Metabolic Engineering 3, 301–312 (2001)
doi:10.1006/mben.2001.0197, available online at http://www.idealibrary.com on IDEAL®

Glycerol Export and Glycerol-3-phosphate Dehydrogenase, but Not Glycerol Phosphatase, Are Rate Limiting for Glycerol Production in *Saccharomyces cerevisiae*

F. Remize,*,[1] L. Barnavon,*,[2] and S. Dequin*,[3]

*UMR Sciences pour l'Œnologie, Microbiologie et Technologie des Fermentations, INRA, 2 Place Viala, F-34060 Montpellier Cedex 1, France

Received January 11, 2001; accepted August 13, 2001

Glycerol, one of the most important by-products of alcoholic fermentation, has positive effects on the sensory properties of fermented beverages. It was recently shown that the most direct approach for increasing glycerol formation is to overexpress *GPD1*, which encodes the glycerol-3-phosphate dehydrogenase (GPDH) isoform Gpd1p. We aimed to identify other steps in glycerol synthesis or transport that limit glycerol flux during glucose fermentation. We showed that the overexpression of *GPD2*, encoding the other isoform of glycerol-3-phosphate dehydrogenase (Gpd2p), is equally as effective as the overexpression of *GPD1* in increasing glycerol production (3.3-fold increase compared to the wild-type strain) and has similar effects on yeast metabolism. In contrast, overexpression of *GPP1*, encoding glycerol 3-phosphatase (Gpp1p), did not enhance glycerol production. Strains that simultaneously overexpress *GPD1* and *GPP1* did not produce higher amounts of glycerol than a *GPD1*-overexpressing strain. These results demonstrate that GPDH, but not the glycerol 3-phosphatase, is rate-limiting for glycerol production. The channel protein Fps1p mediates glycerol export. It has recently been shown that mutants lacking a region in the N-terminal domain of Fps1p constitutively release glycerol. We showed that cells producing truncated Fps1p constructs during glucose fermentation compensate for glycerol loss by increasing glycerol production. Interestingly, the strain with a deregulated Fps1 glycerol channel had a different phenotype to the strain overexpressing *GPD* genes and showed poor growth during fermentation. Overexpression of *GPD1* in this strain increased the amount of glycerol produced but led to a pronounced growth defect.

Key Words: **glycerol production; fermentation; metabolic engineering; glycerol-3-phosphate dehydrogenase; glycerol 3-phosphatase; *FPS1*; MIP protein; *Saccharomyces cerevisiae*.**

INTRODUCTION

In *S. cerevisiae*, glycerol is produced from dihydroxyacetone phosphate (DHAP),[4] a three-carbon intermediate of the glycolytic pathway, by a two-step process. Glycerol-3-phosphate dehydrogenase (GPDH), encoded by *GPD1* and *GPD2*, catalyzes the reduction of DHAP. Glycerol 3-phosphatase (GPP), encoded by *GPP1* and *GPP2,* subsequently dephosphorylates the resultant intermediate. Glycerol is mainly used for osmoregulation and to maintain the redox balance in yeast cells. During osmotic stress, *GPD1*, *GPP1*, and *GPP2* are induced, whereas redox imbalance increases the expression of *GPD2* and *GPP1* (Albertyn *et al.*, 1994; Akhtar *et al.*, 1997; Ansell *et al.*, 1997; Norbeck and Blomberg, 1997; Rep *et al.*, 2000).

The first step of glycerol formation, catalyzed by GPDH, is rate-limiting. Overexpression of *GPD1* significantly enhances glycerol production and reduces ethanol production (Nevoigt and Stahl, 1996; Michnick *et al.*, 1997; Remize *et al.*, 1999). The amount of intracellular glycerol is controlled both by its biosynthetic pathway and by a regulated transmembrane transport system. Glycerol can cross the plasma membrane by passive diffusion or be transported through the MIP protein channel, Fps1p, by facilitated diffusion. Fps1p can transport glycerol in both directions, however, the main physiological role of this glycerol facilitator is to regulate glycerol export rather than its uptake (Luyten *et al.*, 1995; Tamas *et al.*, 1999). Glycerol export is increased in response to hypoosmotic stress and decreased in response to hyperosmotic stress. The opening and closing of the Fps1p channel is responsible for changes in glycerol permeability during osmotic adaptation (Luyten *et al.*, 1995; Sutherland *et al.*, 1997). The long N-terminal

[1] Present address: Laboratoire de Microbiologie, ENSBANA, 1 Esplanade Erasme, Université de Bourgogne, F-21000 Dijon, France.

[2] Present address: Plant Biochemistry, Division of Cell Sciences, University of Southampton, Southampton SO16 7PX, UK.

[3] To whom correspondence and reprint requests should be addressed at UMR Sciences pour l'Œnologie, Microbiologie et Technologie des Fermentations, INRA-IPV, 2 Place Viala, F-34060 Montpellier Cedex 1, France. Fax: 33 4 99 61 28 57. E-mail: dequin@ensam.inra.fr.

[4] Abbreviations used: DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphosphate; GPDH, glycerol-3-phosphate dehydrogenase; MIP, major intrinsic protein; YPD, yeast extract/peptone/dextrose; MS, synthetic medium simulating grape must.

1096-7176/01 $35.00

domain of Fps1p is involved in the regulation of glycerol export: deletion of amino acid residues 150–231 in this domain results in constitutive, unregulated glycerol export (Tamas *et al.*, 1999).

Recent advances on the regulation of glycerol synthesis and transport in *S. cerevisiae* led to the development of metabolic engineering approaches to enhance glycerol production during industrial fermentation processes. As glycerol affects the quality of wines by providing sweetness and fullness (Noble and Bursick, 1984), increasing glycerol production is an important target for improving wine yeasts (Eustace and Thornton, 1987; Pretorius and Westhuizen, 1991; Degré, 1992; Ciani and Ferraro, 1996; Pretorius, 2000). The overproduction of the glycerol-3-phosphate dehydrogenase isoform Gpd1p significantly enhances glycerol production during fermentation (Nevoigt and Stahl, 1996; Michnick *et al.*, 1997; Remize *et al.*, 1999). However, the control exercised on glycerol flux by the glycerol phosphatase step is unknown. Conversely, as the Fps1p facilitator tightly regulates the intracellular accumulation of glycerol, it was possible that wine yeast strains expressing a constitutively "open" transporter compensate for glycerol loss by overproduction.

In this study, strains were constructed in which the genes encoding the glycerol-3-phosphate dehydrogenase and the glycerol 3-phosphatase were overexpressed, independently or simultaneously. Other strains expressing a deregulated glycerol channel alone or in combination with *GPD1* overexpression were also designed. The physiological consequences of glycerol overproduction mediated by these different approaches were studied during fermentation. We found that the overexpression of *GPD1* or *GPD2* enhanced glycerol production and had identical consequences on yeast metabolism, indicating that these isoforms fulfil a similar role. In contrast, overexpression of *GPP1* did not increase glycerol production in the wild-type strain or in *GPD1*-overexpressing strain. In addition, we showed that the expression of a truncated form of the glycerol channel, *FPS1*, enhances glycerol production. However, strains expressing the mutated channel exhibited different kinetics of glycerol overproduction to strains overexpressing *GPD* genes and have altered growth and fermentation rates.

