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Engineering of *Saccharomyces cerevisiae* for the production of L-glycerol 3-phosphate

H.T.T. Nguyen,[a,b] A. Dieterich,[a] K. Athenstaedt,[c] N.H. Truong,[b] U. Stahl,[a] and E. Nevoigt[a,*]

[a] *Institut für Biotechnologie, Fachgebiet Mikrobiologie und Genetik, Technische Universität Berlin, Seestrasse 13, D-13353 Berlin, Germany*
[b] *Institute of Biotechnology, Vietnam National Centre for Natural Science and Technology, Hoang Quoc Viet-Cau Giay, Hanoi, Viet Nam*
[c] *Institut für Biochemie, Technische Universität Graz, Petersgasse 12/2, A-8010 Graz, Austria*

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Abstract

L-glycerol 3-phosphate (L-G3P) was accumulated in *Saccharomyces cerevisiae* by pathway engineering. Intracellular concentration of this metabolic intermediate could be increased more than 20 times compared to the wild type by overexpressing *GPD1* encoding the glycerol 3-phosphate dehydrogenase in a *gpp1*Δ*gpp2*Δ mutant which lacks both isoenzymes of glycerol 3-phosphatase. Investigation of cellular pattern of triacylglycerols and glycerophospholipids did not reveal considerable changes due to accumulation of their precursor L-G3P. Hyperosmotic stress did not affect the L-G3P pool in the *gpp1*Δ*gpp2*Δ mutant overexpressing *GPD1* despite an about 4-fold increase of specific GPD activity. In contrast, oxygen limitation improved intracellular L-G3P concentration by enhancing the availability of cytosolic NADH. The reduction of pyruvate decarboxylase activity by deleting *PDC2* led to an additional increase. In fact, the triple mutant *gpp1*Δ*gpp2*Δ *pdc2*Δ overexpressing *GPD1* accumulated 17 mg L-G3P/g dry weight during glucose batch fermentation under oxygen limitation. This value corresponds to an about 100-fold increase compared to that found in the wild type.

Keywords: Yeast; *Saccharomyces cerevisiae*; Metabolic engineering; L-glycerol 3-phosphate

1. Introduction

The L-enantiomer of glycerol 3-phosphate (L-G3P) is a promising starting material for the enzymatic synthesis of monosaccharides and glycerophospholipids, substances which are of growing interest for the development of novel pharmaceuticals used, e.g., for the treatment of cancer and infections (for reviews see Koeller and Wong, 2000; Dove, 2001; Unger and Eibl, 2001). Using L-G3P, monosaccharides can be enzymatically synthezised via dihydroxyacetone phosphate (DHAP) taking advantage of the stereospecific aldol condensation performed by aldolases (for reviews see Takayama et al., 1997; Koeller and Wong, 2001). In addition, L-G3P is a precursor for the biosynthesis of phosphatidic acid, the key intermediate for the formation of all glycerolipids. Thus, this biocatalytic approach could, in the future, be an alternative to the chemical synthesis of glycerophospholipids which starts from the toxic compound phosphorus oxychloride as reported by Unger and Eibl (2001).

L-G3P is currently produced by several methods which are, however, laborious, expensive or result in the formation of racemic mixtures. The pure L-enantiomer can be generated by chemical synthesis starting from D-acetone glycerol. However, this process requires many steps for protection and deprotection of hydroxyl groups (Baer and Fischer, 1939). In addition, a simple chemical phosphorylation of glycerol using polyphosphoric acid is possible but leads to complex mixtures of G2P, D- and L-G3P (Cherbuliez and Weniger, 1946). Glycerol can also be enzymatically phoshorylated by glycerol kinase which results in the desired L-enantiomer of G3P (Crans and Whitesides, 1985). However, this method requires an ATP-regenerating system. The two most common systems based on acetyl phosphate/acetate kinase and

*Corresponding author.
E-mail address: e.nevoigt@lb.tu-berlin.de (E. Nevoigt).

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phospoenolpyruvate/pyruvate kinase show disadvantages which relate to the stability, expense, or requisite chemical synthesis of the high-energy phosphoryl donors as well as product inhibition in the case of pyruvate kinase (Resnick and Zehnder, 2000). Alternatively, enzymatic phosphorylation of glycerol can be catalyzed by phosphatase, which uses pyrophosphate as a co-substrate (Pradines et al., 1988; Schoevaart et al., 2000). However, these procedures lead to racemic mixtures of D- and L-G3P.

This work aims at gaining access to L-G3P via using an engineered yeast strain. This approach entails a few obvious advantages, e.g., the specific generation of the desired L-enantiomer of G3P and the use of cheap and environmentally sustainable raw materials such as molasses.

