Appl Microbiol Biotechnol (1998) 50: 434–439

ORIGINAL PAPER

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Improved ethanol production by glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*

Received: 16 February 1998 / Received revision: 16 March 1998 / Accepted: 1 June 1998

Abstract The anaerobic performance of *gpd1*Δ and *gpd2*Δ mutants of *Saccharomyces cerevisiae* was characterized and compared to that of a wild-type strain under well-controlled conditions by using a high-performance bioreactor. There was a 40% reduction in glycerol level in the *gpd2*Δ mutant compared to the wild-type. Also the *gpd1*Δ mutant showed a slight decrease in glycerol formation but to a much lesser degree. As a consequence, ethanol formation in the *gpd2*Δ mutant was elevated by 13%. In terms of growth, the *gpd1*Δ mutant and the wild-type were indistinguishable. The *gpd2*Δ mutant, on the other hand, displayed an extended lag phase as well as a reduced growth rate under the exponential phase. Even though glycerol-3-phosphate dehydrogenase 2 (GPD2) is the important enzyme under anaerobic conditions it can, at least in part, be substituted by GPD1. This was indicated by the higher expression level of *GPD1* in the *gpd2*Δ mutant compared to the wild type. These results also show that the cells are able to cope and maintain redox balance under anaerobic conditions even if glycerol formation is substantially reduced, as observed in the *gpd2*Δ mutant. One obvious way of solving the redox problem would be to make a biomass containing less protein, since most of the excess NADH originates from amino acid biosynthesis. However, the *gpd2*Δ mutant did not show any decrease in the protein content of the biomass.

Introduction

The production of glycerol by *Saccharomyces cerevisiae* under anaerobic conditions with glucose as a carbon source is a necessity for maintaining the intracellular redox balance and a sustained conversion of sugar into ethanol. This is due to the fact that biosynthesis, and especially amino acid synthesis, results in a net formation of NADH (Albers et al. 1996). In addition, formation of organic acids, e.g. acetic, pyruvic and succinic acid, also results in a surplus of NADH. The main pathway used by *S. cerevisiae* for regeneration of NAD^+ is glycerol production, since ethanol formation from glucose is a redox-neutral process. However, formation of glycerol represents an unwanted loss of carbon if the aim is to produce maximum amounts of ethanol. Therefore, attempts have been made to decrease the glycerol yield, e.g. by using different nitrogen sources in order to reduce the surplus of NADH due to amino acid synthesis (Albers et al. 1996), or to use microaerobic conditions (Franzén et al. 1994). An alternative strategy, used in this study, would be to minimize glycerol formation by employing mutants in the glycerol-producing pathway.

In *S. cerevisiae* glycerol is formed from dihydroxyacetone phosphate by the consecutive action of glycerol-3-phosphate dehydrogenase (GPD), to yield glycerol-3-phosphate, and glycerol-3-phosphatase (GPP) to yield glycerol. There are two isogenes encoding different forms of GPD, *GPD1* first described by (Larsson et al. 1993) and *GPD2* described by (Eriksson et al. 1995). Even though the two isoenzymes show a strong homology, it seems as if they play distinctively different roles in the cellular machinery. *GPD1* is induced under osmotic stress (Albertyn et al. 1994; Andre et al. 1991; Ansell et al. 1997) whereas *GPD2* is induced under anaerobic conditions and is suggested to be important for redox regulation under these conditions (Ansell et al. 1997). The rationale for osmotic induction is that glycerol is the main osmoregulator in *S. cerevisiae* under hyperosmotic conditions (Blomberg and Adler 1992). The glycerol-3-phosphatase also exists in two isoforms encoded by *GPP1* and *GPP2*, with the latter being osmotically induced (Norbeck et al. 1996). In this study we chose to reduce glycerol production by using GPD

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mutants in order to avoid a possible detrimental accumulation of glycerol-3-phosphate. It is, however, as stated above, not possible to eliminate all glycerol production completely, because of redox constraints. This has been verified by the inability of a double *gpd1gpd2* mutant to grow under anaerobic conditions while aerobically growth was feasible, though at a reduced growth rate (Björkqvist et al. 1997). Furthermore, this mutant showed an immediate reduction in fermentation rate when the conditions changed from aerobic to anaerobic. However, addition of acetoin, which could serve as an acceptor of reducing equivalents by its reduction to butanediol, restored or even increased the aerobic fermentation rate (Björkqvist et al. 1997).