MATERIALS AND METHODS

Strains and Culture Conditions

Escherichia coli DH5α was used for cloning experiments. The *S. cerevisiae* strain used in this study was V5 (ScV5M, *MAT* **a**, *ura3*) derived from a Champagne wine strain. *E. coli* was cultivated as previously described (Sambrook *et al.*, 1989). *S. cerevisiae* was maintained and grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose). Transformants carrying a replicating plasmid were selected and grown on minimal medium (6.7 g/liter yeast nitrogen base without amino acids, 2% glucose) or on YPD medium supplemented with 150 g/ml of geneticin or 100 g/ml of phleomycin.

Batch fermentation experiments were carried out in MS synthetic medium that simulated a standard grape juice as described previously (Michnick *et al.*, 1997). The glucose content was 200 g/liter and nitrogen was in the form of 80 mg/liter ammonium and 120 mg/liter α amino acid nitrogen. The working volume of the fermenters was either 200 ml or 1.1 liter (no differences in the results due to the different cultivation volumes were observed). They were equipped with fermentation locks and CO_2 release was determined by online measurement of fermenter weight loss (Bely *et al.*, 1990). A linear correlation has been established between ethanol and sugar concentrations and the volume of CO_2 released (El Haloui *et al*, 1988). The fermentation rate (r_{CO_2}) was calculated by polynomial smoothing of the last 10 measurements of CO_2 release.

Cells were precultured for 36 h at 28° C in 50-ml flasks without agitation. The fermenters were inoculated by adding 1×10^6 cells per milliliter. Fermentation was carried out at 28° C with permanent stirring (350 rpm).

Fermentation experiments were performed in duplicate or triplicate and one representative experiment is shown.

DNA Manipulation, Cloning Techniques, and Transformation Methods

Restriction and modification enzymes were used according to the manufacturers' instructions. Cloning experiments were carried out by standard techniques (Sambrook *et al.*, 1989). *S. cerevisiae* was transformed by the LiAc procedure (Schiestl and Gietz, 1989).

Plasmid Constructions

The multicopy plasmid pVT100U-ZEO-GPD2 was constructed by cloning *GPD2* into the *Xho*I and *Xba*I restriction sites of pVT100U-ZEO (Michnick *et al.*, 1997). Restriction sites were incorporated into the 5′ and 3′ ends of *GPD2* by PCR using the *Dra*I/*Pst*I *GPD2* fragment cloned in a pUC19 vector (Eriksson *et al.*, 1995) as a template and the oligonucleotides 5′-CGCTCGAGTCC-TAAGAAGATCATTATT-3′ and 5′-GCTCTAGATCA-GAGGGGGAG-3′ corresponding to a region upstream of the ATG start codon (nucleotides −42 to −23) and to a region 40 nucleotides downstream of the stop codon, respectively. The *Hpa*I/*Hpa*I fragment (1.2 kb) containing the *Tn5ble* gene was then eliminated, resulting in pVT100-U-GPD2.

Metabolic Engineering **3**, 301–312 (2001)
doi:10.1006/mben.2001.0197

An 868-bp fragment containing *GPP1* and two flanking *Not*I restriction sites was amplified by PCR and then cloned into pFL61 (Minet *et al.*, 1992) cut with *Not*I, resulting in the vector pFL-GPP1. The fragment was obtained using V5 genomic DNA as a template. The oligonucleotides 5′-CCGCGGCCGCTATACAAACCATCGCAATG-3′ and 5′-CCGCGGCCGCTTGGTCGGAAATGGAGG-3′, complementary to a region immediately upstream the ATG start codon (nucleotides −16 to +3) and to a region 60 nucleotides downstream the stop codon, respectively, were designed to add a *Not*I site to each extremity of the PCR product. The positive selection marker, kanR (Tn903 from *E. coli*), was isolated by digesting pFA6-kan-MX4 (Wach *et al.*, 1994) with *Bam*HI and *Sac*I. The purified fragment was subsequently cloned into the *Bam*HI and *Sac*I sites of pFL61 and pFL-GPP1 to give pFL-kan and pFL-kan-GPP1, respectively.

Multicopy plasmids carrying a 3.8-kb *Sal*I/*Hin*dIII fragment containing *FPS1* (Luyten *et al.*, 1995) or truncated versions of *FPS1* were derived from YEplac195 (Gietz and Sugino, 1988) and kindly provided by M. Tamas (Tamas *et al.*, 1999).

The pFR-GPD1-ZEO vector was obtained by deleting a *Bgl*II/*Bgl*II fragment containing the *URA3* gene from pVT100-U-ZEO-GPD1 (Remize *et al.*, 1999).

Northern Analysis

Total RNA was isolated by use of the Trizol Reagent (GIBCO BRL). Two cells samples (45 DO units) were broken in 400 μl Trizol reagent plus 300 μl of glass beads for 4 min. The two aqueous phases were pooled and the total volume was adjusted to 8 ml with Trizol reagent. Chloroform extraction and RNA precipitation were performed according to the manufacturer's instructions. RNA was separated by electrophoresis as described by Sambrook *et al.* (1989). Blots were hybridized with ^{32}P-labeled *FPS1* probe by the method described by Church and Gilbert (1984). *FPS1* was generated by PCR amplification of V5 total DNA with the oligonucleotides 5′-AACGGTCCGCCGAGTGCAAG-3′ and 5′-AGCGCCTGTGAAGGCACCGA-3′ corresponding to the regions 388 to 407 and 1136 to 1155 of the *FPS1* gene, respectively. An actin probe, obtained using the primers 5′-ATGGATTCTGGTATGTTCTA-3′ and 5′-TTAGAAACACTTGTGGTGAA-3′ corresponding to the regions immediately downstream of the start codon and upstream of the stop codon of the *ACT1* gene, was used as a loading control.

Enzymes Assays

Cell-free extracts were prepared as follows: 10-ml samples were harvested by centrifugation, washed twice with 27 mM imidazole buffer (pH 7) and concentrated in 0.5 ml of 27 mM imidazole buffer (pH 7) containing 2 mM $MgCl_2$ and 1 mM DTT. After adding glass beads (0.5 mm diameter), cells were disrupted by vortexing for 5 min at 0° C. Unbroken cells and debris were removed by centrifugation for 3 min at 16,000*g*. The supernatant was immediately used to assay glycerol 3-phosphatase activity (Norbeck *et al.*, 1996). Inorganic phosphate released by the reaction was measured (Ames, 1966).

Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

One unit (U) is defined as the amount of enzyme catalyzing the conversion of 1 mol of glycerol 3-phosphate/min at 25° C. Specific activity is expressed as mU/mg protein.

Analytical Methods

Glycerol concentration was measured enzymatically using a Boehringer kit. Ethanol was assayed by HPLC as described by Michnick *et al.* (1997). Acetaldehyde concentration was determined enzymatically according to the method of Lundquist (1974).