L-G3P in *Saccharomyces cerevisiae* is formed from sugars by the reduction of the glycolytic intermediate DHAP concomitant with NADH oxidation via cytosolic glycerol 3-phosphate dehydrogenase (GPD) (E.C.1.1.1.8), an enzyme encoded by two isogenes, i.e., *GPD1* and *GPD2* (Albertyn et al., 1992). The subsequent dephosphorylation of L-G3P leads to the formation of glycerol. This reaction is catalyzed through the activity of glycerol 3-phosphatase (GPP) (E.C. 3.1.3.21), which is also encoded by two isogenes referred to as *GPP1* and *GPP2* (Norbeck et al., 1996) (Fig. 1). Moreover, the intermediate L-G3P serves as a precursor for the biosynthesis of triacylglycerols (TAG) and glycerophospholipids (Fig. 1). The first step towards

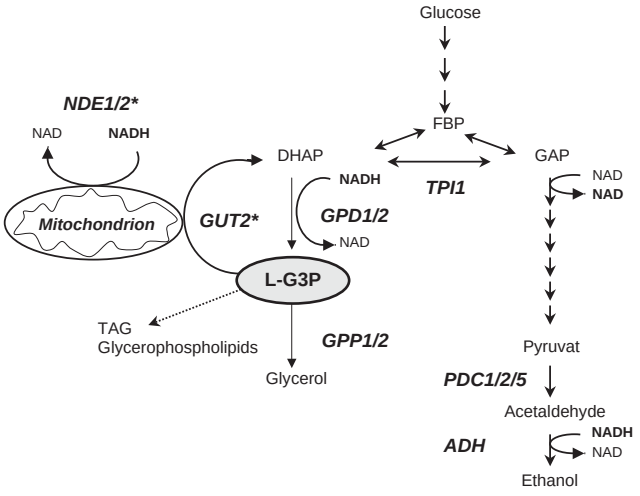

Fig. 1. Scheme of metabolic pathways and enzymes of *S. cerevisae* which are relevant to this study. Abbreviations: L-G3P, L-glycerol 3-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; FBP, fructose 1,6-bisphosphate; TAG, triacylglycerols; *TPI1*, triosephosphate isomerase; *GPP1/2*, cytosolic glycerol 3-phosphatase; *GPD1/2*, glycerol 3-phosphate dehydrogenase; *GUT2*, mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase; *PDC1/2/5*, pyruvate decarboxylase; *NDE1/2*, external mitochondrial NADH dehydrogenase; *ADH1*, alcohol dehydrogenase. *These enzymes transmit reducing equivalents to the mitochondrial respiratory chain, hence only being relevant in the presence of oxygen.

formation of these lipids is catalyzed by L-G3P acyltransferase (Kent, 1995).

In order to create a *S. cerevisiae* strain which overproduces L-G3P, it is important to hamper further metabolization of this intermediate as well as to increase its biosynthesis. On the one hand, dephosphorylation of L-G3P to glycerol can be completely blocked by coincident deletion of *GPP1* and *GPP2* (Påhlman et al., 2001). On the other hand, there are many successful strategies to redirect yeasts' central carbon metabolism towards glycerol for which L-G3P serves as the precursor. First advances in this context have been reported by Neuberg and Reinfurth, (1918). Cells of *S. cerevisiae* were forced to overproduce glycerol by the addition of sulfite, which traps acetaldehyde, the most important electron acceptor for the reoxidation of NADH formed during glycolysis (Fig. 1). Later, recombinant DNA technology was alternatively used to re-route carbon flux. These approaches have focused on minimizing NADH consumption in the ethanol production pathway simulating the classical sulfite process, i.e., by reducing the specific activities of key enzymes such as alcohol dehydrogenase (ADH) (Drewke et al., 1990) and pyruvate decarboxylase (PDC) (Nevoigt and Stahl, 1996). Moreover, the isomerization of DHAP to glycerolaldehyde 3-phosphate can be inhibited by deleting the *TPI1* gene encoding triosephosphate isomerase (Fig. 1) which leads to a considerable elevation of the glycerol yield (Compagno et al., 1996, 1998, 2001). Another genetic approach to accelerate glycerol formation was to overproduce GPD, the key enzyme of the glycerol biosynthetic pathway (Nevoigt and Stahl, 1996; Michnick et al., 1997; Remize et al., 2001; Nevoigt et al., 2002).

In this study we combined overexpression of *GPD1* with the deletion of *GPP1* either alone or together with the deletion of *GPP2* in order to accumulate the metabolic intermediate L-G3P in *S. cerevisiae*. Furthermore, a triple mutant *gpp1*Δ*gpp2*Δ *pdc2*Δ overexpressing *GPD1* was constructed to study the effect of *PDC2* deletion on the L-G3P pool.

2. Materials and methods

2.1. Strains

The yeast strains used in this study are listed in Table 1. *Escherichia coli* DH-5α was used for amplification of plasmids.

