YEAST VOL. 12: 1331–1337 (1996)

Reduced Pyruvate Decarboxylase and Increased Glycerol-3-phosphate Dehydrogenase [NAD$^+$] Levels Enhance Glycerol Production in *Saccharomyces cerevisiae*

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Received 13 December 1995; accepted 3 June 1996

This investigation deals with factors affecting the production of glycerol in *Saccharomyces cerevisiae*. In particular, the impact of reduced pyruvate-decarboxylase (PDC) and increased NAD-dependent glycerol-3-phosphate dehydrogenase (GPD) levels was studied. The glycerol yield was 4·7 times (a *pdc* mutant exhibiting 19% of normal PDC activity) and 6·5 times (a strain exhibiting 20-fold increased GPD activity resulting from overexpression of *GPD1* gene) that of the wild type. In the strain carrying both enzyme activity alterations, the glycerol yield was 8·1 times higher than that of the wild type. In all cases, the substantial increase in glycerol yield was associated with a reduction in ethanol yield and a higher by-product formation.

The rate of glycerol formation in the *pdc* mutant was, due to a slower rate of glucose catabolism, only twice that of the wild type, and was increased by *GPD1* overexpression to three times that of the wild-type level. Overexpression of *GPD1* in the wild-type background, however, led to a six- to seven-fold increase in the rate of glycerol formation. The experimental work clearly demonstrates the rate-limiting role of GPD in glycerol formation in *S. cerevisiae*.

KEY WORDS — pyruvate decarboxylase; glycerol-3-phosphate dehydrogenase; glycerol production; *Saccharomyces cerevisiae*

INTRODUCTION

Glycerol is formed as a by-product when *Saccharomyces cerevisiae* ferments sugar to ethanol. The production of ethanol from glucose is a redox-neutral process. The role of NADH-consuming glycerol formation is in maintaining cytosolic redox balance especially under anaerobic conditions, compensating for cellular reactions that produce NADH (van Dijken and Scheffers, 1986).

Substantial overproduction of glycerol can be observed during alcoholic fermentation, when reoxidation of glycolytically formed NADH is restricted. This is the case, for example, when the intermediate product, acetaldehyde, is trapped by bisulphite, so that it can no longer serve as an electron acceptor for cytosolic NADH (Neuberg and Reinfurth, 1918).

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CCC 0749–503X/96/131331–07
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Glycerol also plays an essential role as a compatible solute during osmoregulation in yeasts (Blomberg and Adler, 1992; Mager and Varela, 1993). In response to decreased extracellular water potentials *S. cerevisiae* greatly increases its rate of glycerol formation (Blomberg and Adler, 1989). A marked rise in the level of cytosolic NAD-dependent glycerol-3-phosphate dehydrogenase (GPD), a key enzyme in glycerol formation, has been observed following osmotic shock (Edgley and Brown, 1983; Blomberg and Adler, 1989; Andre *et al.*, 1991). The osmotic induction of GPD occurs on a transcriptional level via a specific signal transduction pathway and seems to be important for the accumulation of glycerol during osmoregulation (Varela *et al.*, 1992; Albertyn *et al.*, 1994; Brewster *et al.*, 1993). The *GPD1* gene coding for GPD in *S. cerevisiae* has been cloned and sequenced (Larsson *et al.*, 1993; Albertyn *et al.*, 1994).

The aim of the current paper was to investigate in more detail the metabolic prerequisites for glycerol overproduction in *S. cerevisiae*. For this purpose, we analysed the impact of both restricted alcoholic fermentation (decreasing the activity of pyruvate decarboxylase; PDC) and increased GPD activity. The corresponding genetic modifications (deletion of the *PDC2* gene, overexpression of the *GPD1* gene) were studied both independently and in combination. The quantity of relevant fermentation products formed after depletion of glucose as well as the specific rates of glucose consumption, ethanol and glycerol formation were determined for the genetically modified strains. All investigations were carried out under oxygen-limiting conditions so that oxidation of glucose and NADH reoxidation via respiration could be excluded.

