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Metabolic Engineering of Glycerol Production in *Saccharomyces cerevisiae*

Karin M. Overkamp,[1] Barbara M. Bakker,[2] Peter Kötter,[3] Marijke A. H. Luttik,[1] Johannes P. van Dijken,[1] and Jack T. Pronk[1]*

Kluyver Laboratory of Biotechnology, Delft University of Technology, NL-2628 BC Delft,[1] and Molecular Cell Physiology, Free University Amsterdam, NL-1081 HV Amsterdam,[2] The Netherlands, and Institut für Mikrobiologie, J. W. Goethe Universität Frankfurt, 60439 Frankfurt, Germany[3]

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Inactivation of *TPI1*, the *Saccharomyces cerevisiae* structural gene encoding triose phosphate isomerase, completely eliminates growth on glucose as the sole carbon source. In *tpi1*-null mutants, intracellular accumulation of dihydroxyacetone phosphate might be prevented if the cytosolic NADH generated in glycolysis by glyceraldehyde-3-phosphate dehydrogenase were quantitatively used to reduce dihydroxyacetone phosphate to glycerol. We hypothesize that the growth defect of *tpi1*-null mutants is caused by mitochondrial reoxidation of cytosolic NADH, thus rendering it unavailable for dihydroxyacetone-phosphate reduction. To test this hypothesis, a *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ quadruple mutant was constructed. *NDE1* and *NDE2* encode isoenzymes of mitochondrial external NADH dehydrogenase; *GUT2* encodes a key enzyme of the glycerol-3-phosphate shuttle. It has recently been demonstrated that these two systems are primarily responsible for mitochondrial oxidation of cytosolic NADH in *S. cerevisiae*. Consistent with the hypothesis, the quadruple mutant grew on glucose as the sole carbon source. The growth on glucose, which was accompanied by glycerol production, was inhibited at high-glucose concentrations. This inhibition was attributed to glucose repression of respiratory enzymes as, in the quadruple mutant, respiratory pyruvate dissimilation is essential for ATP synthesis and growth. Serial transfer of the quadruple mutant on high-glucose media yielded a spontaneous mutant with much higher specific growth rates in high-glucose media (up to 0.10 h^{-1} at 100 g of glucose · $liter^{-1}$). In aerated batch cultures grown on 400 g of glucose · $liter^{-1}$, this engineered *S. cerevisiae* strain produced over 200 g of glycerol · $liter^{-1}$, corresponding to a molar yield of glycerol on glucose close to unity.

Glycerol is used to synthesize many products, ranging from cosmetics to lubricants. Its current annual production of ca. 600,000 tonnes is mainly recovered as a by-product of soap manufacturing or produced from propylene (55). Alternatively, glycerol can be produced by microbial fermentation, using sustainable carbohydrate feedstocks. Although this may involve a wide range of microorganisms, including algae and bacteria, research has mostly focused on yeasts (1, 55; M. J. Taherzadeh, L. Adler, and G. Lidén, submitted for publication). In yeasts, glycerol is produced by the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P), a reaction catalyzed by cytosolic NAD^+-dependent G3P dehydrogenase. G3P is subsequently dephosphorylated by a glycerol-3-phosphatase (1). Especially during anaerobic growth, glycerol production serves as a redox sink to maintain the cytosolic redox balance (5, 50). Glycerol also functions as an osmolyte, thus enabling yeast growth at high osmolarity (1, 36). Consistent with the latter role, the highest glycerol yields reported to date have been achieved with osmotolerant yeast strains (Table 1).