2.2. Medium and culture conditions

Wild type (W303-1A), *gpp1*Δ (YA101) and *gpp1*Δ*gpp2*Δ (YA103) mutant strain of *S. cerevisiae*

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Source or reference
W303-1A[a]	*MATa*	Thomas and Rothstein (1989)
YA101[a]	*MATα gpp1*Δ::kanMX4	Påhlman et al. (2001)
YA103[a]	*MATa gpp1*Δ::kanMX4 *gpp2*Δ::*HIS3*	Påhlman et al. (2001)
YHN1[a]	*MATα gpp1*Δ::kanMX4 *pdc2*Δ::*TRP1*	This study
YHN2[a]	*MATα gpp1*Δ::kanMX4 *gpp2*Δ::*HIS3 pdc2*Δ::*TRP1*	This study
YSH306	*MATa leu2–3/112 trp1–92 ura3–52 pdc2*Δ::*TRP1 SUC GAL mal*	Hohmann et al. (1993)

[a] These strains harbor additional mutations as follows: *leu2–3/112 ura3–1 trp1-1 his3–11/15 ade2–1 can1–100 GAL SUC2 mal0*.

were grown in Erlenmeyer flasks at 30°C on a rotary shaker at 200 rpm in YEPD medium (2% peptone, 1% yeast extract, 2% glucose). The same strains containing the plasmids YEpKm^R *GPD1* or YEpKm^R were grown in synthetic yeast nitrogen base (YNB) medium (Difco, USA) supplemented with 2% glucose as well as histidine, adenine, tryptophane and uracil (final concentration of 120 μg/ml each). This medium is referred to as YNB (Glc/leu$^-$) medium. Strains deleted in *PDC2* grow very slowly on glucose and were therefore precultured in YEPEG medium (2% peptone, 1% yeast extract, 2% (v/v) glycerol, 2% (v/v) ethanol). The YEPEG medium was supplemented with 200 μg/ml Geneticin G418 when *pdc2*Δ deletion strains containing one of the plasmids mentioned above were pre-cultured. In order to initiate batch fermentation, cells were precultured as specified, harvested, centrifuged and then transferred to fresh glucose medium.

Cell density was estimated by measuring the optical density at 600 nm in 1 cm cuvettes (OD_{600}) using a Shimadzu photometer (UV-160A, Shimadzu Europa GMBH).

E. coli was cultivated in Luria–Bertani medium (LB) at 37°C as previously described (Sambrook et al., 1989).

2.3. Recombinant DNA techniques

Standard techniques were used for transformation of *E. coli*, isolation of plasmid DNA and PCR (Sambrook et al., 1989). Yeast chromosomal DNA was isolated according to the protocol of Hoffman and Winston (1987). Mating and sporulation of *S. cerevisiae* were carried out using standard protocols (Sprague, 1991; Kassir and Simchen, 1991). Isolation of spores was performed according to Adams et al. (1997).

The insertion of a *PDC2* deletion into the *gpp1*Δ single mutant (YA101) was achieved by the one-step gene disruption method (Rothstein, 1983). A set of primers (5′-CGAACGTCCTCTGTCCCACTAT-3′ and 5′-AGCCTGTGTTACCAGGTAAG-3′) was used to amplify a *pdc2*Δ::*TRP1* disruption cassette from the genomic DNA of an existing *pdc2*Δ mutant YSH306 (Hohmann, 1993), kindly provided by the author. The PCR product of 2.4 kb in length was introduced into the strain *gpp1*Δ (YA101) by electroporation (see below). Transformants carrying the disruption cassette were selected on YNB agar medium supplemented with 2% ethanol and 2% glycerol as well as amino acids and nucleic bases as required but lacking tryptophan. The deletion of *PDC2* was confirmed by diagnostic PCR using the primers (i) 5′-CTGGAGTCCAATGATGCAGCC-3′ and (ii) 5′-CAGGATAGATCAAAGAGCAGTC-3′.

To obtain the triple mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ (YNH2), we crossed the strains *gpp1*Δ *pdc2*Δ (YNH1) and *gpp1*Δ *gpp2*Δ (YA103) on a YEPEG agar plate. The resulting diploid strain (*gpp1*Δ/*gpp1*Δ*GPP2*/*gpp2*Δ*pdc2*Δ/*PDC2*) was sporulated on the standard sporulation medium (Sherman, 1991), replacing the glucose by 0.05% (v/v) ethanol and 0.05% (v/v) glycerol. Triple mutants *gpp1*Δ *gpp2*Δ *pdc2*Δ were selected by spreading the spores on YNB agar medium supplemented with 2% ethanol, 2% glycerol, amino acids and nucleic bases as required but lacking histidin and tryptophan. The haploid status of the selected mutants *gpp1*Δ *gpp2*Δ *pdc2*Δ was confirmed by checking their mating type as described by Sprague (1991). Deletions of *GPP2* and *PDC2* in the selected triple mutant were verified by two diagnostic PCRs using the following sets of primers: (i) 5′-ACACAGCCCAATCCTGTGAT-3′ and 5′-AGATTTCTGCAGTGTTCCGT-3′ (deletion of *GPP2*), (ii) 5′-CTGGAGTCCAATGATGCAGCC-3′ and 5′- CAGGATAGATCAAAGAGCAGTC-3′ (deletion of *PDC2*).

