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Modulation of Glycerol and Ethanol Yields During Alcoholic Fermentation in *Saccharomyces cerevisiae* Strains Overexpressed or Disrupted for *GPD1* Encoding Glycerol 3-Phosphate Dehydrogenase

SUMIO MICHNICK[1], JEAN-LOUIS ROUSTAN[1], FABIENNE REMIZE[1], PIERRE BARRE[1] AND SYLVIE DEQUIN[1]*

[1]*Laboratoire de Microbiologie et Technologie des Fermentations, INRA-IPV, 2 Place Viala, F-34060 Montpellier cedex 01, France*

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The possibility of the diversion of carbon flux from ethanol towards glycerol in *Saccharomyces cerevisiae* during alcoholic fermentation was investigated. Variations in the glycerol 3-phosphate dehydrogenase (GPDH) level and similar trends for alcohol dehydrogenase (ADH), pyruvate decarboxylase and glycerol-3-phosphatase were found when low and high glycerol-forming wine yeast strains were compared. GPDH is thus a limiting enzyme for glycerol production. Wine yeast strains with modulated *GPD1* (encoding one of the two GPDH isoenzymes) expression were constructed and characterized during fermentation on glucose-rich medium. Engineered strains fermented glucose with a strongly modified [glycerol] : [ethanol] ratio. *gpd1*Δ mutants exhibited a 50% decrease in glycerol production and increased ethanol yield. Overexpression of *GPD1* on synthetic must (200 g/l glucose) resulted in a substantial increase in glycerol production (× 4) at the expense of ethanol. Acetaldehyde accumulated through the competitive regeneration of NADH via GPDH. Accumulation of by-products such as pyruvate, acetate, acetoin, 2,3 butane-diol and succinate was observed, with a marked increase in acetoin production. © 1997 by John Wiley & Sons, Ltd.

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KEY WORDS — *Saccharomyces cerevisiae*; alcoholic fermentation; glycerol; glycerol 3-phosphate dehydrogenase; redox balance; metabolic engineering

INTRODUCTION

Glycerol, quantitatively the most important by-product of alcoholic fermentation, is synthesized by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate (G3P) by NAD-dependent glycerol 3-phosphate dehydrogenase (GPDH) followed by dephosphorylation of G3P to glycerol by means of a specific glycerol 3-phosphatase (G3Pase).

During alcoholic fermentation, the major role of glycerol formation is to maintain the redox balance. Although ethanol production, which ensures reoxidation of the NADH formed during the oxidation of glyceraldehyde 3-phosphate, is a redox-equilibrated process, excess NADH is produced during biomass formation. Glycerol is mainly produced to counterbalance this surplus of NADH and may be considered to form a redox valve (Nordstöm, 1968; Lagunas and Gancedo, 1973; Oura, 1977; Van Dijken and Scheffers, 1986). In addition, glycerol production may play a role in balancing the ratio of free to bound phosphate in the cytosol, as has been suggested recently (Luyten *et al.*, 1995).

Another aspect is the essential role of glycerol as a compatible solute during hyperosmotic stress. The yeast *Saccharomyces cerevisiae* responds to increased external osmolarity by enhanced production and intracellular accumulation of glycerol to

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counterbalance the osmotic pressure (Blomberg and Adler, 1989, 1992; Mager and Varela, 1993). The NAD-dependent GPDH is strongly induced in these conditions (André *et al.*, 1991; Albertyn *et al.*, 1994a). GPD1, one of the two isogenic genes for GPDH, is directly involved in osmotic-stress response and its expression is partly controlled via the high-osmolarity glycerol response MAP kinase pathway (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b). On the other hand, GPD2, recently characterized, is not subjected to osmotic regulation but assumed to be involved in redox balancing during anaerobiosis (Ericksson *et al.*, 1995; Ansell *et al.*, 1995).

Since formation of both glycerol and ethanol plays a role in balancing the redox state of the cell, it might be assumed that limitation or amplification of the steps specifically involved in the production of these compounds (catalysed by pyruvate decarboxylase, PDC; alcohol dehydrogenase, ADH; GPDH and G3Pase) would result in a change in the [glycerol] : [ethanol] ratio. In agreement with this hypothesis, the amount of glycerol increases, for example, if acetaldehyde is trapped with sulfite. Furthermore, strains with low ADH specific activity or mutants deficient in ADHI produce more glycerol during fermentation (Ciriacy, 1975; Wills and Phelps, 1975; Johansson and Sjöström, 1984). On the other hand, a relationship between GPDH activity and the ability of yeast strains to produce glycerol had been put forward (Radler and Schütz, 1982). Finally, a strain of *Saccharomyces diastaticus* disrupted for GPD1 (previously named DAR1) displays a 75% decrease in glycerol production on 8% glucose medium (Wang *et al.*, 1994).