RESULTS

GPD2 Overexpression Diverts the Carbon Flux toward Glycerol

To compare the effects of *GPD2* overexpression with the effects of overexpressing *GPD1* gene, V5 cells were transformed with pVT100-U (Vernet *et al.*, 1987), pVT100-U-GPD1 (Michnick *et al.*, 1997) or pVT100-U-GPD2. Five independent clones were checked for glycerol production in MS medium (data not shown). One representative transformant each of *GPD1* and *GPD2* was selected and further characterized. Strains overexpressing *GPD1* or *GPD2* produced 24.8 and 24.3 g/liter glycerol, respectively, corresponding to a 3.3-fold increase compared to the control strain (Fig. 1A). As a result of the diversion of carbon flux, the *GPD*-overexpressing strains produced 10–12 g/liter less ethanol than the wild-type strain (Fig. 1B). The kinetics of glycerol and ethanol production during the fermentation were similar for *GPD1*- and *GPD2*-overexpressing strains (data not shown). As the formation of glycerol requires NADH, glycerol overproduction causes a bottleneck in the acetaldehyde reduction step, resulting in a transient accumulation of acetaldehyde (Remize *et al.*, 1999). The acetaldehyde production of

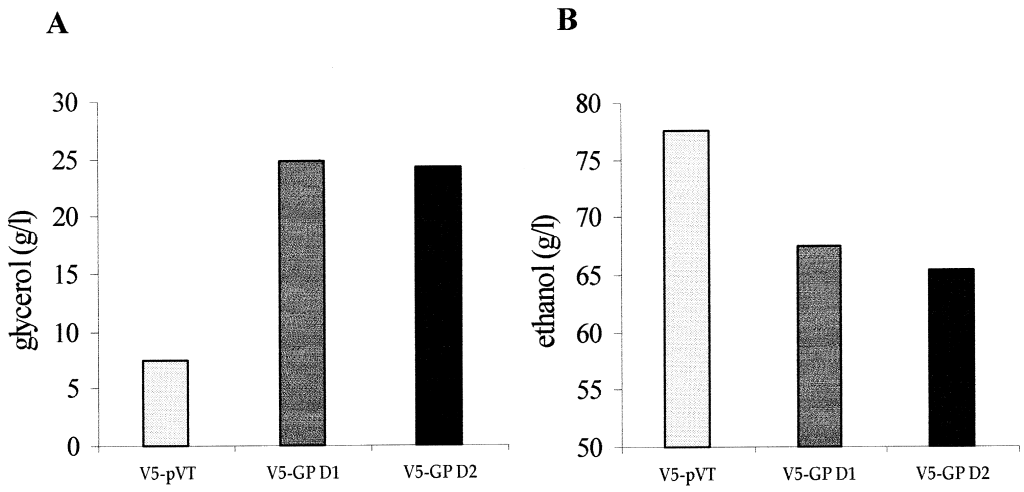

FIG. 1. Effects of *GPD1* and *GPD2* overexpression on glycerol (A) and ethanol (B) production. Fermentation was performed in 1.1-liter fermenters filled with MS medium as described under Materials and Methods. Glycerol concentrations in the medium were determined after complete sugar exhaustion. The experiment was repeated three times with similar results.

GPD1- and *GPD2*-overexpressing strains was markedly increased during the growth phase and the total production was largely above that of the control strain (Fig. 2A). It has been shown that *GPD1*-overexpressing strains exhibit a marked decrease in final biomass, which may be due to a cytotoxic effect of acetaldehyde (Michnick *et al.*, 1997; Remize *et al.*, 1999). A similar decrease (2-fold) in final biomass was observed for *GPD1*- and *GPD2*-overexpressing strains compared to the control strain (Fig. 2B). The maximal fermentation rate was also markedly decreased and the fermentation duration was 60% longer than for the control strain (Fig. 2C).

Glycerol 3-Phosphatase Amplification Does Not Result in Increased Glycerol Production

It is well established that the first glycerol production step, catalyzed by GPDH, is rate-limiting for glycerol production. However glycerol production might also be limited by the amount of glycerol 3-phosphatase. Moreover, this

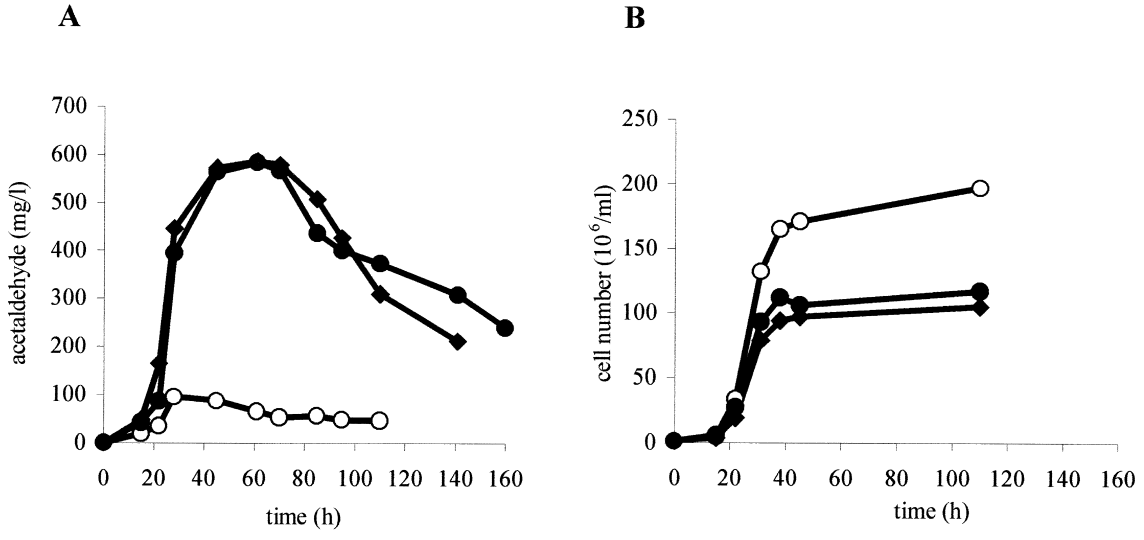

FIG. 2. Effects of *GPD1* and *GPD2* overexpression on acetaldehyde production (A), growth (B), and CO_2 production rate (C). V5-pVT (○), V5-*GPD1* (♦), V5-*GPD2* (●). Cell concentrations were determined by use of a Coulter counter. Fermentation kinetics were monitored by the online measurement of CO_2 release as described under Materials and Methods.

Metabolic Engineering **3**, 301–312 (2001)
doi:10.1006/mben.2001.0197

TABLE 1

Glycerol Production of *S. Cerevisae* Strains Overexpressing *GPP1* or *GPP1* and *GPD1*[a]

Strain	Glycerol production[b] (g/liter)
V5/pFL61	4.8±0.2
V5/pFL-GPP1	4.9±0.3
V5/pVT100U-GPD1-ZEO+pFL-kan	11.7±2.2
V5/pVT100U-GPD1-ZEO+pFL-kan-GPP1	12.0±3.0

[a] Fermentation was performed in 12-ml tubes filled with 10 ml MS medium at 28° C.

[b] Mean and standard deviation for 10 independent transformants.

step may constitute a bottleneck for glycerol production in strains amplified for the GPDH. To examine this and to try to further increase glycerol production in a GPDH-amplified strain, the *GPP1* gene was overexpressed in a wild-type and in a *GPD1*-overexpressing strain. The multicopy plasmids, pFL-GPP1 and pFL-kan-GPP1, which carry *GPP1* under the control of the strong glycolytic *PGK1* promoter and the *URA3* and KanR genes as selection markers, respectively, were constructed.