The amount of excess NADH, and hence the requirement for glycerol formation for amino acid synthesis and formation of organic acids, can vary depending on the preference for NAD^+ or $NADP^+$ as co-factor (Albers et al. 1996). In several metabolic steps NAD^+ as well as $NADP^+$ can be utilized and there are additional biosynthetic pathways for several of the amino acids. However, it seems as if the metabolic machinery is adjusted to produce a minimum amount of excess NADH and the amount of glycerol formed is only just enough to balance this surplus (Albers et al. 1996). If this supposition is true, it would be difficult or even impossible to reduce glycerol formation and hence improve the ethanol yield by means of genetic manipulation of the GPD genes, unless the cells can be forced to produce lower amounts of organic acids but also, more importantly, to manage with a lower protein content. On the other hand, step-change experiments from aerobic to anaerobic conditions showed that a *gpd2* mutant did produce less glycerol than did the wild type without concomitant reduction in protein content (Björkqvist et al. 1997).

The purpose of this investigation was to characterize the anaerobic growth performance and fermentation properties of *gpd1*Δ and *gpd2*Δ strains of *S. cerevisiae* under well-controlled conditions in a bioreactor, the eventual goal being a reduction of glycerol formation and an improved ethanol production.

Materials and methods

Yeast strains and media

The *S. cerevisiae* strains used were all derived from W303-1A (*ade2-1*°, *his3-11*, *leu2-3*, *112trp1-1a*, *ura3-1*, *can100*°) referred to as wild type (ATCC 200060); *gpd1*Δ (*ade2-1*°, *his3-11*, *leu2-3*, *ura3-1*, *can*100°, *gpd1*Δ::TRP1); *gpd2*Δ (*ade2-1*°, *his3-11*, *leu2-3*, *112trp1-1a*, *can100*°, *gpd2*Δ::URA3). The medium was a defined CBS medium (Albers et al. 1996) with 7.5 g/l $(NH4)_2SO_4$ as nitrogen source and 20 g/l glucose as carbon and energy source. The concentration of the required bases and amino acids was 120 mg/l, except for leucine where 240 mg/l was used.

Growth conditions

Cultivations were carried out under anaerobic conditions in a fermentor (Belach Bioteknik AB, Stockholm, Sweden) with a working volume of 2.5 l. The temperature was 30 °C, stirring rate 400 rpm, and the pH was kept constant at 5.0 by automatic addition of 1 M NaOH. To ensure anaerobic conditions, the fermentor was continuously flushed with nitrogen gas at a rate of 37.5 l/h.

Gas analysis

Carbon dioxide evolution was continuously analysed by a carbon dioxide and oxygen monitor (type 1308, Bruel and Kjaer, Naerum, Denmark).

Microcalorimetry

The heat production rate (dQ/dt) was measured by a flow-through microcalorimeter (thermal activity monitor; Thermometric AB Järfälla, Sweden). The effective volume of the measuring cell was 0.52 ml.

Growth determinations

Growth was followed by measuring the absorbance of the cultures at 610 nm in a Beckman B spectrophotometer.

Measurement of glucose, ethanol, glycerol and acetate

Two samples (1.5 ml each) were centrifuged for 5 min at 15 000 *g* and the resulting supernatants were frozen (−20 °C) until analysis. The concentrations were determined by using enzyme combination kits from Boehringer Mannheim (Biochemica test combination; Boehringer Mannheim, GmbH, Germany).

Determination of dry weight

Two samples (5 ml each) were centrifuged at 5000 *g* for 10 min and washed twice with water, and subsequently the pellets were kept at 110 °C for 24 h before temperature equilibration and weighing.