To overexpress *GPD1*, the 2 μm derived plasmid YEpKm^R *GPD1* (Nevoigt and Stahl, 1996) was transformed into the in *S. cerevisiae* strains W303-1A, YA101, YA103 and YHN2. The plasmid YEpKm^R referred to here as empty vector was used to obtain reference strains lacking overexpression of *GPD1*. Both plasmids carry two selectable genetic markers for yeast: (i) Km^R conferring resistance against Geneticin G418 in yeast and (ii) the *LEU1* gene.

Transformation of *S. cerevisiae* was performed by electroporation (Becker and Guarente, 1991). Yeast transformants were selected on YNB (Glc/leu$^-$) medium

supplemented with 1 M sorbitol except when working with strain YHN2. For this strain, the glucose in YNB (Glc/leu$^-$) medium was replaced by 2% ethanol and 2% glycerol.

2.4. Determination of intracellular L-G3P in S. cerevisiae

To extract the intracellular L-G3P from *S. cerevisiae*, we slightly modified the method of Boles et al. (1993). An aliquot of cultures containing approximately 1.5×10^9 cells was mixed with the same volume of pre-cooled methanol (−70°C) to quench metabolism. The cells were rapidly collected on cellulose acetate filters (pore size 0.45 μm, Sartorius, Göttingen, Germany). The filter membrane with the collected cells was transferred quickly into a round-bottomed flask containing a mixture of 5 ml ethanol/3 ml chloroform/0.15 ml formic acid that was pre-cooled at −70°C. The flask was briefly shaken until the cells were separated from the filter and then stored at −20°C for at least 2 h. Cells were completely dried in a rotary evaporator at 60°C for 5–10 min. The dry residues were resuspended in 50 mM imidazole buffer whose pH was adjusted to 9.0 with NaOH. The resulting suspension was cleared by centrifugation at 13,000*g* for 10 min and the L-G3P was measured using an enzymatic assay as described by Bergmeyer (1974).

2.5. Determination of yeast dry weight

Dry weight was determined by filtering 50 ml of the culture using pre-weighted nitrocellulose filters (pore size 0.45 μm). The filters with the cells were washed with distilled water and dried until the weight reached a stable value.

2.6. Enzyme assays

To measure the specific activity of GPD in *S. cerevisiae*, cells were grown in shake-flasks in YNB (Glc/leu$^-$) medium to an OD_{600} of 1.5. Preparation of cell extracts and measuring of specific enzyme activity in vitro were carried out according to Nevoigt and Stahl (1996).

2.7. Determination of lipids

Lipids of whole yeast cells were extracted after disruption of cells with glass beads by the procedure of Folch et al. (1957). To analyze the neutral lipids, extracts were applied to Silica gel 60 plates (Merck, Germany) with the aid of a sample applicator (Linomat IV; CAMAG, Muttenz, Switzerland), and chromatograms were developed in an ascending manner using the solvent system light petroleum/diethyl ether/acetic acid (25:25:1) for the first third of the total distance. The plates were then briefly dried and further developed towards the top of the plate by using the solvent system light petroleum/diethyl ether (49:1). Quantification of ergosterol and ergosteryl esters was carried out by densitometric scanning at 275 nm with ergosterol as a standard. TAG were visualized on thin-layer chromatography (TLC) plates by post-chromatographic staining using a chromatogram immersion device (CAMAG, Muttenz, Switzerland). Plates were dipped for 6 s in a developing reagent consisting of 0.63 g $MnCl_2 \cdot 4H_2O$, 60 ml water, 60 ml methanol, and 4 ml of concentrated sulfuric acid, briefly dried, and heated to 100°C for 30 min. TAG were quantified via densitometric scanning at 400 nm with triolein (NuCheck, Inc., Elysian, MN) as a standard. Individual phospholipids were separated by two-dimensional TLC on Silica gel 60 plates (Merck, Germany), using chloroform/methanol/25% NH_3 (65:35:5) as the first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) as the second, developing solvent. Phospholipids were visualized on TLC plates by staining with iodine vapor, scraped off the plate, and quantified by the method of Broekhuyse (1968).

3. Results

3.1. Accumulation of L-G3P by engineering the glycerol biosynthetic pathway

The deletion of *GPP1* slightly affected the L-G3P pool in *S. cerevisiae*, whereas deletion of both isogenes of GPP resulted in a strong increase of intracellular L-G3P pool (Påhlman et al., 2001). These findings were confirmed by our results (Fig. 2; empty vector). We used the strains (W303-1A, YA101, YA103) bearing the empty vector (YEpKm^R) as a control instead of the untransformed strains in order to enable direct comparison with the same strains bearing the plasmid for *GPD1* overexpression (YEpKm^R *GPD1*). The multicopy transformants exhibited specific GPD activity about 10-fold higher than the strains carrying the empty vector. Although there was no change in the L-G3P level caused by overexpression of *GPD1* in the wild-type background, an obvious increase was obtained in strains whose GPP activity was reduced (*gpp1*Δ mutant) or completely absent (*gpp1*Δ *gpp2*Δ mutant) (Fig. 2). As shown in Fig. 3, overexpression of *GPD1* caused, however, a reduction in growth rate and biomass production in all strains investigated.