Modulation of the [glycerol] : [ethanol] ratio during glucose fermentation would be interesting for industrial purposes. Improvement of wine yeast strains for glycerol production would be advantageous in the case of wines that are lacking in body. Moreover, glycerol contributes to the taste of wine by providing sweetness (Hinreiner *et al.*, 1955; Noble and Bursick, 1984). In addition, utilization of a yeast that overproduces glycerol at the expense of ethanol for the elaboration of beverages with a low ethanol content, for instance wines and half-fermented beverages such as '*pétillant de raisin*' would be an alternative to physical techniques for alcohol removal that do not always conserve the organoleptic characteristics of the product. On the other hand, a sugar-fermenting yeast that produces a small quantity of glycerol but a higher alcohol yield would be of great value for the distilling industry.

In this study, we have investigated the possibility of constructing *S. cerevisiae* strains exhibiting modified glycerol and ethanol yields during alcoholic fermentation. Evidence that GPDH is a rate-limiting enzyme in glycerol production under enological fermentation conditions is reported and sustained by demonstration that disruption and overexpression of *GPD1*, one of the two isogenic genes for GPDH, strongly alter the [glycerol] : [ethanol] ratio. The strains overexpressed for *GPD1* exhibited a four-fold increase in glycerol production under conditions similar to those of wine-making. The study of the metabolic modifications caused by the strains overproducing glycerol to cope with carbon diversion and redox imbalance is presented.

MATERIALS AND METHODS

Strains and culture conditions

Escherichia coli DH5α was used for cloning experiments. The *S. cerevisiae* strains used in this study were V5 (ScV5M, *MAT***a**, *ura3*) derived from a Champagne wine strain (variety *bayanus*) and the enological strains S61 and S267 (variety *cerevisiae*). *E. coli* cultivation and media were as described previously (Sambrook *et al.*, 1989). *S. cerevisiae* was maintained and grown in YPD medium (1% bacto yeast extract, 2% bactopeptone, 2% glucose).

Batch fermentation experiments were carried out in minimal synthetic medium YNB (0·67% yeast nitrogen base without amino acid, 10% glucose, pH 3·3 with hydrochloric acid and supplemented with 0·5% casamino acids) or in MS synthetic must medium simulating a standard grape juice containing 18–20% glucose, as described by Bely *et al.* (1990), but without proline. Nitrogen was in the form of 80 mg/l ammoniacal-nitrogen (NH_4Cl) and 120 mg/l α amino acid-nitrogen.

The inoculum was grown for approximately 36 h at 28°C in 50 ml flasks without agitation. Fermentations were realized by inoculation of precultured cells at a density of 1×10^6 per ml in fermentors with a working volume of 200 ml or 1,1 l (no differences in the results due to different cultivation volumes were observed) equipped with fermentation locks. Fermentations were carried out at 28°C with permanent stirring (500 rpm).

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CO_2 release was determined by automatic measurement of fermentor weight loss each 20 min (Bely *et al.*, 1990). Fermentation was characterized by fermentation progress expressed as $1\text{-}S/S_0$ (S=glucose concentration, S_0=initial concentration).

Preparation of polyclonal serum against Gpd1p protein, protein and Western blot analyses

To produce antibodies against Gpd1p, GPDH protein was purified as described (Michnick, 1995) from the enological strain S61. A major protein exhibiting GPDH activity had a molecular weight of 43 kDa. This protein was isolated from Coomassie-stained sodium dodecyl sulphate (SDS)–polyacrylamide gel and the dried acrylamide band was used for a rabbit immunization, performed by Eurogentec S. A. For Western blot studies, proteins from whole cell extracts were analysed by SDS–polyacrylamide gel electrophoreses (PAGE; Sambrook *et al.*, 1989). Gels were stained with 0·25% Coomassie blue R-250 (Sigma) in 40% methanol and 7% acetic acid. Analyses were carried out using the polyclonal serum and the alkaline phosphatase detection method (Sambrook *et al.*, 1989). The protein concentration in crude extracts was determined with the BCA Protein Assay Reagent (Pierce).

DNA manipulation, cloning techniques and transformation methods

Restriction and modification enzymes were used according to the manufacturer's instructions. *E. coli* plasmid DNA was prepared using standard protocols (Sambrook *et al.*, 1989). Oligonucleotides were synthesized by Eurogentec. *E. coli* transformation was carried out by the $CaCl_2$/$RbCl_2$ method (Hanahan, 1985). Transformation of *S. cerevisiae* was performed using the LiAc procedure (Schiestl and Gietz, 1989).