Protein determination

Two samples (10 ml each) were centrifuged at 5000 *g* for 5 min and washed twice with 0.9% (w/v) NaCl. The pellet was resuspended in 3 ml NaOH and total protein was determined by a modified biuret method (Verduyn et al. 1991) using bovine serum albumin as a standard.

Northern blot analysis

Northern blot analysis of *GPD1* expression was performed according to (Ansell et al. 1997).

Results

Growth characteristics

The wild type and *gpd1*Δ showed an almost indistinguishable growth pattern with close to identical heat production and growth rates (Fig. 1, Table 1). The *gpd2*Δ strain, on the other hand, had a prolonged growth period, resulting in a reduced rate of heat production. This was due to an extended lag phase, but also a reduced growth rate was obtained with *gpd2*Δ com-

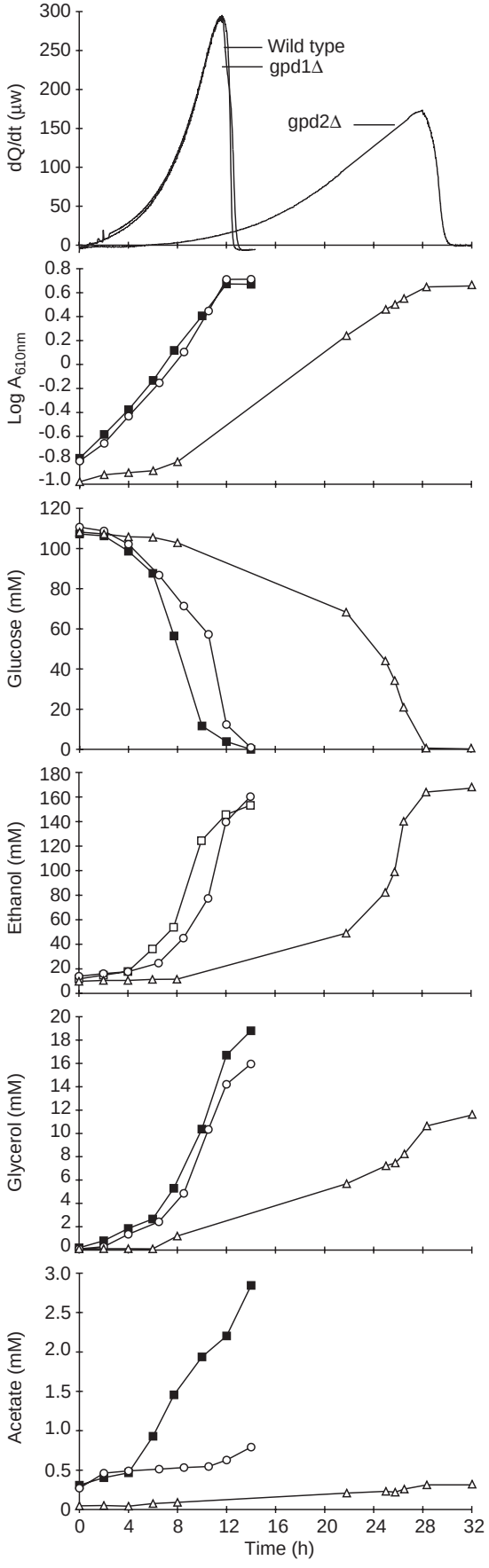

Table 1 A comparison of growth and ethanol production rates of *Saccharomyces cerevisiae* wild type, *gpd1*Δ and *gpd2*Δ respectively, during anaerobic batch growth on 2% glucose

Strain	Growth rate (h^{-1})	Ethanol production (mmol g^{-1} h^{-1})	Ethanol production (mmol l^{-1} h^{-1})
Wild type	0.31 ± 0.04	22.6 ± 3.5	11.0 ± 0.9
*gpd1*Δ	0.32 ± 0.07	17.6 ± 1.5	11.5 ± 0.1
*gpd2*Δ	0.17 ± 0.02	7.4 ± 0.8	5.2 ± 0.2

pared to that of the wild type and *gpd1*Δ. However, the biomass concentration at the end of the growth period was very similar, i.e. the growth yield was comparable for all strains (Table 2).