3.2. Accumulation of L-G3P only results in minor changes in the pattern of cellular lipids

L-G3P is a key intermediate in biosynthesis of all glycerolipids (Fig. 1). We analyzed the pattern of neutral

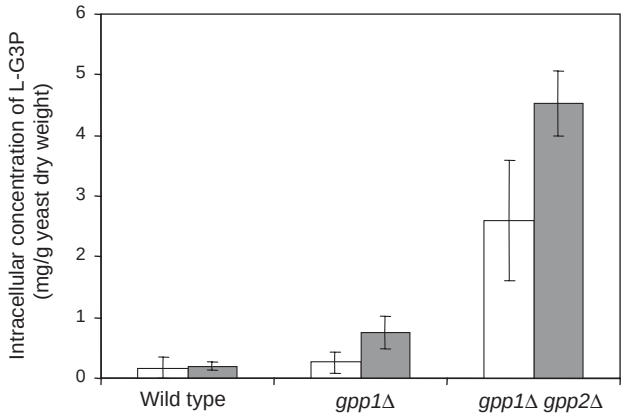

Fig. 2. Intracellular accumulation of L-G3P in wild type (W303-1A), *gpp1*Δ single (YA101) and *gpp1*Δ*gpp2*Δ double mutant (YA103) of *S. cerevisiae* transformed with either *GPD1* multicopy plasmid YEpKm^R*GPD1* (gray bars) or the empty vector YEpKm^R (open bars). Cells were cultivated in YNB (Glc/leu$^-$) in shake-flasks and harvested for extraction of L-G3P in the mid-log phase of growth. Data shown are the average values from two or three independent experiments including the standard deviations.

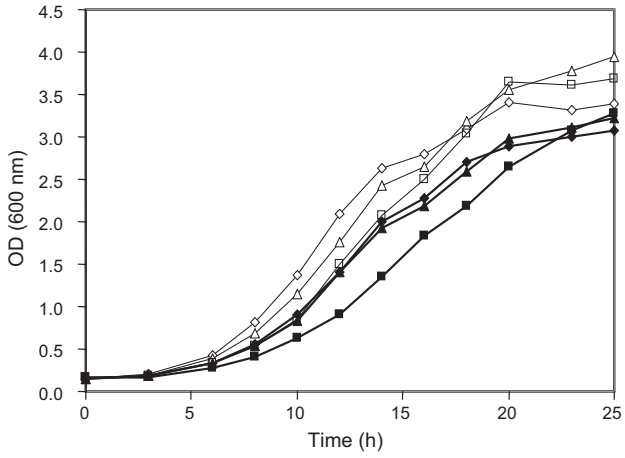

Fig. 3. Growth of the wild type (W303-1A) (◇/◆), *gpp1*Δ single mutant (YA101) (△/▲) and *gpp1*Δ*gpp2*Δ double mutant (YA103) (□/■) during shake-flask cultivation in YNB (Glc/leu$^-$) medium. The open symbols are referred to strains transformed with the empty vector. The closed symbols represent the same strains transformed with the *GPD1* multicopy plasmid.

lipids, i.e., TAG, ergosterol and ergosterol esters, as well as of glycerophospholipids in the *gpp1*Δ*gpp2*Δ double mutant strain overexpressing *GPD1* and the isogenic wild type bearing the empty vector. No differences concerning the cellular concentration of neutral lipids could be detected between both strains (data not shown). Analysis of the glycerophospholipid pattern, however, revealed a slight, but significant, increase in cardiolipin in *gpp1*Δ*gpp2*Δ double mutant strain overexpressing *GPD1* (5.78 + 1.13% of total phospholipids) compared to wild type (4.15 + 0.70% of total phospholipids). The amount of the other glycerophospholipid species was the same in both strains.

3.3. Limited oxygen supply but not osmotic stress is able to improve L-G3P accumulation in the engineered strain

It is well known that osmotic stress induces glycerol biosynthesis in *S. cerevisiae* mainly by increased transcription of *GPD1*. However, the activity of other enzymes is also affected (for reviews see Nevoigt and Stahl, 1997; Blomberg, 2000; Hohmann, 2002). In order to study whether hyperosmotic stress can further increase the L-G3P pool in strains, which overexpress *GPD1*, cells were grown in non-salinity YNB (Glc/leu$^-$) medium until the mid-log phase and then shifted to the same medium supplemented with 0.5 M NaCl. Surprisingly, there was no obvious increase of L-G3P accumulation caused by osmotic stress, however specific GPD activity was strongly induced (Table 2).