Overexpression and disruption of GPD1

Polymerase chain reaction (PCR) cloning of the *GPD1* gene and introduction of *Xho*I and *Bam*HI sites respectively at the 5′ and 3′ ends of the coding region was achieved using the oligonucleotides CGCTCGAGCCCCTCCACAAACACA, complementary to a region upstream of the ATG start codon (nucleotides − 34 to − 18) and GC GGATCCGGGGAAGTATGATATGTT, corresponding to a region located 17 nucleotides downstream of the stop codon (Albertyn *et al.*, 1994b). The 1260 bp DNA fragment (including sites) defined by the two primers was amplified with total DNA from V5 strain as a template and directly cloned into the pGEM-T vector (Promega) to give the pGEM-T-*GPD1* vector. The insert was partially sequenced to verify the identity of the gene. For construction of the *GPD1* expression vector, the PCR fragment was cut with *Xho*I and *Bam*HI and cloned into the *Xho*I and *Bam*HI sites of the yeast expression vector pVT100-U (Vernet *et al.*, 1987) to give the pVT100-U-*GPD1* vector used to transform V5 strain.

Disruption of *GPD1* was obtained by internal deletion of the *Bsm*I-*Xba*I 800 bp fragment of pGEM-T-*GPD1*, giving pGEM-T-*gpd1*Δ. The *URA3 Bgl*II 1·2 kb fragment from pUT332 (Cayla) was then inserted in the *Bgl*II site of pGEM-T-*gpd1*Δ. The *gpd1*Δ gene was isolated by digestion of pGEM-T-*gpd1*Δ by *Xho*I and *Bam*HI. The resulting 1·6 kb *Xho*I-*Bam*HI fragment was used to transform V5 strain. Replacement of the chromosomic *GPD1* allele by the inactivated copy was confirmed by PCR and southern blot analysis of genomic DNA.

Enzyme assays

Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) and glycerol 3-phosphatase (EC 3.1.3.21) were assayed according to the method of Gancedo *et al.* (1968), alcohol dehydrogenase (EC 1.1.1.1) as described by Millan *et al.* (1987) and pyruvate decarboxylase (EC 4.1.1.1) according to Schmitt and Zimmermann (1982) in crude extracts obtained by vortexing yeast cells with glass beads (0·5 mm in diameter) for 4 min at 4°C. Specific activities were expressed as μmol or nmol substrate degraded per min and mg protein.

Analytical methods

Yeast cells were counted using an electronic particle counter (ZM, Coultronics). Glucose, glycerol, ethanol, pyruvate, acetate and succinate were analysed by HPLC on an HPX-87H Aminex column (BioRad). Elution was performed at 45°C with 8 mM-H_2SO_4 at a flow rate of 0·6 ml/min. Detection was performed by means of dual detection: refractometer (Shimadzu) and UV detector (Shimadzu SDD-2A, λ=214 nm). Quantification was performed using external standards prepared from pure compounds (Sigma) and an HP 3365 integration system. Acetaldehyde was determined enzymatically according to the method of

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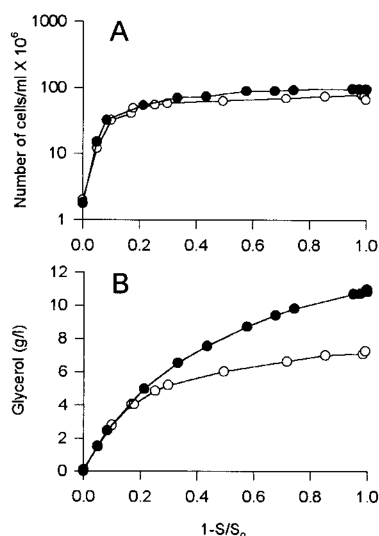

Figure 1. The kinetics of glycerol production by *S. cerevisiae* strains S61 and S267 during alcoholic fermentation. (A) Growth curve; (B) glycerol production; S61 (open circles); S267 (closed circles). Fermentation was carried out on MS medium containing 180 g/l glucose, pH 3·3.

Lundquist (1974). Acetaldehyde-ammoniac trimer (Aldrich) was used as standard. Acetoin and 2,3 butane-diol were determined by gas chromatography (GC). They were extracted as described by Hagenauer-Hener *et al.* (1990). Samples (2 ml) containing an internal standard (hexanol 0·4% v/v in methanol) were saturated with 2·5 g K_2CO_3 and extracted by chloroform (2 ml). The organic phase was dried with Na_2SO_4. 1 µl of a dilution 1/1 (v/v) with methanol was injected on an HP 5890 apparatus fitted with a DBWAX megabore column (Jandel). Injector and detector temperatures were respectively 240°C and 250°C. Oven temperature was kept at 80°C for 2 min and then programmed from 80°C to 200°C at 10°C/min. (Meso) 2,3-butane-diol was separated from optically active forms. Enantiomers of butane-diol and acetoin were not resolved.

RESULTS

Gpd1p is a limiting enzyme for glycerol production

When grown anaerobically on synthetic must (180 g/l glucose), S61 and S267 strains produced 7·3 and 11 g/l of glycerol respectively (Figure 1B). The glycerol production kinetics were biphasic in both strains, with a high production rate during the growth phase and a slowing after the cells entered the stationary phase (Figure 1A), until complete depletion of sugar. Differences in glycerol levels between the two strains were mainly achieved during this second phase of production.

