaining bubble-free sample was passed through the bottom connection. Samples were taken via a computer-controlled pump, which started 100 s before the desired injection time, to ensure that representative samples were taken. At the time of injection, the six-port valve turned and the sample in the loop was carried by an eluent stream of degassed pure water to the mixing chamber. The resulting absorbance peak was recorded in a computer, and the peak height was stored. For aerobic growth conditions, the peak height was linearly related to the biomass concentration up to a concentration of 3 g (dry weight)/liter with a prediction error of less than 3%. However, in step change experiments, deviation from linearity was observed in the anaerobic part for some mutants, resulting in prediction errors of up to 10%. Dry weight was determined from duplicate 10-ml samples that were centrifuged, washed once with distilled water, and dried in an oven at 105°C for no less than 24 h.

(ii) GPD1 activity. A 50-ml volume of whole broth was pumped directly from the fermentor to a beaker placed in an ice bath. The sample was centrifuged at 3,300 × *g* for 3 min at 4°C. The cell pellet was resuspended in 15 ml of ice-cold TrED buffer solution (10 mM triethanolamine, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5). The sample was centrifuged again, resuspended in 1 ml of TrED buffer in an Eppendorf tube, and centrifuged again, whereafter the supernatant was discarded and the pellet was frozen in liquid nitrogen. Cell extracts were prepared and desalted as described previously (2). NAD^+-dependent G-3-P dehydrogenase (EC 1.1.1.8) was assayed as described in reference 4, except that MES buffer [20 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0] was used to assay GPD1 activity. GPD2 activity was not found with the assay described above but could be found by adjusting the buffer to pH 6.5 and adding 10 mM $MgCl_2$. However, the reproducibility of the GPD2 assay was poor and results are not reported.

(iii) Protein analyses. Cellular protein was determined with the modified biuret method of Verduyn et al. (20). A 5.0-ml volume of culture broth was withdrawn directly from the bioreactor into an ice-cooled beaker. The sample was washed with 0.9% ice-cooled NaCl and centrifuged, after which the sample was frozen in liquid nitrogen and stored in a freezer before analysis.

HPLC analyses. Glucose, glycerol, ethanol, pyruvate, acetate, succinate, acetoin, and butanediol were assayed by HPLC. A Shodex SH-1011H column at 56°C with an eluent of 5 mM H_2SO_4 and a flow rate of 1 ml/min was used together with two detectors in series. Glucose, glycerol, ethanol, and butanediol were detected by a refractive index detector (Waters 410), whereas pyruvate was detected by measurement of UV absorption at 210 nm (Waters 586 detector). Succinate, acetate, and acetoin were detected by both refractive index and UV absorption measurements.

RESULTS

Aerobic batch cultures. On-line FIA measurements of the cell concentrations of aerobic batch cultures are shown in Fig. 1. No major differences in growth during the respirofermentative phase were observed for the different mutants, with the exception of the double-mutant strain, which showed a specific growth rate only half as high as the other mutants (Table 1). Well before 30 h, the respirofermentative phase had been completed by all of the mutants; i.e., there was no residual glucose in the medium (Fig. 2), except for the slow-growing double mutant strain, for which complete glucose consumption did not occur until after 50 h. By plotting the measured ethanol and glycerol concentrations versus the glucose concentration, product yields were calculated for the respirofermentative phase (Table 1). The glycerol yields were clearly different for the studied mutants, with no glycerol at all formed by the *gpd1*Δ *gpd2*Δ mutant and substantial reductions in the glycerol yielded by both the *gpd1*Δ (−69%) and *gpd2*Δ (−54%) mutants. The ethanol yields were, however, not significantly different.

Another observation, that was apparent from the biomass and carbon dioxide evolution rate measurements, was that the exponential growth rate decreased before complete exhaustion of all glucose. This was presumably due to exhaustion of some medium component in the late phase of growth, although this component was not identified. Therefore, in the step change experiments described below, the time of change to anaerobic conditions was carefully chosen to take place well before any decrease in the exponential growth rate could be seen.