The amounts of glycerol that are naturally produced by *Saccharomyces cerevisiae* as a response to anaerobiosis and/or osmotic stress are relatively small (42, 44, 51). Much effort has been invested in attempts to redirect sugar metabolism in this yeast towards glycerol production. The first successful attempt was the sulfite process, devised by Neuberg and Reinfurth (34). In this process, sulfite added to fermenting *S. cerevisiae* cultures forms an adduct with acetaldehyde, thus making the latter compound unavailable as an electron acceptor for the reoxidation of glycolytic NADH. Instead, NADH is reoxidized by glycerol production. This early example of redirection of metabolic fluxes has been called metabolic engineering avant la lettre (13). Theoretically, the sulfite process can lead to the formation of equimolar amounts of glycerol, carbon dioxide, and sulfite-acetaldehyde adduct. However, the theoretical yield of glycerol on glucose of 0.51 g · g^{-1} has not been achieved, not even in modern adaptations of the sulfite process (Table 1). Moreover, the presence of by-products (ethanol, acetate, sulfite-acetaldehyde adduct, and biomass) poses problems during glycerol recovery (1).

Over the past decade, research on glycerol production by *S. cerevisiae* has shifted to true metabolic engineering, i.e., the application of recombinant DNA technology for a rational reprogramming of cellular metabolism (4). Several approaches were aimed at minimizing the reduction of acetaldehyde to ethanol, thus mimicking the sulfite process. Indeed, reduced expression of pyruvate decarboxylase (35) and deletion of alcohol dehydrogenase genes (12) led to an increased production of glycerol, but the glycerol concentration did not exceed 5 g · $liter^{-1}$ (Table 1). Other strategies focused on overexpression of the key enzymes of the glycerol pathway in *S. cerevisiae* (33, 39, 44). Overproduction of the *GPD1*-encoded cytosolic G3P dehydrogenase led to an increased production of glycerol

* Corresponding author. Mailing address: Kluyver Laboratory of Biotechnology, Delft University of Technology, Julianalaan 67, NL-2628 BC Delft, The Netherlands. Phone: 31 15 278 3214. Fax: 31 15 278 2355. E-mail: j.t.pronk@tnw.tudelft.nl.

TABLE 1. Some representative yeast processes used for glycerol production

Strain and process	Glycerol concn in broth ($g \cdot l^{-1}$)	Yield of glycerol on glucose ($g \cdot g^{-1}$)	Avg productivity ($g \cdot liter^{-1} \cdot day^{-1}$)	Reference or source
S. cerevisiae				
Sulfite batch	45	0.23	9.0	15
Sulfite; fed batch under vacuum	82	0.25	32.5	27
GPD1 overproduction; batch	25	0.12	4.3	44
ADH deletion; shake flask	4.6	0.26	2.5	12
*pdc2*Δ mutant; shake flask	2.9	0.16	2.1	35
*tpi1*Δ mutant; shake flask (0–24 h)	36	0.46	36	9
with extra glucose (24–56 h)	43	0.20	5.3	
*tpi1*Δ mutant; as above (0–44 h)	63	0.44	35	10
*tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ mutant, aerated batch	219	0.50	57.6	This study
Osmotolerant yeasts				
Candida magnoliae I_2B; batch	80[a]	0.32	15.6	46
Saccharomyces strain LORRE Y8; fed batch	260	0.47	29.8	19
Pichia farinosa; fed batch with solid glucose	300[a]	0.46	37.5	53
Candida glycerinogenes; batch	127	0.64	40.6	56

[a] Total polyol concentration.

(33, 44) (Table 1), whereas overproduction of glycerol phosphatase had no effect on glycerol production (39, 44). Increasing glycerol export by deregulation of the Fps1p channel protein increased glycerol production but, in combination with overproduction of G3P dehydrogenase, negatively affected growth (44).

The highest glycerol yield and productivity reported to date for metabolically engineered *S. cerevisiae* were observed with a *tpi1*Δ deletion mutant (9, 10) (Table 1). Apparently, in *tpi1*Δ mutants, which lack the glycolytic enzyme triose phosphate isomerase, accumulation of DHAP is prevented by its conversion to glycerol (Fig. 1). The maximum theoretical yield of this process is 1 mol of glycerol · mol of glucose^{-1} if all glucose is metabolized via glycolysis. However, *S. cerevisiae tpi1*Δ mutants are unable to grow on glucose as the sole carbon source (9–11). Therefore, biomass was pregrown on glucose-ethanol mixtures, followed by a bioconversion of glucose to glycerol (9, 10). During the bioconversion phase, glycerol productivity decreased strongly with time (Table 1).