The glycerol production of ten independent transformants was studied on 10 ml MS medium. No significant difference in glycerol production was observed compared to the control strain, V5/pFL61 (Table 1). To determine whether the phosphatase becomes limiting in a strain amplified for the GPDH, the strain V5 was cotransformed by pVT100U-ZEO-GPD1 (Remize *et al.*, 1999) and pFL-kan or pFL-kan-GPP1. Analysis of ten independent transformants showed that the overexpression of *GPP1* did not increase the glycerol production of strains amplified for GPDH and producing around 12 g/liter of glycerol. The glycerol 3-phosphatase activity, tested for two transformants, was shown to be 7- to 24-fold higher than for the wild-type (Table 2). The same results were obtained by overexpressing *GPP1* in the industrial wine yeast strain, K1, and in K1 overexpressing *GPD1* (data not shown).

TABLE 2

Glycerol-3-phophatase Specific Activity of Control and *GPP1*-Overexpressing Strains during Fermentation on MS Medium[a]

Strain[b]	Glycerol-3-phophatase activity (U/mg)	
	Early stationary phase (30 h)	Stationary phase (48 h)
V5/pFL61	0.8	0.7
V5/pFL-GPP1 A	5.6	11.2
V5/pFL-GPP1 B	6.5	17.2

[a] Fermentation was performed in a 1.1-liter fermenter on MS medium at 28° C with agitation.

[b] A and B are two independent transformants.

Deregulation of Glycerol Efflux Leads to Increased Glycerol Production

FPS1 was constitutively expressed during fermentation on MS medium (Fig. 3). It was recently shown (Tamas *et al.*, 1999) that the N-terminal extension of Fps1p is required for the opening/closing of the Fps1p channel. The sequences required for regulation are located in a segment close to the first transmembrane domain (amino acid residues 150–231). To determine whether the expression of a constitutively "open" glycerol channel forces the cell to increase its glycerol production to counterbalance the lack of intracellular glycerol, five different truncated versions of Fps1p (Tamas *et al.*, 1999) were expressed in the V5 strain carrying a wild-type copy of *FPS1*. A multicopy vector carrying *FPS1* (YEp*FPS1*) was used as a control. For each vector, five independent transformants were tested for glycerol production on MS medium. As predicted, only the strains in which the deletions were outside residues 150–231 of Fps1 and exhibiting an abolished channel regulation significantly increased their glycerol production (Table 3). The glycerol production of the Fps1p-modified strains was 2.3- to 2.7-fold higher than in the control strain, V5/YEp*FPS1*.

Physiological Effects of the Expression of a Deregulated Fps1 Glycerol Channel

To examine the consequences of glycerol overproduction obtained by deregulating the glycerol efflux, fermentation experiments were conducted with V5/YEp*fps1-Δ*1 (V5-*Δ*1) and the control strain V5/YEp*FPS1* (V5-*FPS1*) on MS medium. V5-*Δ*1 produced 16 g/liter of glycerol and V5-*FPS1* produced 7.5 g/liter (Fig. 4A). V5-*Δ*1 exhibited a lag phase of approximately 15 h, grew slowly until 80 h fermentation, and produced 30% less final biomass than the control strain (Fig. 4B). The fermentation kinetics of V5-*Δ*1 were considerably altered, especially during the first phase. Complete sugar degradation was achieved 20 h later than in the control strain (Fig. 4C). The growth rate, population, glycerol production, and fermentation kinetics of V5-*FPS1* were identical to those of the untransformed V5 strain (data not shown).

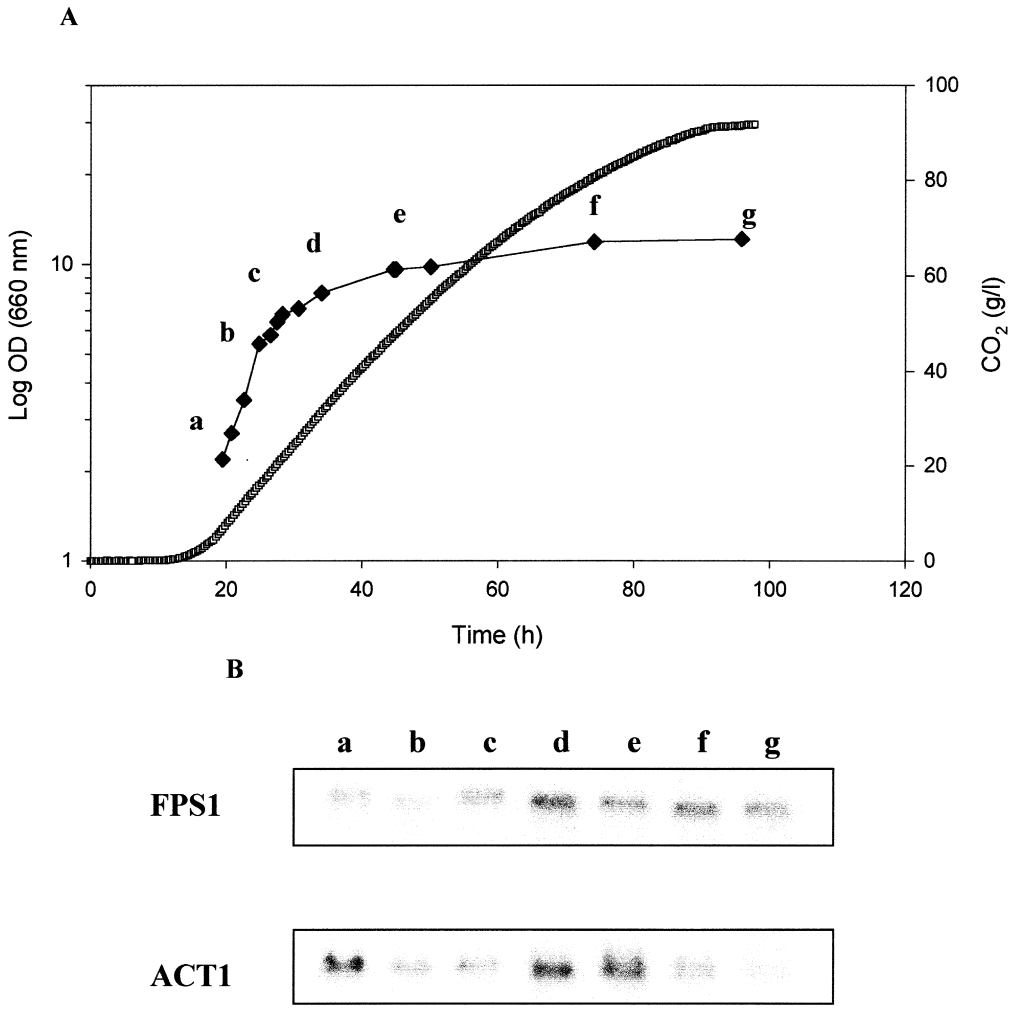

FIG. 3. Fermentation of strain V5 and Northern blot analysis of *FPS1*. (A) Growth (♦) and CO_2 production (□) during fermentation in 1.1-liter fermenters on MS medium. Cells were sampled and RNA was extracted at the indicated times (a to g). (B) Amount of *FPS1* mRNA at the different time points. Actin mRNA was used as internal control.