The activities of enzymes specifically involved in ethanol (PDC and ADH) or in glycerol (GPDH and G3Pase) production were monitored during this second phase (Figure 2) in order to determine whether these differences may be related to a limiting step either in ethanol or glycerol pathways. Although the low (S61) and high (S267) glycerol-forming strains exhibited similar PDC, ADH and G3Pase specific activities, significant variations between the two strains were found with GPDH. These data suggest that the level of GPDH activity may have some importance in the capacity of yeast to form glycerol during fermentation.

In order to estimate the amount of GPDH protein in S61 and S267, this protein was purified from strain S267, which displayed the highest glycerol yield (Michnick, 1995). The purified GPDH protein was shown to correspond to Gpd1p since its internal sequence was identical to the protein sequence deduced from the nucleotidic sequence of *GPD1* (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b). Antibodies were raised against the purified protein. GPDH production was monitored during fermentation on YNB medium containing 180 g/l of glucose, pH 3·3 (Figure 3). Proteins from crude cell extracts were resolved by SDS–PAGE and analysed by Western blotting. For both strains, GPDH protein was detected at the expected molecular weight of 43 kDa. The maximum amount of GPDH protein was detected towards the end of fermentation for both strains (Figure 3), in general agreement with the increase in GPDH activity observed at this stage (Figure 2D). GPDH protein in S61 strain was found to be induced a little later than expected from GPDH activity data, but the results obtained with S267 are in full agreement with the variation in GPDH activity. In addition, the amount of GPDH was generally smaller for the strain with a low glycerol yield (S61) in comparison with strain S267.

The glycerol formation capacity may thus be related to variations in the amount of GPDH. Since two GPDH isoenzymes which exhibit 69% identity in amino acid sequence are found in *S. cerevisiae*, encoded by *GPD1* and *GPD2* genes (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b; Ericksson *et al.*, 1995), both may have been detected. However, results obtained with the

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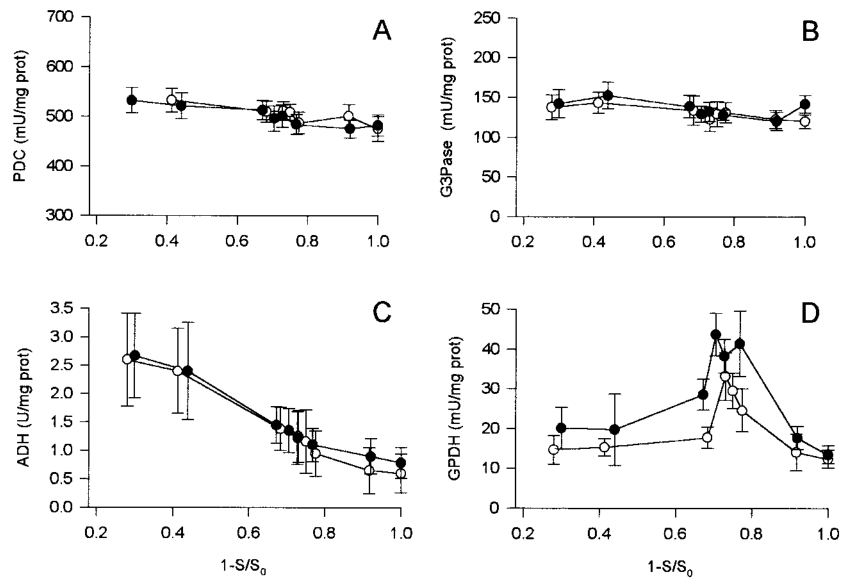

Figure 2. Variations in GPDH activity between S61 and S267 strains. Specific activities of (A) PDC, (B) G3Pase, (C) ADH and (D) GPDH were monitored during the stationary phase. S61 (open circles); S267 (closed circles). Three individual determinations were performed. Fermentation was carried out under the same conditions as described in Figure 1.