The biochemical mechanism responsible for the inability of

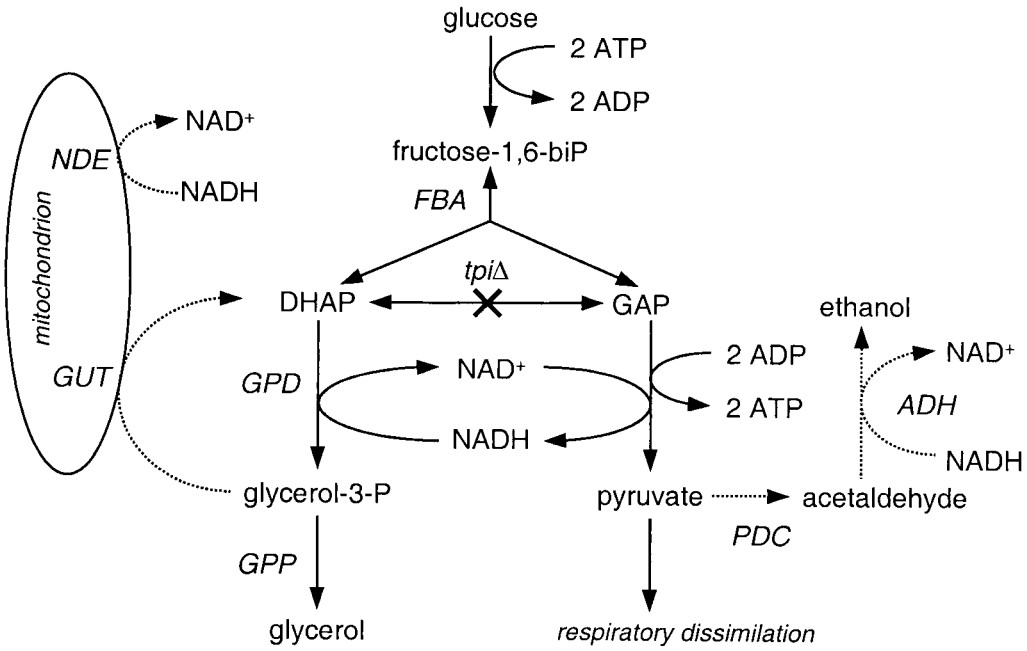

FIG. 1. Hypothetical pathway (solid lines) for glucose dissimilation by triose phosphate isomerase-negative (*tpi1*Δ) *S. cerevisiae*. DHAP is detoxified by its reduction to G3P and subsequent dephosphorylation to glycerol. This requires that all NADH generated in the glyceraldehyde-3-phosphate dehydrogenase reaction be available for DHAP reduction. Oxidation of either cytosolic G3P or cytosolic NADH by other processes (dotted lines) will lead to accumulation of DHAP. Abbreviations: GAP, glyceraldehyde-3-phosphate; FBA, fructose-1,6-biphosphate aldolase; GPD, cytosolic G3P dehydrogenase; GPP, glycerol phosphatase; GUT, mitochondrial flavin adenine dinucleotide-dependent G3P dehydrogenase; NDE, external mitochondrial NADH dehydrogenase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase.