TABLE 3

Glycerol Production of Strains Expressing Different Truncated Versions of Fps1p during Fermentation on MS Medium[a]

Strain	Glycerol production[b] (g/liter)
V5/YEp*FPSI*	5.8±1.0
V5/YEp*fps1-Δ*1 (aa 12–231)	14.6±1.7
V5/YEp*fps1-Δ*2 (aa 12–70)	4.8±0.8
V5/YEp*fps1-Δ*3 (aa 75–231)	15.8±1.4
V5/YEp*fps1-Δ*4 (aa 12–145)	4.6±0.4
V5/YEp*fps1-Δ*5 (aa 150–231)	13.2±2.1

[a] Fermentation was performed in 12 ml tubes filled with 10 ml MS medium at 28° C.

[b] Mean and standard deviation for five independent transformants.

Overexpression of GPD1 in a Strain Expressing a Deregulated Fps1 Channel Results in an Even Higher Increase in Glycerol Production

GPD1 was overexpressed by use of the pFR-*GPD1*-ZEO vector containing Tn5*ble* gene conferring resistance to phleomycin as a single marker, while YEp*fps1-Δ*1 was maintained by uracil auxotrophy selection. The V5 strain was successively transformed by pFR-*GPD1*-ZEO and by either YEp*FPS1* or YEp*fps1-Δ*1, resulting in V5-*GPD1-FPS1* and V5-*GPD1-Δ*1, respectively.

Overexpression of *GPD1* in V5-*Δ*1 resulted in a higher glycerol production (+7.1 g/liter) compared to V5-*Δ*1 (Fig. 5A). However, when *GPD1* is overexpressed the growth rate and final population decreased (Fig. 5B). The maximal

Metabolic Engineering **3**, 301–312 (2001)
doi:10.1006/mben.2001.0197

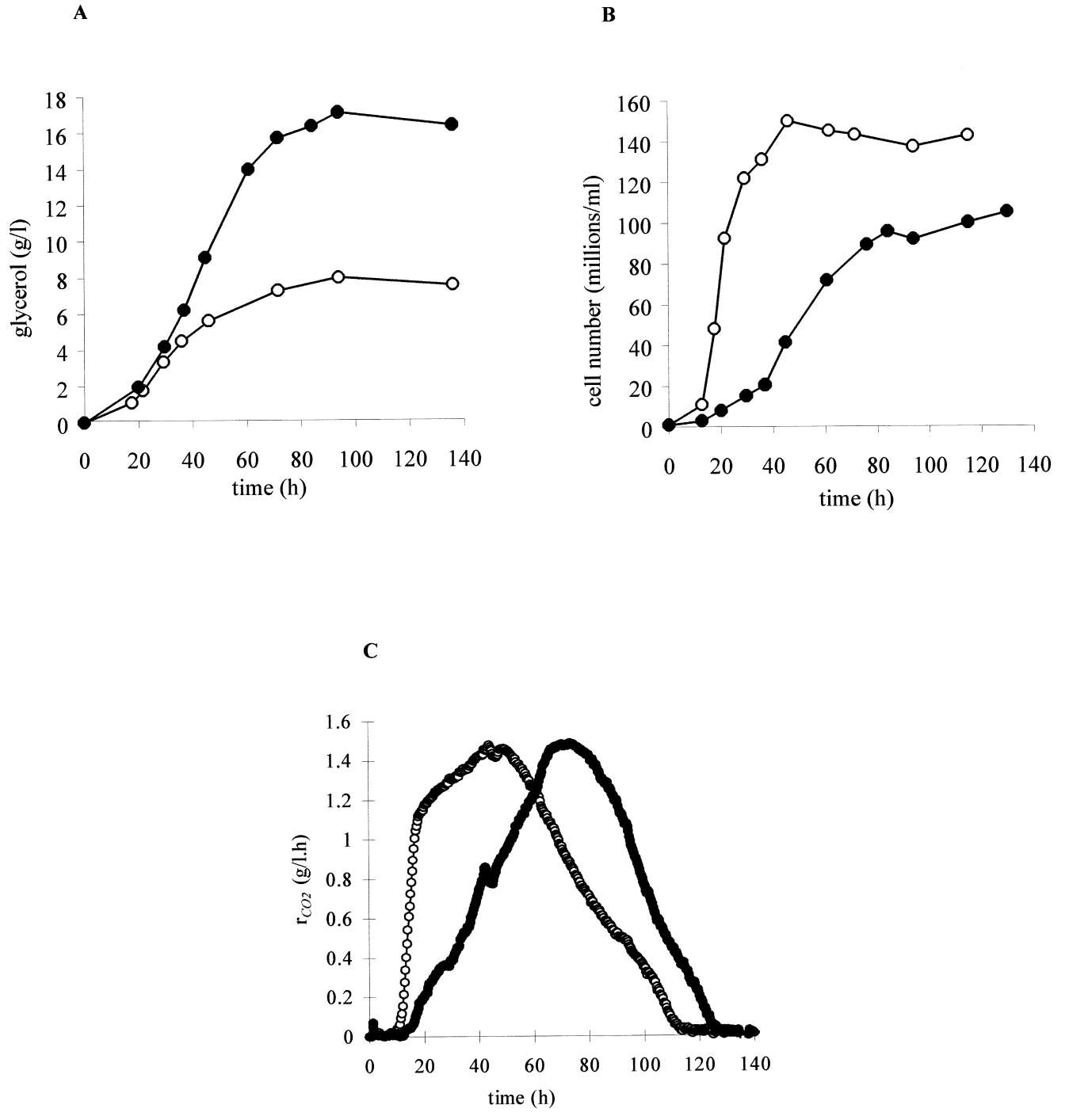

FIG. 4. Effects of the expression of truncated forms of Fps1p on glycerol production (A), growth (B), and CO_2 production rate (C) during fermentation. V5-*FPS1*, control strain (○), V5-*Δ*1 strain (●). Fermentations were performed in 1.1-liter fermenters filled with MS medium. Growth and fermentation rates were determined as described in the legend to Fig. 2.