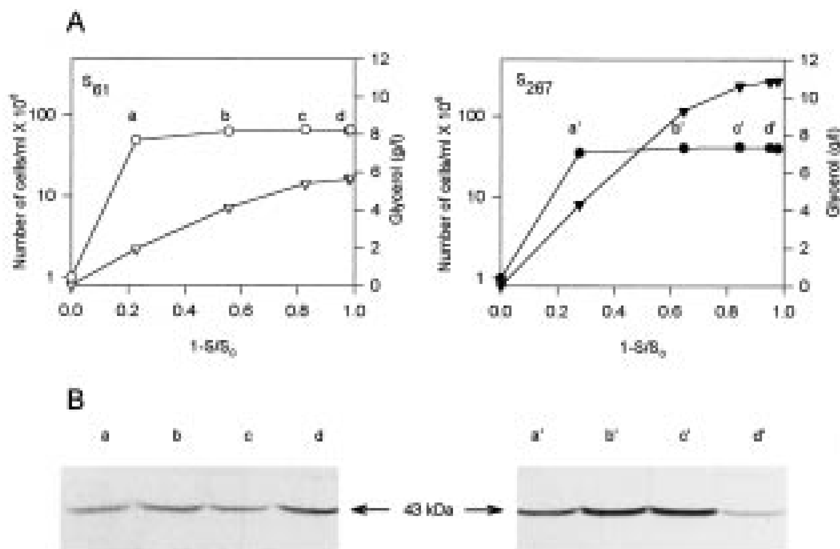

Figure 3. Expression level of Gpd1 protein in S61 and S267 strains during alcoholic fermentation. (A) Growth (circles) and glycerol production (triangles). (B) Western blot analysis. 10 μg of protein extracted from S61 and S267 at the times indicated was analysed by immunoblotting using anti-Gpd1p polyclonal serum. Fermentation was carried out on YNB medium containing 180 g/l of glucose, pH 3·3.

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*gpd1*Δ mutant (this study) strongly suggest that under the conditions used for the determination of GPDH activity, Gpd2p activity was not detected and that the antibodies directed against Gpd1p were specific for this isoform. This suggests that most of the GPDH activity and most of the protein detected corresponded to Gpd1p. Surprisingly, the *in vitro* Gpd1p activity level and amount were highest during the stationary phase when glycerol production is the lowest. This indicates that GPDH activity is regulated *in vivo*. Nevertheless, these results strongly suggest that the amount of Gpd1p is one of the limiting factors for glycerol production in *S. cerevisiae* under enological conditions, with the possibility of a diversion of the carbon flux towards glycerol or ethanol by modulation of the level of expression of the *GPD1* gene.

Overexpression and disruption of GPD1 *strongly modify the yield of glycerol and ethanol*

The consequences of overexpression and deletion of *GPD1* on glycerol production were studied on minimal medium YNB with 100 g/l of glucose, pH 3·3. Transformants carrying *GPD1* under the control of the *ADH1* promoter and terminator on a multicopy plasmid (V5/*GPD1*) displayed high GPDH specific activity during the growth phase with a maximum level observed when the cells reached the stationary phase, corresponding to a 15-fold increase compared to the control strain (Figure 4A). In contrast, GPDH specific activity for the control strain (V5/pVTU) was low and constant during the growth phase; it then increased and reached a peak of 80 mU/mg protein during the second part of fermentation ($1\text{-}S/S_0=0{\cdot}6$, Figure 4A). Western blot analysis was performed on protein from whole cell extracts (Figure 4B). Gpd1 protein was shown to be produced in large amounts in V5/*GPD1* in agreement with the high level of activity, whereas it was only detected in the sample displaying the highest GPDH activity ($1\text{-}S/S_0=0{\cdot}6$) in V5/pVTU (data not shown). Although the activity was found to decrease during the stationary phase, the protein was always detected in large amounts, suggesting *in vivo* regulation mechanisms. GPDH activity (related to Gpd1 isoenzyme) was not detected in the *gpd1*Δ mutant when grown under the same conditions as those described in Figure 4, and Gpd1 protein was not detected by Western blot analysis (data not shown).

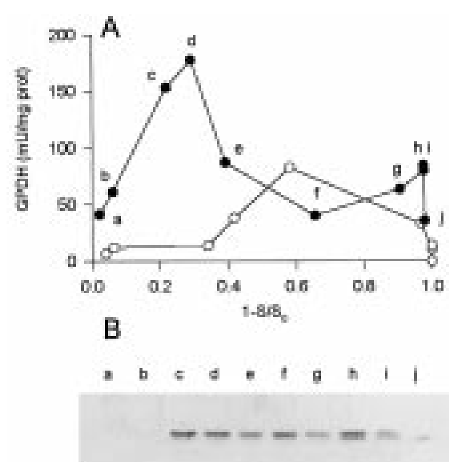

Figure 4. GPDH activity and Western blot analysis in *S. cerevisiae* overexpressing *GPD1*. (A) Specific GPDH activity in crude extracts of V5/*GPD1* (closed circles) and in V5/pVTU (open circles) cells. (B) 10 μg of proteins extracted from V5/*GPD1* cells was analysed by immunoblotting using anti-Gpd1p antiserum. Fermentation was carried out on minimal medium YNB containing 100 g/l glucose, pH 3·3.

As a result of overproduction of Gpd1 protein, the increase in the glycerol yield was more than three times greater in V5/*GPD1* in comparison with the control strain (Table 1). The large increase

Table 1. Glycerol and ethanol yields in V5/pVTU and V5/*GPD1*. The cells were grown on YNB medium containing 100 g/l glucose, pH 3·3. Metabolites were analysed after complete glucose depletion.