Aerobic-anaerobic step change experiments. The most dramatic effects seen in the step change experiments was that both the growth (Fig. 3) and the metabolic activity, as evidenced by the carbon dioxide evolution rate, CER (Fig. 4), of the double-

TABLE 1. Respirofermentative yields and growth rates for aerobic batch cultures of GPD mutants[a]

Strain	μ_{max}[b] (h^{-1})	$Y_{Glyc/S}$[c] (g/g)	$Y_{Etoh/S}$[d] (g/g)	$Y_{X/S}$[e] (g/g)
W303-1A parent	0.51	0.013	0.38	0.15
*gpd1*Δ mutant	0.48	0.004	0.38	0.15
*gpd2*Δ mutant	0.46	0.006	0.37	0.14
*gpd1*Δ *gpd2*Δ mutant	0.27		0.36	0.15

[a] Glucose (20 g/liter) was the carbon and energy source.
[b] μ_{max}, maximum growth rate.
[c] $Y_{Glyc/S}$, glycerol yield (grams of glycerol/gram of glucose).
[d] $Y_{Etoh/S}$, ethanol yield (grams of ethanol/gram of glucose).
[e] $Y_{X/S}$, biomass yield (grams of biomass/gram of glucose).

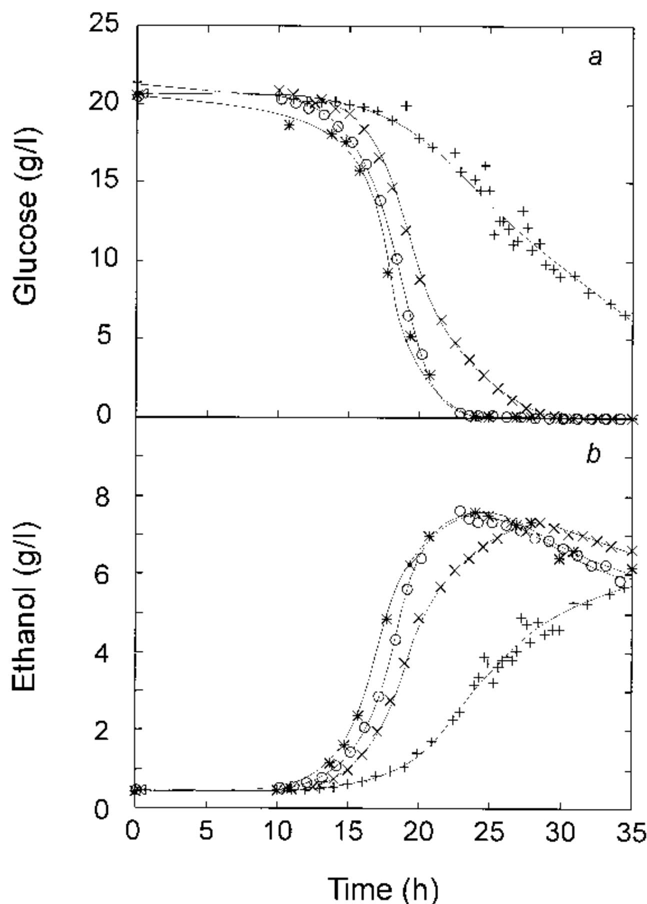

FIG. 2. Glucose (a) and ethanol (b) measured in aerobic batch cultures of the W303-1A parental strain (*), the *gpd1*Δ mutant (×), the *gpd2*Δ mutant (○), and the *gpd1*Δ *gpd2*Δ mutant (+). Glucose (20 g/liter) was used as the carbon and energy source.

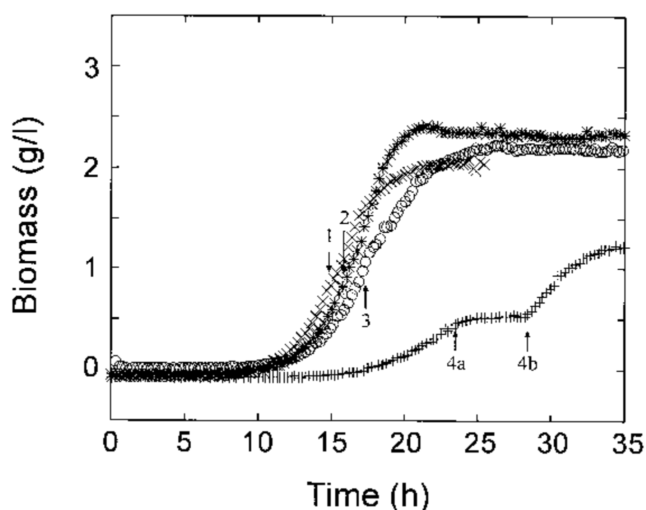

FIG. 3. Biomass concentration measured by on-line FIA in batch cultures of the W303-1A parental strain (*), the *gpd1*Δ mutant (×), the *gpd2*Δ mutant (○), and the *gpd1*Δ *gpd2*Δ mutant (+) in which step changes to anaerobic conditions were made. Glucose (20 g/liter) was used as the carbon and energy source. Arrows 1, 2, 3, and 4a indicate times when changes from aerobic to anaerobic conditions were made. At the time shown by arrow 4b, addition of acetoin (0.5 g/liter) to the *gpd1*Δ *gpd2*Δ mutant was done under anaerobic conditions.