MATERIALS AND METHODS

Yeast strains

The Δ*pdc2* mutant YSH 306 (*MAT***a** *leu2-3/112 ura3-52 trp1-92 pdc2*Δ*::TRP1*; Hohmann, 1993) and the isogenic wild-type YSH 1.1.-6B (*MAT*α *leu2-3/112 ura3-52 trp1-92*; Hohmann and Zimmermann, 1986) were kindly provided by S. Hohmann, Laboratorium voor Moleculaire Celbiologie, Katholieke Universiteit Leuven, Leuven-Heverlee, Belgium.

Construction of plasmids

DNA methods and transformation of *Escherichia coli* were performed by standard techniques (Sambrook *et al.*, 1989).

The 2 µm plasmid (YEp*GPD1* containing the entire *GPD1* gene of *S. cerevisiae* and the *LEU2* gene (Albertyn *et al.*, 1994) was also provided by S. Hohmann (see above). To create the plasmid YEp-Km^R*GPD1*, the kanamycin resistance GenBlock from the transposon Tn*903* (Pharmacia, Sweden), which confers resistance to G418 in yeast, was restricted with *Bam*HI and ligated into the *Bam*HI site (multiple cloning site) of YEp*GPD1*. The plasmid YEpKm^R (reference plasmid without *GPD1*) was obtained by removing the 5·2-kb *Eco*RI fragment from YEpKm^R*GPD1*.

Transformations of *S. cerevisiae* with plasmids were performed by electroporation (Becker and Guarente, 1991). Yeast transformants were selected on YNBleu$^-$ medium containing yeast nitrogen base (0·67%), amino acids as required (Rose *et al.*, 1990), and either 2% glucose (YSH 1.1-6B) or 3% glycerol (YSH 306) as a carbon source.

The use of Km^R, a dominant selective marker conferring resistance to G418 in yeast, made it possible to perform all batch fermentations in YEPD medium.

Media and culture conditions

Yeast cells were cultured at 30°C. YEP medium (2% peptone, 1% yeast extract) was used as a basic medium which was supplemented with 1·8% glucose (YEPD) for preculturing of wild-type strain. The *pdc* mutant which grows very slowly on glucose was precultured in YEP medium containing 2% ethanol and 2% glycerol (YEPEG) and then incubated with 1·8% glucose for 3–5 h to induce glycolytic enzymes. For cultivation of *S. cerevisiae* transformants carrying a plasmid (YEpKm^R*GPD1* or YEpKm^R), the appropriate medium was supplemented with 100 µg/ml G418.

Precultured cells were adapted to anaerobic conditions for 3 h and washed with 0·85% NaCl before inoculation into the fermentation medium at a density of 5×10^7 to 1×10^8 cells per ml. The batch fermentations were performed in 500 ml Erlenmeyer flasks containing 500 ml of YEPD medium and 100 µg/ml G418. The cultures were stirred continuously at 500 rpm after closing the vessels with air-locks which ensured the exclusion of oxygen but allowed the release of gases.

Enzyme assays

In vitro enzyme activities were, in general, determined 3 h after starting the batch fermentations. Yeast cells were broken by vortexing with glass beads (0·5 mm in diameter) for 15 min at 4°C in accordance with a previously described method (Ciriacy, 1975). Approximately 5×10^8 cells were harvested for the PDC assay and homogenized in 1 ml imidazole buffer (Seehaus, 1986) with 0·5 g glass beads and 0·2 mM-phenylmethyl-sulphonylfluoride. In order to assay GPD, approximately 3×10^9 cells were harvested and homogenized in 3 ml triethanolamine buffer (Blomberg and Adler, 1989; Andre *et al.*, 1991) containing 0·2 mM-phenylmethyl-sulphonylfluoride and 2 g glass beads. The homogenate was centrifuged in each case at 12 000 *g* and 4°C for 15 min. The supernatant was used either immediately for enzyme assay (PDC) or after desalting (GPD) by passage through a Sephadex G-25 column

(Pharmacia PD-10, Pharmacia Fine Chemicals, Sweden).