TABLE 2. *S. cerevisiae* strains used in this study

Strain	Genotype[a]	Source or reference
CEN.PK113-7D	*MAT***a**	31
CEN.PK122	*MAT***a**/*MAT*α	31
CEN.PK167-2B	*MAT***a** *nde1* (41–1659)::*loxP-kanMX4-loxP nde2*(51–100)::*loxP-kanMX4-loxP*	31
CEN.PK225-2C	*MAT***a** *gut2*(41–2010)::*loxP-kanMX4-loxP*	37
CEN.PK536-5B	*MAT*α *tpi1*(41–707)::*loxP-kanMX4-loxP nde1*(41–1659)::*loxP-kanMX4-loxP nde2* (51–100)::*loxP-kanMX4-loxP*	This study
CEN.PK530-1B	*MAT*α *tpi1*(41–707)::*loxP-kanMX4-loxP*	This study
CEN.PK530-1C	*MAT***a** *tpi1*(41–707)::*loxP-kanMX4-loxP*	This study
CEN.PK546-12B	*MAT***a** *tpi1* (41–707)::*loxP-kanMX4-loxP nde1* (41–1659)::*loxP-kanMX4-loxP nde2*(51–100)::*loxP-kanMX4-loxP gut2*(41–2010)::*loxP-kanMX4-loxP*	This study
CEN.PK546-AHG	Spontaneous mutant of CEN.PK546-12B, selected for AHG concn	This study

[a] The numbers in parentheses indicate the deleted nucleotides (ATG = 1).

*S. cerevisiae tpi1*Δ mutants to grow on glucose as the sole carbon source is unknown. Since conversion of glucose to equimolar amounts of pyruvate and glycerol is neutral in terms of ATP (Fig. 1), growth of *tpi1*Δ mutants should depend on the respiratory dissimilation of pyruvate. Glucose repression of the synthesis of key enzymes of respiratory glucose dissimilation (16, 50) may therefore prevent growth of *tpi1*Δ mutants on glucose in batch cultures. However, *tpi1*Δ mutants also failed to grow in aerobic, glucose-limited chemostat cultures, in which glucose repression is alleviated (11). As in batch cultures, growth on glucose in chemostat cultures required the inclusion of ethanol in the medium feed (11). This indicated that glucose repression of respiration is not the only factor that prevents growth of *tpi1*Δ mutants on glucose as the sole carbon source.

Complete conversion of DHAP to glycerol in a *tpi1*Δ mutant requires that all NADH generated in the glyceraldehyde-3-phosphate dehydrogenase reaction be used to reduce DHAP to glycerol (Fig. 1). If other reactions were to compete for NADH with glycerol production, this would lead to accumulation of DHAP, which in turn can be converted to methylglyoxal, a cytotoxic compound that can inhibit growth (25, 32). We hypothesize that oxidation of cytosolic NADH by the mitochondrial respiratory chain is such a competing mechanism and thus contributes to the inability of *tpi1*Δ mutants to grow on glucose as the sole carbon source (Fig. 1). Two mechanisms are involved in the mitochondrial oxidation of cytosolic NADH in *S. cerevisiae*: mitochondrial external NADH dehydrogenase and a G3P shuttle (29, 31, 37, 48). At low specific growth rates, either of these two systems is sufficient to sustain respiratory growth (37). The *NDE1* and *NDE2* genes encode two isoenzymes of the external NADH dehydrogenase (31, 48), whereas the *GUT2* gene encodes a mitochondrial respiratory-chain-linked G3P dehydrogenase, a key enzyme of the G3P shuttle (29, 45). The latter enzyme might also directly affect growth of *tpi1*Δ mutants by reoxidizing G3P to DHAP (Fig. 1).