mutant strain completely stopped under anaerobic conditions. The parental strain and the *gpd1*Δ mutant showed responses similar to each other, whereas the *gpd2*Δ mutant responded differently. The CERs of the *gpd1*Δ mutant and the parental strain demonstrated a low-amplitude, short-duration decrease, whereas the decrease in the CER of the *gpd2*Δ mutant was much larger and lasted longer. The GPD1 activity of the *gpd2*Δ mutant increased from 15 mU/mg of protein at the time of the step change to 20 mU/mg of protein 1 h after the step change and to 24 mU/mg of protein 3 h after the step change. No GPD activity was found in the double-mutant strain under either aerobic nor anaerobic conditions. As previously pointed out, the glycerol yield is normally much higher under anaerobic conditions than under aerobic conditions, and this was also evident in these experiments (Fig. 5). The glycerol yield during anaerobiosis was somewhat lower for the *gpd2*Δ mutant than for the parental strain and the *gpd1*Δ mutant (0.07 g/g of glucose compared with 0.09 and 0.10 g/g, respectively).

To examine the reason behind the difference in glycerol formation between the two single mutants, additional step change experiments were done in which the total cellular protein concentrations were measured (Fig. 6). Differences in total protein concentrations could not explain the difference in glycerol formation. However, less acetic acid was formed by the *gpd2*Δ mutant. The difference in final acetic acid concentration between the two mutants was 0.16 g/liter and the difference in final glycerol concentration was 0.3 g/liter. If it is assumed that 1 mol of NADH is formed per mol of acetic acid (18), 80% of the extra glycerol formed by the *gpd1*Δ mutant could be accounted for.

Acetoin addition experiments. To examine if a redox problem caused the halt in metabolic activity, pulse additions of acetoin (3-hydroxy-2-butanone) to the *gpd1*Δ *gpd2*Δ double mutant were made under anaerobic conditions. A small pulse of acetoin resulted in quickly regained metabolic activity, as well as an increase in the cell concentration (Fig. 3). A separate experiment was carried out in which culture fluorescence tuned for the detection of NAD(P)H was measured, and on-line HPLC analyses of ethanol, acetoin, and butanediol were

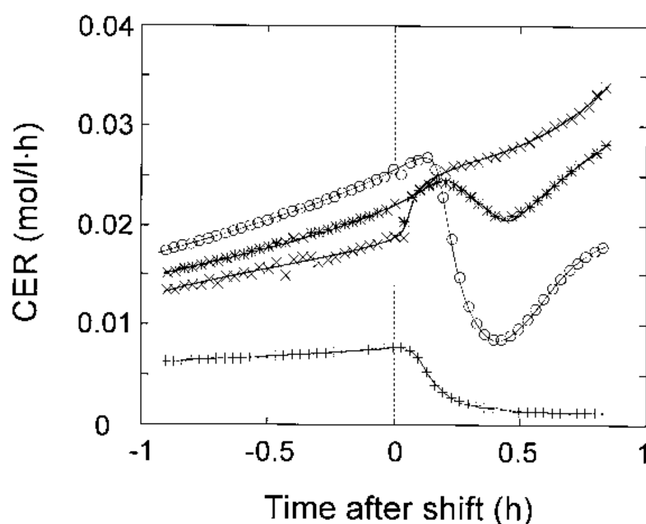

FIG. 4. CER following step changes to anaerobic conditions in batch cultures of the W303-1A parental strain (*), the *gpd1*Δ mutant (×), the *gpd2*Δ mutant (○), and the *gpd1*Δ *gpd2*Δ mutant (+). The inlet gas was changed from air to nitrogen at the time indicated by the dashed line.