Moreover, we ascertained whether anaerobiosis could improve L-G3P accumulation. Respiratory processes to reoxidize cytosolic NADH that transmit the reducing equivalents from NADH into the mitochondrial respiratory chain are blocked when oxygen is limited. These processes are comprised by the external NADH dehydrogenases encoded by *NDE1/2* (Luttik et al., 1998; Small and McAlister-Henn, 1998) and the DHAP/L-G3P shuttle involving *GUT2* gene product (Larsson et al., 1998) (Fig. 1). Hence, the glycerol pathway is the only remaining route to reoxidize the surplus of cytosolic NADH formed during the synthesis of biomass and oxidized by-products (for reviews see van Dijken and Scheffers, 1986; Bakker et al., 2001). As can

Table 2
Impact of hyperosmotic stress and oxygen limitation on intracellular L-G3P accumulation in the *gpp1*Δ*gpp2*Δ mutant containing the plasmid YEpKm^R*GPD1* (overexpression of *GPD1*)

Conditions of cultivation	Specific activity of GPD (U/mg protein)	Intracellular concentration of L-G3P (mg/g yeast dry weight)
Reference[a]	2.3 ± 0.8	4.5 ± 0.5
Hyperosmotic stress[b]	9.0 ± 2.1	5.2 ± 0.7
Oxygen limitation[c]	2.7 ± 0.3	7.2 ± 1.3

Cells were pre-grown in shake flasks with YNB (Glc/leu$^-$) medium until the mid-log phase. They were then shifted to fresh YNB (Glc/leu$^-$) medium and incubated for 4 h as indicated in the table before measuring specific GPD activities and extracting L-G3P. Data shown are the average values from at least two independent experiments including the standard deviation.

[a] Glucose batch fermentation in shake flask.

[b] Medium was supplemented with 0.5 M NaCl.

[c] Glucose batch fermentation under oxygen limitation was performed in Erlenmeyer flasks sealed with air-locks which ensures the exclusion of oxygen but allows the release of gases. The cultures were stirred continuously at 350 rpm.

be seen from Table 2, specific GPD activity in the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1* was only slightly increased during oxygen limitation. This small increase is caused by the induction of the *GPD2* isogene which is known to be positively regulated by anaerobiosis (Ansell et al., 1997; Påhlman et al., 2001) in contrast to *GPD1*, the abundant isogene in the multicopy transformant investigated here. Nevertheless, oxygen limitation enhanced the intracellular L-G3P pool by about 60% (Table 2).

3.4. Deletion of the PDC2 gene increases intracellular L-G3P accumulation

Ethanol production is the main route of central carbon catabolism in *S. cerevisiae* during growth on glucose or other sugars. In contrast, the glycerol biosynthesis is rather an auxiliary pathway. Moreover, the main part of glycolytic NADH is reoxidized by ADH, the key enzyme of ethanol production pathway. In order to further increase L-G3P production, the flux towards ethanol was reduced by deleting the *PDC2* gene (Fig. 1) simulating the classical sulfite process which is known to enhance glycerol production pathway in yeast (see Introduction). *PDC2* encodes a positive transcriptional regulator of the structural genes *PDC1* and *PDC5* in *S. cerevisiae* (Hohmann, 1993). *PDC2* deletion in the *gpp1*Δ *gpp2*Δ background via one-step gene disruption method was not directly possible. Therefore, we first deleted *PDC2* in the *gpp1*Δ background obtaining strain *gpp1*Δ*pdc2*Δ, which was subsequently crossed with the *gpp1*Δ *gpp2*Δ double mutant strain in order to obtain the triple mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ (Materials and methods).

Growth of the triple mutant in glucose medium was severely inhibited in comparison to the double mutant *gpp1*Δ *gpp2*Δ (Fig. 4)· No growth at all could be detected under these conditions when *GPD1* was simultaneously overexpressed in the triple mutant (data not shown), confirming once more the negative impact of GPD overpoduction on growth of *S. cerevisiae*.

To investigate the impact of *PDC2* deletion on L-G3P accumulation, the *gpp1*Δ *gpp2*Δ double and the *gpp1*Δ *gpp2*Δ *pdc2*Δ triple mutant with and without overexpression of *GPD1* were pre-grown in medium supplemented with non-fermentable carbon sources in order to enable growth of the *pdc2*Δ deletion mutants. Subsequently, batch fermentation was initiated in glucose medium under oxygen limitation. Results shown in Fig. 5 demonstrate that *PDC2* deletion indeed caused an increase of the L-G3P concentration in the *gpp1*Δ *gpp2*Δ mutant background both with and without overexpression of *GPD1* (Fig. 5).

In order to address the question whether accumulation of L-G3P leads to secretion of this intermediate, the culture medium was analyzed for the presence of this compound. Traces of this substance could only be found after prolonged incubation in glucose medium, i.e., after 24 h, especially in the mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ overexpressing *GPD1*. However, it cannot be excluded that this small amount (<20 mg/L) originates from autolyzed cells since this mutant showed no visible growth in glucose medium (see above).