Substrate consumption and product formation

Depletion of glucose and cessation of growth were nicely traced by the rapid decline of the heat production rate (Fig. 1). Both the glucose consumption and ethanol production were very similar in the wild type and the *gpd1*Δ mutant, whereas *gpd2*Δ showed a delayed response. Not only was the response delayed but the ethanol production rate was also reduced in the *gpd2* mutant compared to that in the other two strains (Table 2). As a consequence, the overall ethanol production rate obtained from the fermentor was lower (Table 2). However, the final ethanol concentration was higher in the *gpd2*Δ mutant and the ethanol yield was elevated by 8% (relative to the amount of substrate consumed) or 13% (relative to the biomass formed) compared to the wild type (Table 2). There was a slightly lower glycerol production in *gpd1*Δ than in the wild-type, whereas *gpd2*Δ showed a drastic reduction in the level of this metabolite (Fig. 1, Table 2). Deletion of the *GPD2* gene resulted in a decline in glycerol production, relative to the amount of biomass formed, by almost 40%. Interestingly, the acetate concentrations were much lower in the mutants than in the wild type. In the *gpd2* mutant, acetate production was almost totally abolished but *gpd1*Δ also showed a drastic reduction in acetate concentration and yield (Fig. 1, Table 2). It might be that a decrease in acetate formation is an example of a metabolic adjustment by the cells to minimize the NADH surplus when the glycerol production capacity is hampered.

ATP yields

The ATP yields (Y_{ATP}, g biomass/mol ATP) of 16–17 g/mol (Table 2) for the wild type and *gpd1*Δ were close to the value of 16 g/mol reported for optimal growth of *S. cerevisiae* under anaerobic conditions (Verduyn et al.

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Fig. 1 Changes in measured parameters during anaerobic batch cultures of *Saccharomyces cerevisiae* wild type (■), *gpd1*Δ (○) and *gpd2*Δ (△) respectively, with 2% glucose as carbon and energy source

Table 2 A comparison of different yields, heat production and energy balance of *S. cerevisiae* wild type, *gpd1*Δ and *gpd2*Δ respectively, during anaerobic batch growth on 2% glucose

Parameter	Wild type	*gpd1*Δ	*gpd2*Δ
Growth yield (g biomass/g glucose)	0.110 ± 0.000	0.108	0.110 ± 0.013
Ethanol yield (mol EtOH/mol glucose)	1.32 ± 0.00	1.33	1.43 ± 0.02
Ethanol yield (mmol EtOH/g biomass)	66.74 ± 0.16	68.57	75.15 ± 7.97
Glycerol yield (mol glycerol/mol glucose)	0.175 ± 0.002	0.145	0.106 ± 0.000
Glycerol yield (mmol glycerol/g biomass)	8.85 ± 0.04	7.43	5.58 ± 0.68
Acetate yield (mol acetate/mol glucose)	0.019 ± 0.004	0.005	0.002 ± 0.000
Acetate yield (mmol acetate/g biomass)	0.98 ± 0.22	0.25	0.11 ± 0.01
Heat yield (kJ/g biomass)	4.81 ± 0.61	4.30	5.16 ± 0.78
ATP yield (g biomass/mol ATP)	17.0 ± 0.1	16.3	14.0 ± 2.0
Carbon balance (%)	95.2 ± 0.9	93.7	95.7 ± 1.1

1990). It should be remembered, however, that the required amino acids and bases that are added to the medium represent an additional carbon source and hence influence the ATP yields obtained. Owing to the higher ethanol and lower glycerol yields, the Y_{ATP} was slightly decreased for the *gpd2* mutant. The small increase in heat yield obtained for *gpd2*Δ could also be a reflection of the elevated ethanol production in this strain. On the other hand, the excess NADH and hence the amount of glycerol formed have a drastic influence on the heat yield, i.e. the more glycerol the lower the heat yield (Larsson and Gustafsson 1998). This is due to the fact that glycerol production from glucose is a reductive energy-requiring process.