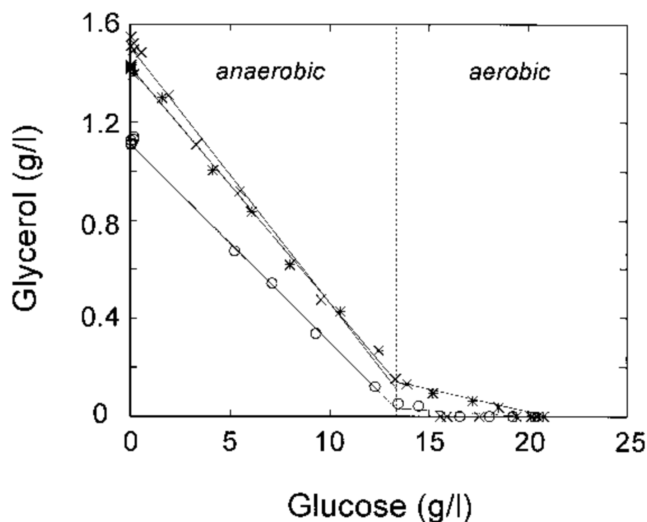

FIG. 5. Glycerol concentrations plotted versus glucose concentrations during aerobic and anaerobic growth of the W303-1A parental strain (*), the *gpd1*Δ mutant (×), and the *gpd2*Δ mutant (○).

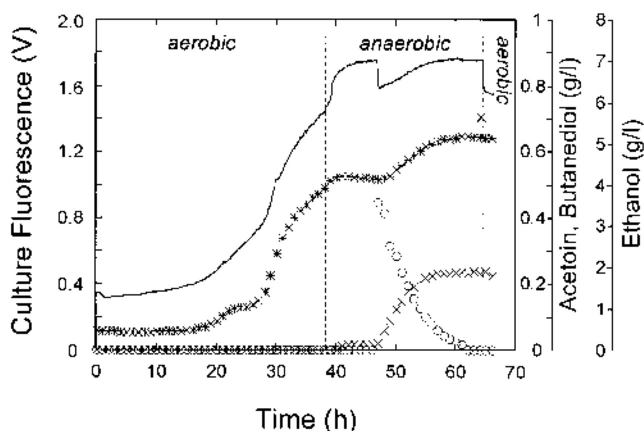

FIG. 7. Culture fluorescence (solid line), ethanol (*), acetoin (○), and butanediol (×) during batch cultivation of the *gpd1*Δ *gpd2*Δ mutant with glucose (20 g/liter) as the carbon and energy source. A vertical dashed line indicates a change from air to nitrogen, or vice versa, in the inlet gas. The arrow indicates addition of acetoin (0.5 g/liter) to the culture. V, volts.

done (Fig. 7). After transfer to anaerobic conditions, a rapid increase in NAD(P)H fluorescence occurred, while the addition of acetoin immediately restored the NAD(P)H value to a seemingly normal level. Concordantly, the added acetoin was consumed and butanediol was produced simultaneously. After complete consumption of the added acetoin, metabolic activity again stopped. The effect of acetoin addition was also tested in fully aerobic batch cultures. It was found that a small addition of acetoin (0.5 g/liter) caused an increase in the specific growth rate of the double mutant from 0.27 to 0.35 h^{-1} (data not shown).