PDC assays were performed in imidazole buffer at pH 6·8 as previously described (Seehaus, 1986). GPD was assayed in imidazole buffer at pH 7·0 in accordance to Gancedo *et al.* (1968).

Protein concentration was measured by the Coomassie blue method (Bradford, 1976), using bovine serum albumin A 3350 (Sigma Chemical Co., St Louis, MO) as a standard.

Determination of glucose and fermentation products

Samples of 10 ml were taken and immediately centrifuged for 5 min at 3500 × *g*. Supernatants were incubated for 10 min at 80°C in tightly closed Falcon tubes to stop enzymatic reactions. Concentrations of glucose, ethanol, glycerol, pyruvate, acetaldehyde and acetate were determined by using either test kits or protocols supplied by Boehringer GmbH, Mannheim, Germany (Boehringer GmbH, 1989).

Dry weights were determined by centrifuging (see above) 10 ml culture medium in pre-weighed tubes. Cells were washed twice with distilled water. Tubes were dried for 48 h at 100°C, cooled in a desiccator and reweighed.

RESULTS

Enzyme activities, fermentation products and fluxes of glucose catabolism were always determined in experiments where the compared strains were cultured and assayed in parallel.

Pyruvate decarboxylase and glycerol-3-phosphate dehydrogenase levels

To study the impact of reduced PDC activity on glycerol production, the strain YSH 306, which carries a disruption in the regulatory gene *PDC2* (Hohmann, 1993), was used. The deletion of *PDC2* leads to diminished transcription of the structural *PDC1* gene and a reduction in specific PDC activity. Values between 10 and 25% of wild-type PDC activity have been determined depending on the conditions used (Hohmann, 1993). Under the cultivation conditions for batch fermentations used in the current work (see Materials and Methods), the Δ*pdc2* mutant exhibited a specific PDC activity of 0·25 ± 0·01 U/mg protein. This value corresponds to 19% of the level measured in the isogenic wild type YSH 1.1.-6B (1·29 ± 0·10 U/mg protein).

To analyse the effect of increased GPD activity, the structural gene for GPD (*GPD1*) was overexpressed in the wild-type YSH 1.1.-6B and also in the Δ*pdc2* mutant where the *GPD1* overexpression was combined with reduced PDC activity. For this purpose, the strains were transformed with the multicopy plasmid YEpKm^R*GPD1* and with the plasmid YEpKm^R, the latter being used as reference strains. Both in wild-type and *pdc*-mutant background, overexpression of *GPD1* led to an approximately 20-fold higher specific GPD activity (1·23 ± 0·12 U/mg protein) compared to the corresponding reference strains (0·06 ± 0·01 U/mg protein).

Fermentation products, carbon and redox balances, and ATP yields

As shown in Table 1, both reduced PDC and increased GPD levels led to marked shifts in the yields of fermentation products. The glycerol yield was 4·7 times (*pdc* mutant) and 6·5 times (*GPD1* overexpression) that of the wild type. In the strain carrying both enzyme activity alterations, the glycerol yield was 8·1 times higher than that of the wild-type. In all cases, the substantial increase in glycerol yield was associated with a reduction in ethanol yield. Furthermore, increased glycerol production was accompanied by increased by-product formation.

The type of by-products formed (pyruvate, acetaldehyde and acetate) depended on the prevailing enzyme modification. In any case, the accumulation of oxidized glycolytic intermediates in equimolar amounts to that of glycerol which is overproduced could be explained as a result of redox balancing.

The observed increase in pyruvate production in the *pdc* mutant fits well with a limited PDC activity. Each mole of pyruvate which is not decarboxylated and hence reduced to ethanol, would result in an equimolar excess of NADH which is oxidized in glycerol formation. The higher levels of acetaldehyde and acetate formed by the wild-type strain overexpressing *GPD1* (instead of pyruvate in the *pdc* mutant) would account for glycerol overproduction provoked by the increased GPD activity.