Strain	Glycerol (g/l)	Ethanol (g/l)	Molar ratio of	
			Glycerol/glucose	Ethanol/glucose
V5/pVTU	4·3	46·8	0·08	1·83
V5/*GPD1*	14	36·6	0·27	1·43
V5*gpol*$_1$Δ	2·4	49·2	0·05	1·92

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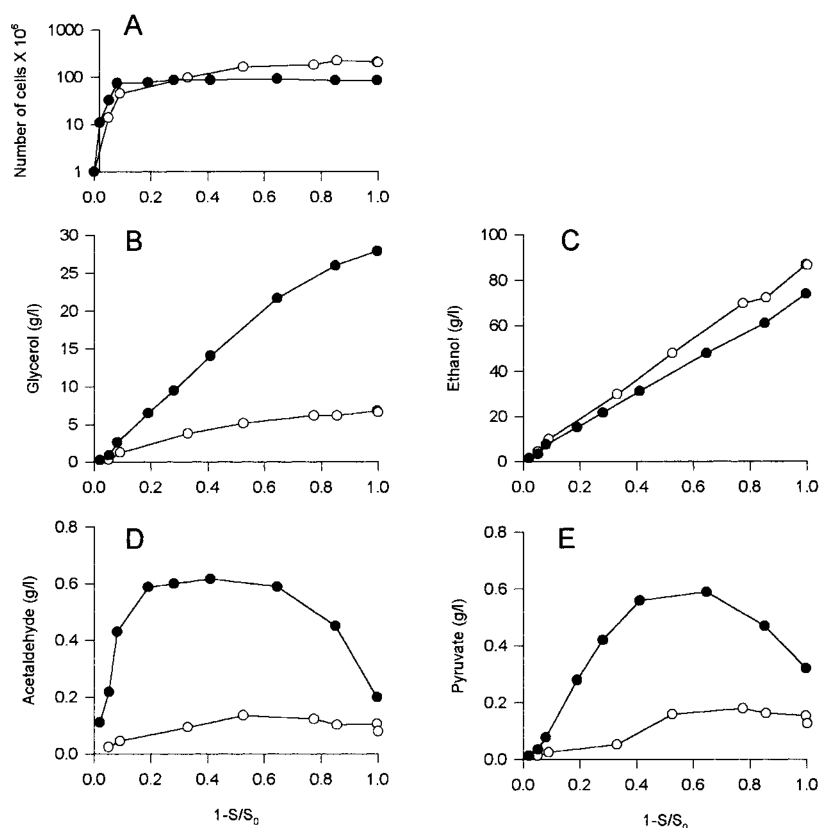

Figure 5. The effects of *GPD1* overexpression on the kinetics of glycerol, ethanol, acetaldehyde and pyruvate production under enological conditions. (A) Growth curves; (B–E) production of glycerol, ethanol, acetaldehyde and pyruvate. V5/pVTU (open circles); V5/*GPD1* (closed circles). Fermentation was carried out on MS medium containing 200 g/l glucose, pH 3·3.

in glycerol production (14 g/l instead of 4·3 g/l) took place simultaneously with a decrease in the production of ethanol, the molar ratio of ethanol to glucose decreasing by 20% for the transformant overproducing glycerol. In contrast, the glycerol production level was reduced for the strain disrupted for *GPD1* whereas the ethanol yield was slightly enhanced (Table 1). Residual glycerol production was 50% for the *gpd1*Δ strain, suggesting that both *GPD1* and *GPD2* genes may contribute to glycerol formation under enological conditions.

Consequences of glycerol overproduction under enological-like conditions

The kinetics of formation of glycerol, ethanol and intermediate metabolites were monitored in the transformant overexpressing *GPD1* during fermentation on synthetic must (glucose 200 g/l) in order to examine how *S. cerevisiae* reacts to a glycolytic flux diverted to glycerol. Growth and production of glycerol, ethanol, acetaldehyde and pyruvate are shown in Figure 5. A strong increase in the glycerol rate was observed, leading to the production of 28 g/l compared to 7 g/l for the control strain (Figure 5B), while ethanol was produced at a lower rate, resulting in a decrease in the final concentration of about 15 g/l (Figure 5C). Glycerol yield was about four times higher and ethanol yield was reduced about 20% for V5/*GPD1* compared with V5 (Table 2). In consequence, acetaldehyde and pyruvate were transiently accumulated in the strain that overproduced glycerol (Figure 5D,E). Peak level was reached for both compounds when the cells entered the stationary phase; the concentration in these compounds subsequently

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Table 2. Concentrations and yields of fermentation products and balances in V5/pVTU (control) and V5/*GPD1*, after anaerobic growth in MS medium containing 200 g/l glucose, pH 3·3.