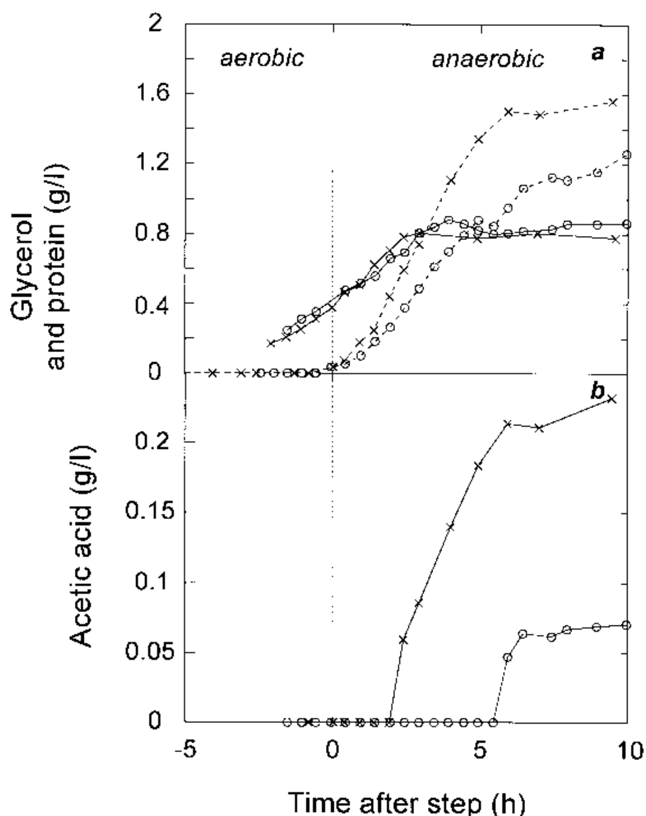

FIG. 6. Glycerol concentrations (dashed lines), protein concentrations (solid lines) (a), and acetate concentrations (solid lines) (b) for step change experiments with the *gpd1*Δ mutant (×) and the *gpd2*Δ mutant (○).

DISCUSSION

It is well established that anabolic biomass formation from glucose by *S. cerevisiae* results in a net production of NADH, despite the fact that biomass is more reduced than glucose. The reason for this is that net consumption of NADPH and production of CO_2 take place concomitantly with the NADH production (6, 11). As is clear from Fig. 3, *S. cerevisiae* is incapable of growth under anaerobic conditions when deprived of its ability to regenerate NAD^+ by the reduction of dihydroxyacetone phosphate to G-3-P. No net generation of NADH results from the strict conversion of glucose to ethanol, so in principle this reaction sequence could still continue even in the absence of growth, provided that the cells could consume the ATP formed. However, the formation of ethanol also stopped in the double-mutant strain under anaerobic conditions (Fig. 7).

Acetoin can react to form butanediol in a reaction, catalyzed by acetoin reductase, in which NADH is consumed (19). As was shown by the acetoin addition experiments (Fig. 3 and 7), it is not glycerol formation per se that is essential for the cell, but rather the consumption of NADH, since acetoin addition restored the anaerobic metabolic activity of the *gpd1*Δ *gpd2*Δ strain. The results presented here strongly suggest that anaerobic conversion of glucose to ethanol by *S. cerevisiae* without formation of glycerol is not possible unless an externally added redox sink is provided by some means. Since acetoin addition increased the growth rate under fully aerobic conditions as well, it is reasonable to assume that an extra "bleed valve" for cytoplasmatically formed NADH is also necessary under aerobic conditions for the cell to be able to maintain a high glycolytic flux.

The immediate preparedness to sustain anaerobic conditions is apparently not as good in strains lacking the GPD2 enzyme as it is in the parental strain and the *gpd1*Δ strain. The *gpd2*Δ mutant is able to overcome the redox problems by

increasing GPD1 activity, probably by synthesizing more GPD1 enzyme. There are also conditions under which the GPD2 enzyme can compensate for a lack of the GPD1 enzyme. Ansell et al. (3) demonstrated by Northern blot analysis that expression of GPD2, in contrast to that of GPD1, is induced during anoxic conditions and observed that anaerobically grown *gpd1*Δ cells were also able to grow in a high-salt medium. After induction, the GPD2 enzyme could thus fulfill the osmoregulatory function of the missing GPD1 enzyme. The mechanisms behind the anaerobic control of *GPD2* expression are not known. Ansell et al. (3) also demonstrated that the gene is not regulated by *ROX1*, which encodes a repressor of hypoxic genes in *S. cerevisiae* (8, 23), or *ROX3* (16, 23), which codes for a nuclear protein that is induced by anaerobic conditions and is assumed to be involved in the control of a number of genes required for growth under anaerobic conditions.

Although anaerobic growth on glucose with no glycerol production is not possible due to redox constraints, the present study shows that a decreased glycerol yield can be attained with single-mutant strains. The *gpd2*Δ mutant had a decreased glycerol yield under anaerobic conditions, and some decrease in the glycerol yield of a *gpd1*Δ mutant strain in an anaerobic batch study has also been reported (22). The difference in glycerol yield between the two single mutants could, to a large extent, be explained by decreased formation of acetic acid. However, the reason behind the difference in acetate yield remains to be understood.