As noted in the legend to Table 1, it is likely that the yield of acetaldehyde is underestimated. Overexpression of *GPD1* in the *pdc* mutant

Table 1. Amounts of main fermentation products, carbon and redox balances, and ATP yield in the wild type (*YSH 1.1.-6B*) and a *pdc* mutant strain (YSH 306) of *S. cerevisiae*, transformed with YEpKm^{R}*GPD1* (overexpression of *GPD1*) and YEpKm^{R} (reference plasmid), after anaerobic growth in YEPD medium.

	Wild type		*pdc* mutant (19% residual PDC activity)	
	Reference plasmid[h]	*GPD1* overexpression (20-fold GPD activity)	Reference plasmid[h]	*GPD1* overexpression (20-fold GPD activity)
Ethanol (mmol/l)[a]	171·1 ± 1·3	111·3 ± 0·7	122·0 ± 3·3	93·0 ± 1·7
Glycerol (mmol/l)[a]	6·8 ± 2·3	44·1 ± 0·1	31·7 ± 1·7	54·9 ± 2·2
Pyruvate (mmol/l)[a]	<1·0	<1·0	24·9 ± 1·7	34·2 ± 0·1
Acetaldehyde (mmol/l)[b]	<0·1	8·2	<0·1	<0·1
Acetate (mmol/l)[a]	3·3 ± 0·2	9·7 ± 1·2	4·8 ± 2·5	6·7 ± 0·2
Biomass formation (g/l)[a,c]	1·59 ± 0·11	1·06 ± 0·14	0·64 ± 0·08	0·46 ± 0·01
Total amount of fermentation products (mmol/l glucose equivalents)[d]	102·3	94·5	96·4	97·8
Initial glucose (mmol/l)[a]	100·0 ± 1·2	100·2 ± 1·2	102·4 ± 2·6	101·6 ± 2·7
Carbon balance (mmol/l glucose equivalents)[e]	+2·2	− 5·7	− 6·0	− 3·8
Redox balance (mmol/l NADH)[f]	− 0·2	− 16·5	+2·8	− 7·3
ATP yield (mmol ATP/mmol glucose)[g]	1·7	0·9	1·2	0·8

[a]Values shown are the average values from two experiments, including standard deviations.
[b]Considering the high volatility of acetaldehyde (boiling point: 21°C), allowance should be made for some losses due to escape; values may therefore be underestimated. The value shown for the *GPD1*-overexpressing wild-type strain is the highest value we could detect after depletion of glucose in the culture supernatant.
[c]Values shown are the differences in dry weight before and after fermentations.
[d]Glucose equivalents, used for biomass formation, are calculated according to the formula of Bruinenberg *et al.* (1983).
[e]It should be noted that, because of the rich medium used, the fermentation products may not be derived exclusively from glucose.
[f]Values shown are calculated from the values of fermentation products whose formation is not redox neutral (glycerol, pyruvate, acetaldehyde[b] and acetate).
[g]Values shown are calculated from the formation of ethanol, acetaldehyde[b], pyruvate and acetate (net ATP gain) as well as glycerol (net ATP loss).
[h]The amounts of fermentation products shown for the strains carrying the reference plasmid YEpKm^{R} are not significantly different from those obtained for the respective untransformed strains.

background was associated with extra pyruvate yields and no acetaldehyde production. Calculation of carbon balances (Table 1) shows that the assayed fermentation products in the modified strains did not account for all of the glucose used. In the strains overexpressing *GPD1*, redox levels were also unbalanced. With reference to the wild-type strain overexpressing *GPD1*, the gaps observed both in carbon and redox balances (Table 1) may result from the escape of acetaldehyde from the culture vessel (see legend in Table 1).

The carbon imbalance of the *pdc* mutant could result from overproduction of fermentation products which were not analysed in this study. However, formation of these products does not seem to be associated with the production or consumption of NADH, since redox levels were balanced in this strain. In contrast, the gap in carbon balance in the *pdc* mutant overexpressing *GPD1* was associated with a corresponding gap in the redox balance (Table 1).