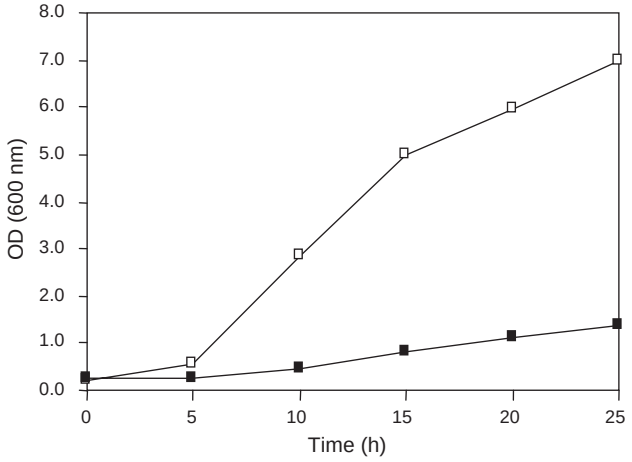

Fig. 4. Growth of *gpp1*Δ*gpp2*Δ double (YA103) (□) and *gpp1*Δ*gpp2*Δ*pdc2*Δ triple mutant (YHN2) (■) during shake-flasks cultivation in medium containing 2% glucose. Cells were pre-grown in YEPEG medium before transferring them into YNB (Glc/leu⁻) medium supplemented with 120 mg/L leucine. Data shown are the average values from two independent experiments including the standard deviations.

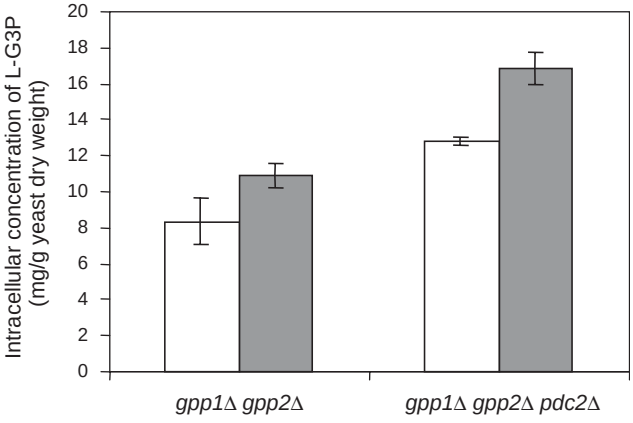

Fig. 5. Intracellular accumulation of L-G3P in *gpp1*Δ*gpp2*Δ (YA103) and *gpp1*Δ*gpp2*Δ*pdc2*Δ mutant (YHN2) transformed with either *GPD1* overexpression plasmid (gray bars) or the reference plasmid (open bars). Cells were pre-grown in YEPEG medium enabling growth of the triple mutant. Then, glucose batch fermentation in YNB (Glc/leu⁻) medium was inoculated at a cell concentration corresponding to an OD600 of about 0.8. Samples for extraction and measurement of L-G3P were taken after 6 h of cultivation under oxygen limitation (see legend of Table 2). Values shown are the average values from two independent experiments including standard deviations.

4. Discussion

This paper aims at accumulating L-G3P in *S. cerevisiae* by pathway engineering. Strains where GPP activity is reduced (deletion of *GPP1*) or completely abolished (deletion of *GPP1* and *GPP2*) have been already shown to accumulate L-G3P to different extents (Påhlman et al., 2001). Our results show that multicopy expression of *GPD1* could further improve the L-G3P pool in both *gpp1*Δ single (2.8-fold) and *gpp1*Δ *gpp2*Δ double (1.7-fold) deletion strain confirming once more the rate-limiting role of GPD in the glycerol biosynthetic pathway of *S. cerevisiae* (Nevoigt and Stahl, 1996; Michnick et al., 1997; Remize et al., 2001; Nevoigt et al., 2002). In contrast, no accumulation of L-G3P could be detected in the wild type overexpressing *GPD1*, indicating that GPP activity is sufficient to metabolize surplus L-G3P resulting from increased GPD activity. This observation matches the finding of Remize et al. (2001) that GPP is not rate limiting for glycerol production in *S. cerevisiae*.

In general, we found that the intracellular level of L-G3P did not increase beyond a certain level during prolonged fermentation of glucose (data not shown). We assume GPD is subjected product inhibition.

A negative correlation between L-G3P accumulation and the growth rate was observed (Figs. 1 and 2). However, the increase of intracellular L-G3P pool cannot be the only reason for the decreased growth rate of the engineered strains. The *gpp1*Δ *gpp2*Δ double deletion strain showed a considerably higher intracellular accumulation of L-G3P, but a similar growth rate and a higher biomass formation than the *gpp1*Δ single deletion strain overexpressing *GPD1*. In fact, there is an additional negative impact of *GPD1* overexpression on growth of all strains investigated (Fig. 2) which can be explained by the net loss of ATP and the accumulation of the toxic intermediate acetaldehyde (Nevoigt and Stahl, 1996; Michnick et al., 1997; Remize et al., 1999; Remize et al., 2001; Nevoigt et al., 2002).