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REFERENCES

1. **Albertyn, J., S. Hohmann, J. M. Thevelein, and B. A. Prior.** 1994. GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. **14:**4135–4144.
2. **André, L., A. Hemming, and L. Adler.** 1991. Osmoregulation in *Saccharomyces cerevisiae*. Studies on the osmotic induction of glycerol production and glycerol 3-phosphate dehydrogenase (NAD^+). FEBS Lett. **286:**13–17.
3. **Ansell, R., K. Granath, S. Hohmann, J. M. Thevelein, and L. Adler.** The two isoenzymes for yeast NAD^+-dependent glycerol-3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. Submitted for publication.
4. **Blomberg, A., and L. Adler.** 1989. Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD^+) in acquired osmotolerance of *Saccharomyces cerevisiae*. J. Bacteriol. **171:**1087–1092.
5. **Blomberg, A., and L. Adler.** 1992. Physiology of osmotolerance in fungi. Adv. Microb. Physiol. **33:**146–212.
6. **Bruinenberg, P. M., J. P. van Dijken, and W. A. Scheffers.** 1983. A theoretical analysis of NADPH production and consumption in yeasts. J. Gen. Microbiol. **129:**953–964.
7. **Christensen, L., U. Schulze, J. Nielsen, and J. Villadsen.** 1995. Acoustic off-gas analyser for bioreactors: precision, accuracy and dynamics of detection. Chem. Eng. Sci. **50:**2601–2610.
8. **Deckert, J., A. M. Rodriguez Torres, J. T. Simon, and R. S. Zitomer.** 1969. Mutational analysis of Rox1, a DNA-bending repressor of hypoxic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**6109–6117.
9. **Eriksson, P., L. André, R. Ansell, A. Blomberg, and L. Adler.** 1995. Cloning and characterization of *GPD2*—a second gene encoding *sn*-glycerol-3-phosphate dehydrogenase (NAD^+) in *Saccharomyces cerevisiae*, and its comparison with *GPD1*. Mol. Microbiol. **17:**95–107.
10. **Holzer, H., W. Bernhardt, and S. Schneider.** 1963. Zur Glycerinbildung in Bäckerhefe. Biochem. Z. **336:**495–509.
11. **Lagunas, R., and J. M. Gancedo.** 1973. Reduced pyridine-nucleotides balance in glucose-growing *Saccharomyces cerevisiae*. Eur. J. Biochem. **37:**90–94.
12. **Larsson, K., R. Ansell, P. Eriksson, and L. Adler.** 1993. A gene encoding *sn*-glycerol-3-phosphate dehydrogenase (NAD^+) complements an osmosensitive mutant of *Saccharomyces cerevisiae*. Mol. Microbiol. **10:**1101–1111.
13. **Nordström, K.** 1966. Yeast growth and glycerol formation. Acta Chem. Scand. **20:**1016–1025.
14. **Oura, E.** 1977. Reaction products of yeast fermentations. Proc. Biochem. **12:**19–21, 35.
15. **Radler, F., and H. Schutz.** 1982. Glycerol production of various strains of *Saccharomyces*. Am. J. Enol. Vitic. **33:**36–40.
16. **Rosenblum-Vos, L. S., L. Rhodes, C. C. J. Evangelista, K. A. Boyake, and R. S. Zitomer.** 1991. The *ROX3* gene encodes an essential nuclear protein involved in *CYC7* gene expression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**5639–5647.
17. **Ryding, P.-O., C. Niklasson, and G. Liden.** 1993. Anaerobic fermentation of xylose by *Pichia stipitis*: the effect of forced cycling of pH. Can. J. Chem. Eng. **71:**911–916.
18. **Taherzadeh, M., G. Liden, L. Gustafsson, and C. Niklasson.** 1996. The effects of pantothenate deficiency and acetate addition on anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **46:**176–182.
19. **Tittel, M. B., and F. Radler.** 1979. Über die Bildung von 2,3-Butanediol bei *Saccharomyces cerevisiae*. Monatsschr. Brau. **11:**5639–5647.
20. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken.** 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J. Gen. Microbiol. **136:**395–403.
21. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken.** 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous culture study on the regulation of respiration and alcoholic fermentation. Yeast **8:**501–517.
22. **Wang, H.-T., P. Rahaim, P. Robbins, and R. R. Yocum.** 1994. Cloning, sequence, and disruption of the *Saccharomyces diastaticus DAR1* gene encoding a glycerol-3-phosphate dehydrogenase. J. Bacteriol. **176:**7091–7095.
23. **Zitomer, R. S., and C. Lowry.** 1992. Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. Microbiol. Rev. **56:**1–11.