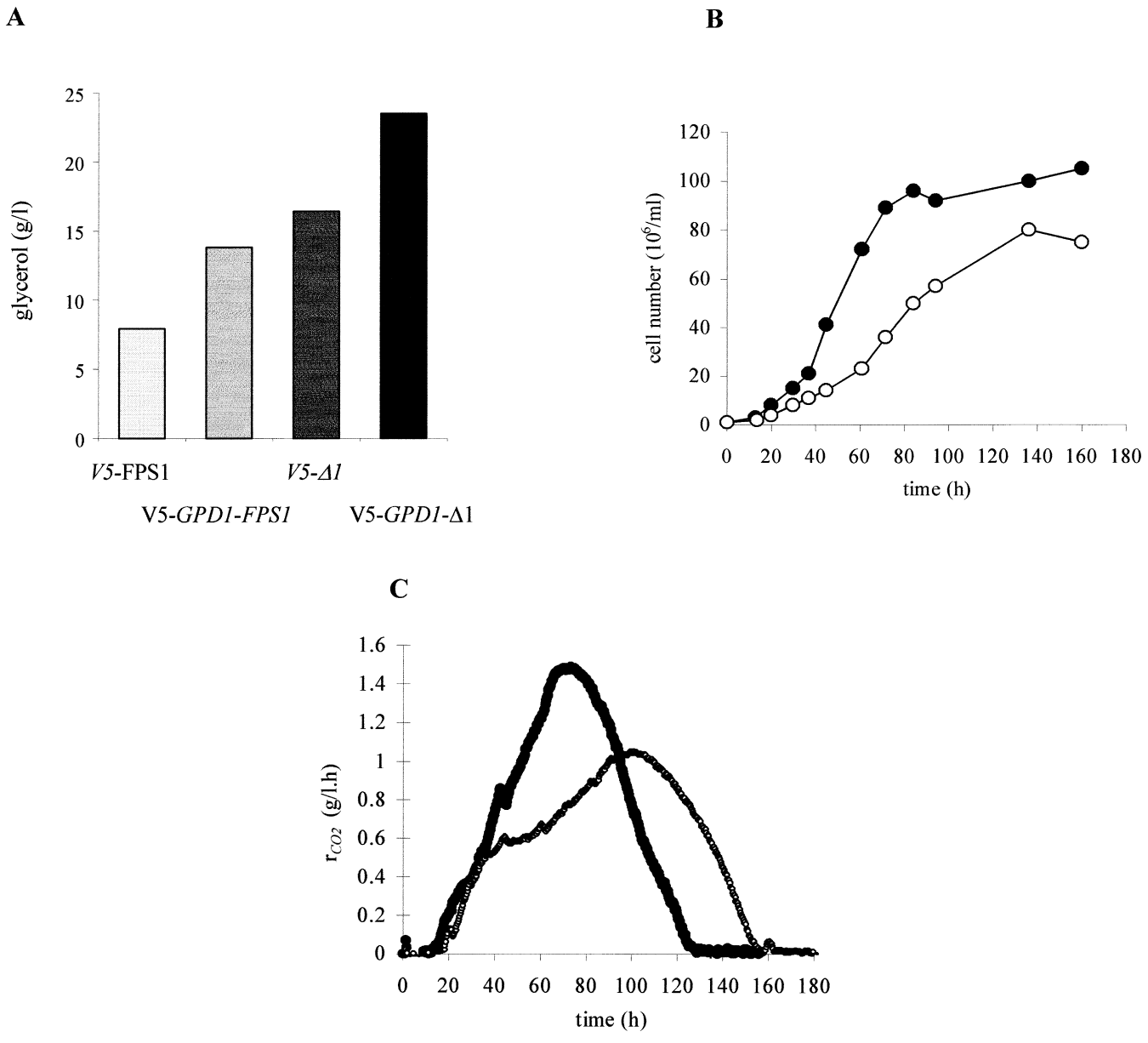

FIG. 5. Effects of expressing the truncated forms of Fps1p on glycerol production (A), growth (B), and CO_2 production rate (C) during fermentation. V5-*Δ1* (●) V5-*GPD1-Δ1* (○). The fermentation conditions were as described in the legend to Fig. 4.

fermentation rate was lower and delayed in the strain overexpressing *GPD1* and the duration of fermentation was increased (Fig. 5C).

DISCUSSION

GPD1 and GPD2 Overexpressions Have Similar Physiological Effects

The amino acid sequences of Gpd1p and Gpd2p share 74% identity and 87% similarity and both proteins are located in the cytosol (Larsson *et al.*, 1993; Albertyn *et al.*, 1994). The enzymatic properties of Gpd1 have been widely studied (Gancedo *et al.*, 1968; Albertyn *et al.*, 1992; Cai *et al.*, 1996; Michnick *et al.*, 1997); however, less is known about Gpd2p. These two isoforms have similar affinity constants for NADH and ATP (Albertyn *et al.*, 1992; Ansell *et al.*, 1997; Michnick *et al.*, 1997). Gpd1 is known to be inhibited by NAD, ATP, ADP, and FBP with inhibitory constants in the range of the physiological concentration of these metabolites (Gancedo *et al.*, 1968; Albertyn *et al.*, 1992; Michnick, 1995). In this study we showed that the