	Value[a] for the strain:	
	V5/pVTU	V5/*GPD1*
Ethanol (g/l)	89·2 ± 3·6	72·5 ± 2·1
(mmol/mol glucose)	1746 ± 70	1417 ± 42
Carbon dioxide (g/l)	94·5 ± 0·07	85·5 ± 0·02
(mmol/mol glucose)	1934 ± 1	1748 ± 58
Glycerol (g/l)	7·1 ± 0·7	28·6 ± 1·4
(mmol/mol glucose)	69 ± 6	279 ± 13
Pyruvic acid[b] (g/l)	0·13	0·32
(mmol/mol glucose)	1·4	3·6
Acetaldehyde[c] (g/l)	<0·1	0·22 ± 0·01
(mmol/mol glucose)		4·5 ± 0·3
Acetate (g/l)	0·52 ± 0·06	1·6 ± 0·1
(mmol/mol glucose)	7·7 ± 1·0	23 ± 1
Acetoin (g/l)	<0·1	6·1 ± 0·8
(mmol/mol glucose)		62 ± 9
2,3 Butane-diol (g/l)	0·9 ± 0·4	1·3 ± 0·1
(mmol/mol glucose)	8·9 ± 4·4	13 ± 1
Succinate (g/l)	0·25 ± 0·06	0·54 ± 0·04
(mmol/mol glucose)	1·9 ± 0·5	4·1 ± 0·3
Biomass formation[d]		
dry weight (g/l)	4·2 ± 0·8	2·55 ± 0·07
Carbon recovery (%)	97·6 ± 2·4	98·2 ± 0·3
Redox balance[e] (%)	95·3 ± 3·6	96·4 ± 0·6

[a]Except as noted, all values are expressed as means derived from two independent experiments, including standard deviations.
[b]Single experiment.
[c]Acetaldehyde loss was always less than 100 mg throughout the fermentation.
[d]The carbon-molar mass of biomass was estimated using the elemental composition ($C_4H_{7\cdot32}O_{2\cdot24}N_{0\cdot68}S_{0\cdot006}$) used by van Dijken and Scheffers (1986).
[e]The redox balance represents the ratio between the reductance degree of fermentation products (including biomass) and the reductance degree of glucose, expressed as a percentage.

decreased until glucose was completely depleted and remained slightly higher than for the control strain at the end of fermentation (Table 2).

Variation in the production of by-products of acetaldehyde and/or pyruvate was investigated by HPLC analysis (Table 2). The acetate level increased (three-fold) for the transformed strain in comparison with the control strain. Smaller but significant accumulation (about two-fold) was also observed for succinate. Large amounts of two other compounds eluted at retention times corresponding to those of acetoin and 2,3 butane-diol were also detected for the *GPD1* strain. The identity of these compounds was further confirmed by GC analysis. Substantial accumulation of acetoin was observed for the *GPD1* strain (6·1 g/l). The level of 2,3 butane-diol, the product of acetoin reduction, was 1·5-fold that of the V5/pVTU strain. The carbon and redox balances were equilibrated satisfactorily for both strains.

The consequences of *GPD1* overexpression on growth are shown in Figure 5A and Table 2. V5/*GPD1* cells reached the stationary phase earlier than the control strain (30 h compared with 45 h), and the final quantity of biomass for the strain overexpressing *GPD1* was 1·6 times lower than that of the V5/pVTU strain.

DISCUSSION

There have been few previous investigations of the regulation of glycerol production under enological

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conditions. In the present study, it was shown that *in vitro* GPDH specific activity and the amount of Gpd1p isoenzyme monitored during fermentation were higher in a high glycerol-forming strain than in a low glycerol-forming one, whereas activities of other enzymes involved in glycerol or ethanol formation were similar for both strains, suggesting that GPDH is a limiting step for glycerol production under enological conditions. The strong modification of glycerol yield for the strains overexpressed or disrupted for *GPD1* supports this assumption.

The rate of glycerol production was shown to be high during the growth phase, in agreement with the role of glycerol in the reoxidation of NADH generated by anabolic processes. Surprisingly, the GPDH synthesis level was shown to be low during this phase and high during the stationary phase when glycerol production slowed down (Figures 2D, 3 and 4A). These results are rather unexpected since a 30–60% variation in GPDH activity during the stationary phase results in a significant difference in glycerol production, as observed for strains S61 and S267. However, regulation at GPDH synthesis level and *in vivo* regulation of its activity are both involved in the control of glycerol production. Enzymatic studies on purified preparations of GPDH (Nader *et al.*, 1979; Albertyn *et al.*, 1992) and of Gpd1p isoform (Michnick, 1995) from *Saccharomyces* strains have shown that GPDH activity is modulated by physiological concentrations of ATP, ADP, NAD and fructose 1,6 biphosphate.