Glycerol formation is an ATP-consuming process. Overproduction of glycerol therefore leads to a net ATP loss. Estimations of ATP yields for the different strains are shown in Table 1. It should be noted that the value shown for the wild-type strain overexpressing *GPD1* is certainly underestimated, again due to the volatility of acetaldehyde. The smaller ATP gain, resulting from glycerol

Table 2. Specific rates[a,b] of glycerol and ethanol formation and glucose consumption during anaerobic growth of wild type (YSH 1.1.-6B) and a *pdc* mutant strain (YSH 306) of *S. cerevisiae* transformed with YEpKm^R*GPD1* (overexpression of *GPD1*) and YEpKm^R (reference plasmid) in YEPD medium.

Strain	Specific rate (mmol/h per g dry weight)		
	Glycerol formation	Ethanol formation	Glucose consumption
Wild type (reference plasmid)[c]	0·8 ± 0·2	13·3 ± 0·1	8·5 ± 1·2
Wild type (*GPD1* overexpression)	5·1 ± 0·2	12·0 ± 0·2	10·9 ± 1·3
pdc mutant (reference plasmid)[c]	1·5 ± 0·1	7·4 ± 0·8	6·2 ± 0·7
pdc mutant (*GPD1* overexpression)	2·5 ± 0·1	2·6 ± 0·2	4·5 ± 0·8

[a]Specific rates were calculated as follows: hourly metabolite concentrations were fitted to a second-order polynomic function. At distinct time intervals the slope of the curve was determined and divided by the corresponding dry weight. The specific rates were constant over a wide range of fermentation times.
[b]The data shown are the average values from two experiments including standard deviations.
[c]The values shown for the strains carrying the reference plasmid YEpKm^R are not significantly different from those obtained for the respective untransformed strains.

overproduction, would, at least in part, account for the decreased biomass formation found in all modified strains (Table 1). In *pdc* mutants, ATP-consuming pyruvate excretion could, in addition, be responsible for diminished biomass formation.

Relevant fluxes of glucose catabolism

In addition to measuring the amounts of fermentation products after depletion of glucose, we also determined the impact of the different enzyme activity alterations on central fluxes of glucose catabolism, i.e. the specific rates of formation of glycerol and ethanol, as well as the rate of glucose consumption.

The specific rate of glycerol formation in the *pdc* mutant was increased to twice that of the wild type but did not match the decrease in the rate of ethanol formation (Table 2). Moreover, the rate of glucose consumption in the *pdc* mutant was slightly decreased (Table 2). The approximately four- to five-fold increase in glycerol yield observed in this strain (Table 1) reflects therefore partly a higher rate of glycerol formation and partly a slower glucose catabolism.

In addition, Table 2 shows that an increased GPD level in the wild-type background was likewise associated with a six- to seven-fold increase in the rate of glycerol formation and a small but reproducible increase in the rate of glucose utilization. An enhanced glucose catabolism supplying the increased flux to glycerol matches the observation that ethanol formation was hardly affected in this strain (Table 2).

Overexpression of *GPD1* in the *pdc* mutant background led to a rate of glycerol formation slightly higher than that of the corresponding reference strain, but only half that of the wild-type strain where *GPD1* was overexpressed. This is due to the much slower rate of glucose catabolism in the *pdc* mutant background (Table 2).

DISCUSSION

The aim of this paper was to characterize in more detail various approaches to enhancing glycerol production in *S. cerevisiae.*

The impact of a limited alcoholic fermentation was studied by using a mutant strain of *S. cerevisiae* (Δ*pdc2*) that exhibits a markedly decreased level of PDC. Considering the glycerol yield after batch fermentation of glucose, the value observed in the *pdc* mutant was 4·7 times that of the wild type. The specific rate of glycerol formation was, however, only doubled. This result can be explained by a lower rate of glucose catabolism in *pdc* mutants.

Glycerol formation in *pdc* mutants has already been investigated by Schmitt and Zimmermann (1982). In their study, no increase in the rate of

glycerol formation was found, and the rate was even slightly less than in wild type. The disagreement of our data could result from the different culture conditions used. Schmitt and Zimmermann (1982) added antimycin A to the culture medium to prevent respiration, whereas our investigations were done under oxygen-limited conditions.