Metabolic Engineering **3**, 301–312 (2001)
doi:10.1006/mben.2001.0197

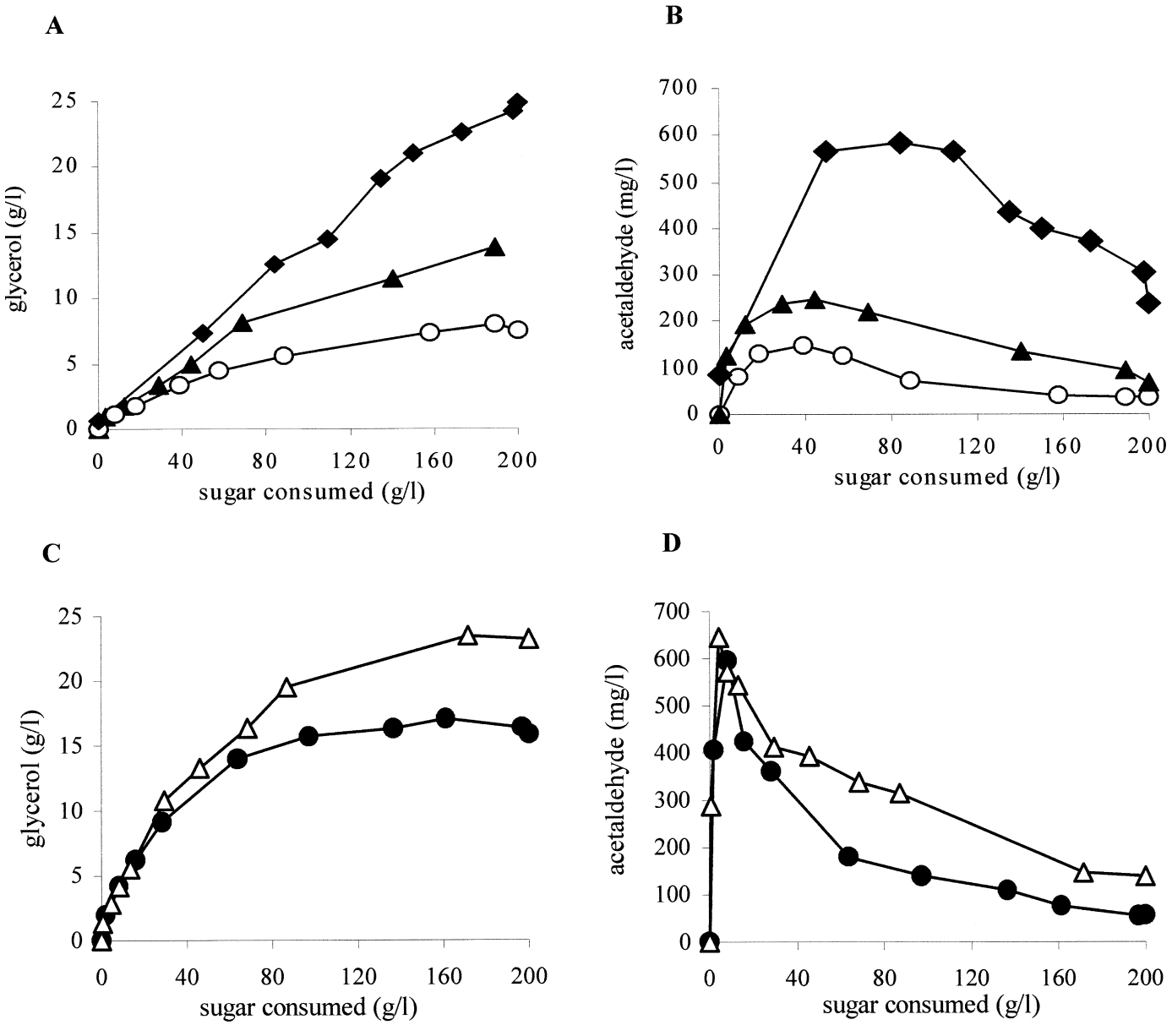

FIG. 6. Comparison of the kinetics of glycerol (A, C) and acetaldehyde (B, D) production mediated by *GPD1*-overexpressing strains (A, B) and by strains expressing the truncated Fps1p (C, D) during fermentation on MS 300. V5-*FPS1*, control strain (○), V5-*GPD1-FPS1* (▲), V5-*Δ*1 (●), V5-*GPD1* (♦), V5-*GPD1-Δ*1 (△). The fermentation conditions were as described in the legend to Fig. 4.

overexpression of *GPD1* and *GPD2,* under the control of the same promoter, leads to the same increase in glycerol production and thus has the same effects on the formation of other fermentation by-products. In our fermentation conditions the two proteins were functionally quivalent. This finding is consistent with previous studies (Ansell *et al.*, 1997; Bjorkqvist *et al.*, 1997), which showed that during osmotic stress the growth of *gpd1Δ* mutants is restored by overexpressing *GPD2*, and inversely that a *gpd2Δ* mutant has a normal anaerobic growth when *GPD1* is overexpressed.

Glycerol 3-Phosphatase Is Not Limiting for Glycerol Production

When Gpd1p is overproduced more than 10-fold by overexpression of the corresponding gene (Michnick *et al.*,

1997) or in response to osmotic stress (Blomberg and Adler, 1989; André *et al.*, 1991), glycerol production only increases three- to fourfold. This may be due to *in vivo* regulation of GPDH or to a metabolic bottleneck at the level of the second enzymatic step catalyzed by glycerol 3-phosphatase. We showed that glycerol 3-phosphatase is not rate-limiting for glycerol production. It was very recently reported that a laboratory strain overexpressing *GPP1* under its own promoter did not produce higher glycerol amount in minimal medium (Pahlman *et al.*, 2000). In addition, we showed that this second step is not rate limiting even though *GPD1* is overexpressed.

Expression of a Constitutively Open Glycerol Channel Deregulates Glycerol Production

We showed that the expression of a mutated form of the glycerol channel, Fps1p, leads to a constitutive glycerol efflux which triggers a 2- to 2.5-fold increase in final glycerol concentration. No difference in the intracellular level of glycerol could be detected between the mutant and the control strains during the first 60 h of fermentation (data not shown). We believe that the cell maintains its intracellular glycerol level by increasing glycerol production. Indeed, Tamas *et al.* (1999) showed that strains carrying a truncated Fps1-*Δ*1 protein accumulated as much intracellular glycerol as the wild-type after 24 h of incubation on a hypersaline medium. This is due to an increased glycerol production rate while a high glycerol leakage was maintained.

The changes in growth of V5-*Δ*1 were more pronounced than we expected based on the increase in the final level of glycerol per se. Indeed, the 3.3-fold increase in glycerol production obtained by overexpressing *GPD* gene altered the growth rate less than when glycerol production was increased 2.5-fold by expressing the Fps1-*Δ*1 protein (Figs. 2B and 4B). When glycerol and acetaldehyde levels are expressed as a function of glucose consumption (Fig. 6), V5-*GPD1* and V5-*Δ*1 exhibit completely different profiles. In strains carrying the truncated Fps1p, the increase in glycerol production starts suddenly and very early, whereas it is more progressive in the case of V5-*GPD1* (Figs. 6A and 6C). A progressive increase, similar to that seen for the control strain, V5-*FPS1*, was observed in strains overexpressing *GPD1* even at higher levels (24.8 g/liter). As a consequence of NADH limitation due to glycerol overproduction, acetaldehyde level was extremely high at the beginning of fermentation for the strain V5-*Δ*1 (Figs. 6B and 6D). The toxic effect of acetaldehyde is exerted at multiple cellular levels (Jones and Greenfield, 1986; Stanley *et al.*, 1993, 1997). High concentrations of acetaldehyde at the beginning of the fermentation process may explain the lag phase and poor growth observed for V5-*Δ*1. A possible explanation for the much-pronounced growth and fermentation defects of V5-*GPD1-Δ*1 is that the acetaldehyde concentration in the medium slowly decreased over time (Figs. 5 and 6D). Thus, the control of intracellular glycerol is of crucial importance for growth during wine fermentation and the regulation of glycerol transport via the Fps1 channel plays a decisive role. Furthermore, the observation that the kinetics glycerol overproduction are very different in strains expressing truncated Fps1 versions and in strains overexpressing *GPD1* or *GPD2* suggests novel regulatory connections.

Industrial strains engineered to produce higher glycerol levels (12 to 18 g/liter) could significantly improve the quality of some wines. This study demonstrated that this can be achieved by engineering Fps1p, however, it also showed that the utilization of the corresponding engineered strain is unrealistic with regard to industrial requirements for fermentation performance. Thus, overexpressing either *GPD1* or *GPD2* looks highly promising. As previously shown, the growth of industrial strains overexpressing *GPD1* and overproducing glycerol in the range of 12–18 g/liter was almost unaffected and these strains even exhibited improved fermentation performance during alcoholic fermentation (Remize *et al.*, 1999). However, the side effects of the metabolites on production remain to be determined.

ACKNOWLEDGMENTS

This work was supported by the European Community in the framework of the Biotechnology–Cell Factory research program "Yeast Glycerol Metabolism BIO4-CT95-0161." We are grateful to Markus Tamas (KU Leuven) and Stefan Hohmann (Göteborg University) for providing the plasmids carrying *FPS1* constructs. We thank Brigitte Cambon for technical assistance in Northern experiments.

REFERENCES

Akhtar, N., Blomberg, A., and Adler, L. (1997). Osmoregulation and protein expression in a *pbs2Δ* mutant of *Saccharomyces cerevisiae* during adaptation to hypersaline stress. *FEBS Lett.* **403**, 173–180.

Albertyn, J., Hohmann, S., Thevelein, J. M., and Prior, B. A. (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.

Albertyn, J., Tonder, A. v., and Prior, B.-A. (1992). Purification and characterization of glycerol-3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. *FEBS Lett.* **308**, 130–132.

Ames, B. (1966). Assay of inorganic phosphate, total phosphate and phosphatase. *Methods Enzymol.* **8**, 115–118.