An intriguing finding is the induction of GPDH during the phase of low glycerol production. Might this synthesis meet a physiological requirement? The existence of NADH-generating metabolism (i.e. via the oxidative branch of the TCA cycle) unconnected with biomass formation at this stage of fermentation cannot be ruled out. However, other mechanisms such as response to environmental conditions might be involved. Since osmotic stress is moderated under enological conditions (Jones and Greenfield, 1985), its participation in the Gpd1p induction observed seems rather unlikely. On the other hand, involvement of regulation mechanisms related to stationary phase (which mainly result from nitrogen and micronutrient starvation under enological conditions; Bely *et al.*, 1990) and to different stresses (high ethanol concentration, low pH) cannot be ruled out. This aspect clearly requires further attention, especially as evidence has been found that the expression of some stress-induced genes is controlled differently under enological conditions (glucose 20%, pH 3·5) in comparison with standard laboratory conditions (glucose 2%, pH 6; Riou *et al.*, 1997).

The contribution and the role of the two GPDH isoenzymes in glycerol production during alcoholic fermentation is not known. Under the conditions used here, *gpd1*Δ displayed a residual glycerol production of 50%, suggesting that both *GPD1* and *GPD2* genes might be involved in glycerol production during anaerobiosis. Although no GPDH activity could be detected for this mutant under our experimental conditions, glycerol was certainly produced by means of Gpd2p, since most of the *GPD1* coding region was deleted and only two GPD isogenes are found in *S. cerevisiae* (genome sequence data). However, the possibility that GPD2 is only expressed in the *gpd1* deletion mutant cannot be ruled out. Expression studies of *GPD1* and *GPD2* under enological conditions might show whether both genes are expressed in a wild-type yeast under enological conditions (in progress).

Strains engineered for *GPD1* presenting strongly modified yields of glycerol and ethanol were obtained. Besides their potential interest for technological processes, these strains provide direct genetic evidence for a main role of GPDH in glycerol formation. The *S. cerevisiae gpd1*Δ strain presented half the glycerol yield and a small increase in ethanol production, in agreement with the results reported for *S. diastaticus gpd1*Δ strain (Wang *et al.*, 1994). Although no increase in glycerol production has been shown previously in *S. cerevisiae* strains amplified for *GPD1* during fermentation on 2% glucose (Albertyn *et al.*, 1994b), amplification of *GPD1* in *S. cerevisiae* under the control of the *ADH1* promoter triggered substantial overproduction of extracellular glycerol during growth on sugar-rich medium. Simple diffusion and facilitated diffusion by means of the channel protein Fsp1p were suggested as being involved in glycerol efflux (Luyten *et al.*, 1995). This efflux seems not to be a limiting factor since V5/*GPD1* did not accumulate more intracellular glycerol than the control strain during the fermentation (data not shown).

Furthermore, study of the strain overexpressed for GPD1 provides strong evidence for the role of glycerol production in adjusting the redox balance. As a result of (i) diversion of the carbon flux towards glycerol and (ii) enhanced utilization of NADH through the glycerol pathway due to the

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15-fold increase in GPDH specific activity, ethanol formation was limited, resulting in transient accumulation of acetaldehyde. Acetaldehyde is a toxic compound with pleiotropic effects that have been described extensively (reviewed by Jones, 1989). Variations in the production level of secondary metabolites (acetate, acetoin, 2,3 butane-diol and succinate) should thus be regarded as a detoxication mechanism with regard to acetaldehyde (for acetoin and 2,3 butane-diol) or as a way in which the cells generate some NADH (for acetate and succinate). The main side-effect observed was a considerable increase in the formation of acetoin, a by-product of carbohydrate metabolism in yeast formed from acetaldehyde-TPP complex (recently reviewed by Romano and Suzzi, 1996). The concentration of 2,3 butane-diol produced by reduction of acetoin was shown to be increased in V5/*GPD1*. This reaction requires NADH and therefore competes with GPDH and ADH activities for the utilization of NADH. In contrast with the accumulation of the latter products, overproduction of acetate and succinate probably results from a NADH requirement since both compounds play a role in balancing the redox state of yeast (Oura, 1977; Gancedo and Serrano, 1989). It should be noticed that similar effects were observed with various strains overexpressed for GPD1, but that a different distribution of by-products was observed depending on the strain (Remize *et al.*, in preparation).

A significant reduction in final biomass was observed for the strains overexpressed for *GPD1*. This is not surprising since glycerol fermentation *per se* results in net consumption of ATP. However, it is likely that this detrimental effect on cells also results from the large amount of acetaldehyde attained at the end of the growth phase. Nevertheless, the ability of the strains engineered for *GPD1* to ferment 200 g/l of sugar was not impaired.

The new properties resulting from the shift of carbon flux towards glycerol might be of technological interest when higher glycerol yields are required or when ethanol concentrations must be limited in the final product. Depending on the industrial application and on the glycerol yield required, the reduction of side-effects such as large amounts of acetaldehyde and derived metabolites will be necessary. Better characterization of the pathways involved in the observed metabolic responses will be helpful in defining new strategies for overcoming those effects. Finally, the use of a strain improved for ethanol yield as a result of a decrease of glycerol production could be advantageous for distillery purposes.

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