The function of the two NAD^+-dependent isoforms of GPD, GPD1 and GPD2

It is clear that GPD2 is the most important enzyme during anaerobic growth (Ansell et al. 1997). This was manifested also in this study, since deletion of *GPD1* resulted in a phenotype more or less indistinguishable from that of the wild type, the only detectable difference being the strong reduction in acetate formation in the mutant (Fig. 1). The *gpd2*Δ strain, on the other hand, was severely affected during anaerobic growth. However, glycerol formation also persisted in the *gpd2*Δ strain, even though the production rate was much lower than the wild-type level (Fig. 1). This suggests that, under anaerobic conditions, GPD2 can, at least in part, be substituted by GPD1. A supposition corroborated by Northern analysis, showing a higher level of expression *GPD1* in *gpd2*Δ than in the wild-type strain (Fig. 2).

Protein content and NADH formation

There was no reduction in the protein content of the *gpd2* mutant. Instead a slightly higher value of 59.9% (w/w) protein was obtained for the mutant whereas the wild type contained 56.7% protein. The acetate production, and hence the accompanying NADH formation, due to this acid was much lower in the mutant. According to Albers et al. (1996) each gram of protein synthesized is accompanied by the production of 17.9–21.2 mmol NADH. The range is due to the existence of multiple biosynthetic pathways for some of the amino acids as well as uncertainties concerning the use of NAD^+ or $NADP^+$ as coenzyme. One must also consider that the strain used in the present study is auxotrophic for leucine, histidine and tryptophan. Hence, there is no NADH formation through synthesis of these amino acids; they are simply taken up from the medium. When a correction has been made for this, by using the amino acid composition given by Albers et al. (1996), a range of 14.7–8.0 mmol NADH/g protein could be calculated for the strain used in the present study. By taking this into account, together with the amount of acetate formed, a surplus of 8.9–11.0 mmol/g NADH could be calculated for the *gpd2*Δ mutant. The observed glycerol level can only compensate for a fraction of this excess NADH (Table 3). There could also be additional NADH formed as a result of RNA synthesis and formation of other metabolites, but this contribution is usually very small (Albers et al. 1996).

Discussion

This study showed that it is indeed possible to obtain a drastic reduction in glycerol formation and a concomitant increase in the production of ethanol by using a

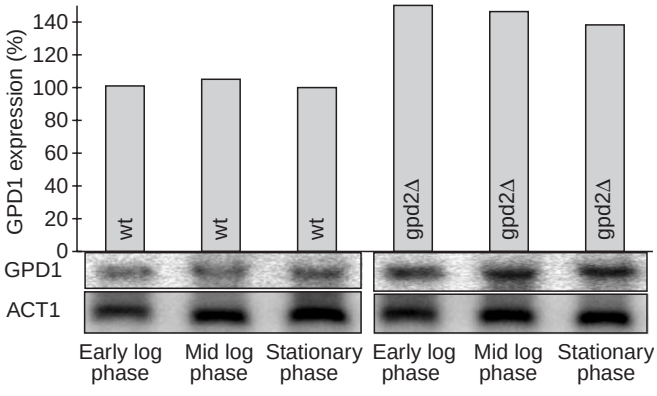

Fig. 2 Northern blot analysis of glycerol-3-phosphate dehydrogenase (*GPD1*) expression in the wild-type and *gpd2*Δ mutant respectively. The expression level at early log phase in the wild type is set to 100%. Samples were taken at early log, mid log and early stationary phase