André, L., Hemming, A., and Adler, L. (1991). Osmoregulation in *Saccharomyces cerevisiae*. Studies on the osmotic induction of glycerol

Metabolic Engineering **3**, 301–312 (2001)
doi:10.1006/mben.2001.0197

production and glycerol-3-phosphate dehydrogenase (NAD+). *FEBS Lett*. **286**, 13–17.

Ansell, R., Granath, K., Homann, S., Thevelein, J.-M., and Adler, L. (1997). The two isoenzymes for yeast NAD+-dependent glycerol-3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. *EMBO J*. **16**, 2179–2187.

Bely, M., Sablayrolles, J.-M., and Barre, P. (1990). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *J. Ferment. Bioeng*. **70**, 246–252.

Bjorkqvist, S., Ansell, R., Adler, L., and Liden, C. (1997). Physiological response to anaerobicity of glycerol-3-phosphate dehydrogenase mutant of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol*. **63**, 128–132.

Blomberg, A., and Adler, L. (1989). Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD+) in acquired osmotolerance of *Saccharomyces cerevisiae*. *J. Bacteriol*. **171**, 1087–1092.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem* **72**, 248.

Cai, J., Piezsch, M., Theobald, U., and Rizzi, M. (1996). Fast purification and kinetic studies of the glycerol-3-phosphate dehydrogenase from the yeast *Saccharomyces cerevisiae*. *J. Biotechnol*. **49**, 19–27.

Church, G., and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.

Ciani, M., and Ferraro, L. (1996). Enhanced glycerol content in wines made with immobilized *Candida stellata* cells. *Appl. Environ. Microbiol*. **62**, 128–132.

Degré, R. (1992). *In* "Wine Microbiology and Biotechnology" (G. H. Fleet, Ed.), pp. 421–449. Harwood Academic Publishers, Chur, Switzerland.

El Haloui, N., Picque D., and Corrieu, G. (1988). Alcoholic fermentation in winemaking: On line measurement of density and carbon dioxide evolution. *J. Food Eng*. **8**, 17–30.

Eriksson, P., André, L., Ansell, R., Blomberg, A., and Adler, L. (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.

Eustace, R., and Thornton, R. J. (1987). Selective hybridization of wine yeasts for higher yields of glycerol. *Can. J. Microbiol*. **33**, 112–117.

Gancedo, C., Gancedo, J. M., and Sols, A. (1968). Glycerol metabolism in yeast. Pathways of utilisation and production. *Eur. J. Biochem*. **5**, 165–172.

Gietz, R. D., and Sugino, A. (1988). New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527–534.

Jones, R. P., and Greenfield, P. F. (1986). Role of water activity in ethanol fermentations. *Biotechnol. Bioeng*. **28**, 29–40.

Larsson, K., Ansell, R., Eriksson, P., and Adler, L. (1993). A gene encoding sn-glycerol-3-phosphate dehydrogenase (NAD+) complements an osmosensitive mutant of *Saccharomyces cerevisiae*. *Mol. Microbiol*. **10**, 1101–1111.

Lundquist, F. (1974). *In* "Methods of Enzymatic Analysis" (H. U. Bergmeyer, Ed.), pp. 1509–1513. Academic Press, New York and London.

Luyten, K., Albertyn, J., Skibbe, W. F., Prior, B. A., Ramos, J., Thevelein, J. M., and Hohmann, S. (1995). Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and it is inactive under osmotic stress. *EMBO J*. **14**, 1360–1371.

Michnick, S. (1995). "Etude de la Glycérol-3-phosphate Déshydrogénase en Relation avec la Production de Glycérol en Fermentation Oenologique chez *Saccharomyces cerevisiae*. Conséquences de la Surexpression et de la Disruption du Gène *GPD1*." Ph.D. thesis, Univ. Montpellier II, France.

Michnick, S., Roustan, J.-L., Remize, F., Barre, P., and Dequin, S. (1997). Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for *GPD1* encoding glycerol-3-phosphate dehydrogenase. *Yeast* **13**, 783–793.

Minet, M., Dufour, M. E., and Lacroute, F. (1992). Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. *Plant J*. **2**, 417–422.

Nevoigt, E., and Stahl, U. (1996). Reduced pyruvate decarboxylase and increased glycerol-3-phosphate dehydrogenase (NAD+) levels enhance glycerol production in *Saccharomyces cerevisiae*. *Yeast* **12**, 1331–1337.

Noble, A. C., and Bursick, G. F. (1984). The contribution of glycerol to perceived viscosity and sweetness in white wine. *Am. J. Enol. Vitic*. **35**, 110–112.

Norbeck, J., and Blomberg, A. (1997). Metabolic and regulatory changes associated with growth of *Saccharomyces cerevisiae* in 1.4 M NaCl. *J. Biol. Chem*. **272**, 5544–5554.

Norbeck, J., Pahlman, A. K., Akhtar, N., Blomberg, A., and Adler, L. (1996). Purification and characterisation of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem*. **271**, 13875–13881.

Pahlman, A. K., Granath, K., Ansell, R., Hohmann, S., and Adler, L. (2000). The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic and oxidative stress. *J. Biol. Chem*. **31**, in press.

Pretorius, I. (2000). Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. *Yeast* **16**, 675–729.

Pretorius, I. S., and Westhuizen, T. J. (1991). The impact of yeast genetics and recombinant DNA technology on the wine industry—A review. *S. Afr. J. Enol. Vitic*. **12**, 3–31.

Remize, F., Roustan, J., Sablayrolles, J., Barre, P., and Dequin, S. (1999). Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. *Appl. Environ. Microbiol*. **65**, 143–149.

Rep, M., Krantz, M., Thevelein, J., and Hohmann, S. (2000). The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J. Biol. Chem*. **275**, 8290–8300.

Sambrook, J., Frisch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, New York.

Schiestl, R. H., and Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet*. **16**, 339–346.

Stanley, G. A., Douglas, N. G., Every, E. J., Tzanatos, T., and Pamment, N. B. (1993). Inhibition and stimulation of yeast growth by acetaldehyde. *Biotechnol. Lett*. **15**, 1199–1204.

Stanley, G. A., Hobley, T. J., and Pamment, N. B. (1997). Effect of acetaldehyde on *Saccharomyces cerevisiae* and *Zymomonas mobilis* subjected to environmental shocks. *Biotechnol. Bioeng*. **53**, 71–78.

Sutherland, F. C. W., Lages, F., Lucas, C., Luyten, K., Albertyn, J., Hohmann, S., Prior, B. A., and Kilian, S. G. (1997). Characteristics of Fps1-dependent and -independent glycerol transport in *Saccharomyces cerevisiae*. *J. Bacteriol*. **179**, 7790–7795.

Tamas, M., Luyten, K., Sutherland, F., Hernandez, A., Albertyn, J.,

Valadi, H., Li, H., Prior, B., Kilian, S., Ramos, J., Gustafsson, L., Thevelein, J., and Hohmann, S. (1999). Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* **31**, 1087–1094.

Vernet, T., Dignard, D., and Thomas, D. Y. (1987). A family of yeast expression vectors containing the phage f1 intergenic region. *Gene* **52**, 225–233.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**, 1793–1808.