Based on his work with *pdc* mutants, Zimmermann (1992) concluded that the strongly enhanced rate of glycerol formation in the presence of bisulfite (Neuberg and Reinfurth, 1918; see Introduction) cannot simply be explained as being a result of restricting alcoholic fermentation by trapping acetaldehyde, but must be triggered by some other mechanism. In this context, a higher level of GPD has to be taken into consideration, which has been reported during fermentation in the presence of bisulfite (Gancedo *et al.*, 1968). By overexpression of *GPD1* in a *pdc* mutant, we have shown that a rise in GPD level markedly increases the glycerol yield in a strain whose alcoholic fermentation is restricted (Table 1).

The dramatic increase in GPD activity which is necessary for the enhanced glycerol production in *S. cerevisiae* as part of osmoregulation has already been described (see Introduction). However, it remained unclear whether a simple increase in GPD activity could alone provoke a substantial increase in flux to glycerol without the influence of any other enzyme activity. By overexpression of *GPD1* in a wild-type strain of *S. cerevisiae* we have provided strong evidence for the control of GPD over glycerol formation. This was the case not only under oxygen-limited conditions but also in glucose fermentations in the presence of oxygen (data not shown). A relatively high flux control coefficient for GPD over the flux from glucose to glycerol has already been estimated by Blomberg and Adler (1989) based on their observations in cells exposed to lower water potentials. Nevertheless, it cannot be excluded that the other less dramatic changes in enzyme activity observed during osmoregulation, such as increased levels of aldehyde dehydrogenase and reduced levels of alcohol dehydrogenase and PDC, as well as of most glycolytic enzymes (Blomberg and Adler, 1989; Blomberg, 1995), also participate in glycerol overproduction in osmotically stressed cells. These enzyme activity alterations may support the metabolic shift towards glycerol, similar to the situation seen in a strain overexpressing the *GPD1* gene in a *pdc*-mutant background (Table 1).

Results obtained with the *GPD1* multicopy wild-type transformant further show that cellular levels of phosphatase, the other key enzyme in glycerol formation, are high enough to account to some extent for an enhanced flux to glycerol. This is in accordance with the finding of Gancedo *et al.* (1968). There was no increase in phosphatase levels in the presence of bisulphite, although flux to glycerol was markedly enhanced under these conditions.

Furthermore, our findings suggest that the increase in the rate of glycerol production in the *GPD1* multicopy wild-type transformant to the value observed in this study was not prevented by the activities of regulatory glycolytic enzymes or glucose uptake. These factors have often been suggested to limit the rate of glucose catabolism in *S. cerevisiae* (Gancedo and Serrano, 1989; Schaaff *et al.*, 1989). It is possible that certain factors specifically associated with the increased flux to glycerol, such as net ATP loss or enhanced release of inorganic phosphate, are responsible for increasing glycolytic flux in the *GPD1* multicopy transformant, as indicated by the higher rate of glucose consumption.

With regard to the possible factors limiting the flux from glucose to glycerol in a strain where rate-limiting GPD is overproduced, consideration should be given not only to the level of phosphatase or the rate of glucose catabolism, but also the efflux of glycerol out of the cell. Recently, a gene coding for a membrane-spanning protein in *S. cerevisiae* (*FPS1*), homologous with the glycerol facilitator in *E. coli*, has been isolated (Luyten *et al.*, 1995). It has been suggested that this channel protein is involved in regulation of both efflux and production of glycerol in yeast. It may be that overexpression of the glycerol transport system could even enhance glycerol overproduction in a strain overexpressing *GPD1*.

ACKNOWLEDGEMENTS

We thank C. Lang for helpful comments on this manuscript, C. A. Dawson for correcting the English, and S. Hohmann for providing us with both yeast strains and the *GPD1* gene. We also thank D. Kattner who performed some of the metabolite determinations. This work was supported by grants from the 'Bundesministerium für Wirtschaft, Arbeitsgemeinschaft für industrielle Forschung (AiF) Otto V. Guericke', Köln, Germany.

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