Table 3 Calculation of NADH formation of *S. cerevisiae* wild type and *gpd2*Δ respectively, during anaerobic batch growth on 2% glucose. The protein content was 56.7% and 59.9% for the wild type and the mutant respectively. The amount of NADH accompanying protein synthesis was calculated by using the range 17.9–21.2 mmol NADH/g protein as suggested by Albers et al. (1996) and compensating for the uptake of leucine, histidine and tryptophan (see Results). The range of values for production during acetate formation is due to the uncertainty whether NAD^+ or $NADP^+$ is used in the aldehyde dehydrogenase step. The final NADH yield is the balance resulting from NADH formation due to protein and acetate formation and NADH consumption due to glycerol production

Strain	Glycerol (mmol NADH/g)	Acetate (mmol NADH/g)	Protein (mmol NADH/g)	NADH (mmol/g)
Wild type	−8.85	+0.98–1.96	+8.3–10.2	+0.4–3.3
*gpd2*Δ	−5.58	+0.11–0.22	+8.8–10.8	+3.3–5.4

*gpd2*Δ strain (Table 1). The decrease in glycerol production was not accompanied by any decline in the protein content of the cells. Most of the NADH surplus, and hence the need for redox adjustment by glycerol formation, originates from amino acid synthesis (Albers et al. 1996). Depending on the co-factor involved, NAD^+ or $NADP^+$, and the existence of multiple biosynthesis pathways for at least some of the amino acids, it is possible to minimize the amount of excess NADH formed. However, when comparing the amount of glycerol formed, it seemed as if the wild-type levels in this study (Table 3) as well as in previous observations (Albers et al. 1996) were just enough to keep up with the redox requirements when NADH formation was minimized. Nevertheless, this study showed that, by using a *gpd2* mutant, glycerol formation could be reduced even further. How the redox balance in the *gpd2*Δ mutant is accomplished is not known; part of the explanation is a decrease in the amount of acetate formed. There must, however, be additional mechanisms as well. One possibility might be that $NADP^+$ can substitute for NAD^+ as a co-factor to a larger extent than expected and/or that additional biosynthetic pathways may exist. A theoretical possibility is also that *S. cerevisiae* has the potential of using other reductive pathways apart from glycerol formation during anaerobic growth on glucose. To summarize, it ought to be impossible for the cells to reduce glycerol formation under anaerobic conditions since not more than what is absolutely required seems to be produced. However, the *gpd2*Δ mutant in some way managed to maintain redox balance and sustained metabolic activity in spite of the drastic reduction in glycerol level, the result being an increase in ethanol production and reduction in, not only glycerol, but also acetate formation.

The two different isoforms of GPD seem to have specific functions but it also appears that, at least in some circumstances, they can replace each other (Ansell et al. 1997). *GPD2* is expressed under anaerobic conditions and its regulation is somehow related to the redox status of the cells. However, redox regulation is not limited to *GPD2*. Aerobically GPD1 was found to be the important enzyme when the cells were challenged with a high redox adjustment demand due to growth on a reduced substrate, i.e. ethanol (Larsson et al. 1998). Under such aerobic conditions GPD2 did not show any activity. This study, on the other hand, confirmed the leading role of GPD2 under anaerobic conditions but also showed the partial ability of GPD1 as a replacement in the *gpd2*Δ mutant. A recent publication by Michnick et al. (1997) showed a slightly different result with respect to GPD1 under anaerobic conditions. In our study, *GPD1* deletion did not influence glycerol production while, in their study (Michnik et al. 1997), the absence of the *GPD1* gene product gave an almost 50% reduction in glycerol formation compared to the wild type. However, it might be that the comparably high glucose concentration used (10%) triggered osmotic induction of *GPD1* since *GPD1* is the isoform of GPD induced under osmotic stress conditions (Ansell et al. 1997).

A striking observation was also the dramatic reduction in acetate production observed in the *gpd1*Δ mutant (Fig. 1). Especially since this was the only parameter measured that was significantly different from that of the wild type. Even though GPD1 is not important for glycerol production under anaerobic conditions, the cells still seem to recognize the reduction in NADH-oxidizing capacity when this protein is lacking. The response being a reduced acetate and hence NADH formation rate.

Acknowledgements Financial support from the Swedish National board for Technical Development is gratefully acknowledged.

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