L-G3P is an important precursor for the synthesis of all glycerolipids, i.e., TAG and glycerophospholipids. Therefore, accumulation of TAG and/or alterations in the phospholipid pattern caused by an increased intracellular L-G3P pool could be envisaged. However, there was no accumulation of TAG, most probably due to the lack of a concomitant excess of fatty acids, the second component needed for TAG synthesis. Påhlman et al. (2001) assumed that despite the accumulation of L-G3P in *S. cerevisiae* strains deleted in *GPP1* and *GPP2*, the flux of this compound towards glycerophospholipids is rather low. However, this hypothesis was never experimentally proven. Indeed, our analyzes of the phospholipid patterns in wild type and *gpp1*Δ *gpp2*Δ mutant overexpressing *GPD1* demonstrated that accumulation of L-G3P has only minor influences. Only a slight, but significant, increase of cardiolipin could be observed. Interestingly, both cardiolipin as well as the enzymes responsible for its biosynthesis are only found in mitochondrial membranes. In fact, there is a specific acyltransferase in yeast mitochondria using DHAP as a substrate to synthesize phosphatidic acid, the precursor of all phospholipids (Athenstaedt et al., 1999). Furthermore, the inner mitochondrial membrane is the site where the FAD dependent glycerol 3-phosphate dehydrogenase (Gut2p) is located (Janssen et al., 2002) which probably oxidizes accumulated L-G3P to DHAP (Fig. 1) directly supporting mitochondrial cardiolipin synthesis. Moreover, the synthesis of cardiolipin, in contrast to all the other glycerophospholipids, consumes 3 molecules of L-G3P instead of only one.

It is well known that both osmotic stress and oxygen limitation leads to strong enhancement of glycerol biosynthesis in wild-type cells of *S. cerevisiae*. As expected, the addition of 0.5 M NaCl caused a further marked increase of the already high GPD activity in the *GPD1* multicopy transformant. In fact, the plasmid used for overexpression contains the natural promoter of *GPD1* known to be induced by hyperosmotic stress. Unexpectedly, the pool of L-G3P was nearly unchanged under these conditions indicating that other factors apart from specific GPD activity hampered further increase in L-G3P pool.

In contrast, oxygen limitation induced an obvious increase in L-G3P accumulation although GPD activity was only slightly affected. During anaerobiosis, cytosolic NADH cannot be reoxidized by either external NADH dehydrogenases or L-G3P/DHAP shuttle since these systems are coupled to the respiratory chain (Fig. 1). Our finding that the L-G3P level can be improved by oxygen limitation suggests that the availability of cytosolic NADH is one limiting factor for the synthesis of L-G3P in the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1*. Alternative to using anaerobic conditions, all genes encoding the enzymes involved in respiratory reoxidation of cytosolic NADH can be deleted. In fact, the additional knockout of *NDE1*, *NDE2* and *GUT2* improved glycerol production in a *tpi1*Δ mutant (Overkamp et al., 2002).

In order to further support L-G3P production in the engineered strain, we envisaged a reduction of metabolic flux in the ethanol biosynthetic pathway. We expected a redirection of carbon flux towards glycerol pathway according to Neuberg and Reinfurth (1918), if the availability of acetaldehyde, the most important electron acceptor for cytosolic NADH reoxidation, is reduced by an appropriate genetic modification in our L-G3P overproducer. *PDC2* deletion was thought to be suitable for this purpose since flux towards ethanol is decreased but not completely blocked (Nevoigt and Stahl, 1996). Indeed, the deletion of *PDC2* led to an increase of L-G3P level in strains *gpp1*Δ *gpp2*Δ both with and

without overexpression of *GPD1*. The triple mutant *gpp1Δ gpp2Δpdc2Δ* overexpressing *GPD1* was the best L-G3P producer under the conditions tested. The level of 17 mg/g yeast dry weight corresponds to an increase of 100 times when compared to the wild-type level during aerobic fermentation of glucose (0.17 mg/g yeast dry weight). However, the deletion of *PDC2* had a detrimental impact on growth in glucose medium. The reason for this behavior which has also been observed by other authors (Hohmann, 1993; Flikweert et al., 1999) is unclear to date. The only plausible explanation is the capacity for reoxidation of glycolytic NADH limits the glycolytic flux at reduced PDC activities. This can be ruled out since the improved reoxidation of NADH via *GPD1* overexpression was not able to rescue *pdc2Δ* mutants from their growth defect (Nevoigt and Stahl, 1996; this study).

However, every severe intervention of central carbon metabolism in *S. cerevisiae* is expected be accompanied by reduction of growth. Hence, it will be inescapable for a practical application of such kind of engineered strains to uncouple the formation of product and the generation of biomass. Moreover, our future work will focus on releasing accumulated L-G3P into the medium in order to prevent product inhibition of GPD and facilitate purification of the product.

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