The aim of this study is to test the hypothesis that mitochondrial reoxidation of cytosolic NADH and/or G3P plays a role in the phenotype of *tpi1*Δ mutants and to investigate whether metabolic engineering of mitochondrial respiration can be used to improve glycerol production by *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains and maintenance. The *S. cerevisiae* strains used and constructed in this study (Table 2) are prototrophic strains belonging to the CEN.PK family (49). Stock cultures were grown at 30°C in shake flasks on YPED medium (10 g of Bacto yeast extract · liter^{-1}, 20 g of peptone · liter^{-1}, 10 ml of ethanol · liter^{-1}, and 0.5 g of glucose · liter^{-1}) except the adaptation to high glucose concentration (AHG) strain, which was grown on 100 g of glucose · liter^{-1} in MMU medium (3.0 g of K_2SO_4 · liter^{-1}, 3.0 g of KH_2PO_4 · liter^{-1}, 3.0 g of urea · liter^{-1}, 0.5 g of $MgSO_4 \cdot 7H_2O$ · per liter) with trace elements and vitamins prepared and sterilized as described previously (52). Urea was added to the medium after separate filter sterilization. When stationary phase was reached, 30% (vol/vol) sterile glycerol was added, and 2-ml aliquots were stored in sterile vials at −80°C.

Construction of null mutants. Standard techniques and media for genetic modification of *S. cerevisiae* were used (3). Deletions in *TPI1* were obtained by the short flanking homology method (54), using pUG6 as a template (21). PCR amplification, yeast transformation, and verification of the correct gene deletion, as well as determination of the mating type, were carried out as described by Luttik et al. (31). The *loxP-kanMX4-loxP* cassette amplified by PCR was used to transform diploid strain CEN.PK122. After tetrad analysis, the G418^R segregants were checked by diagnostic PCR for the correct integration of the *kanMX* cassette (Table 3).

All further double, triple, or quadruple deletion strains were constructed by crossing of the corresponding single, double, or triple deletion strains. The resulting diploid strains were subsequently analyzed by tetrad analysis to obtain the respective segregants and further analyzed by diagnostic PCR to confirm the correct deletion of the corresponding genes. Strain CEN.PK536-5B (*nde1*Δ *nde2*Δ *tpi1*Δ) resulted from crossing of strains CEN.PK167-2B (*nde1*Δ *nde2*Δ) and CEN.PK530-1B (*tpi1*Δ). To obtain the quadruple deletion strain CEN.PK546-12B (*nde1*Δ *nde2*Δ *tpi1*Δ *gut2*Δ), the strains CEN.PK536-5B (*nde1*Δ *nde2*Δ *tpi1*Δ) and CEN.PK225-2C (*gut2*Δ), respectively, were crossed.

Shake flask cultivation. Shake flask cultures were grown in an orbital incubator (200 rpm, 30°C) in spherical flat-bottom flasks (500 ml) containing 100 ml of medium. Precultures were grown on MMU medium supplemented with 5 ml of ethanol · liter^{-1} and 1 g of glucose · liter^{-1}. During the exponential growth phase, the optical density at 660 nm (OD_{660}) was measured with an Amersham Pharmacia Novaspec II spectrophotometer. Exponential-phase cultures were used as the inoculum for further shake flask experiments. Prior to inoculation, cells were centrifuged (4,500 × *g*; 3 min) and washed aseptically with sterile

TABLE 3. Oligonucleotides used for construction of the *TPI1* deletion cassette (S1/S2) and for diagnostic PCR (A1/K1) and (K2/A4) of the deletion strains.[a]

Oligonucleotide	DNA sequence
TPII-S1	5′-ATG GCT AGA ACT TTC TTT GTC GGT GGT AAC TTT AAA TTA ACA GCT GAA GCT TCG TAC GC-3′
TPII-S2	5′-TTA GTT TCT AGA GTT GAT GAT ATC AAC AAA TTC TGG CTT CGC ATA GGC CAC TAG TGG ATC TG-3′
TPII-A1	5′-CTT CTG CGG TAT CAC CCT AC-3′
TPII-A4	5′-CAA TGC AGT CTT CGG TAC AC-3′
K1	5′-GGA TGT ATG GGC TAA ATG TAC G-3′
K2	5′-GTT TCA TTT GAT GCT CGA TGA G-3′

[a] Oligonucleotides used for the construction of the deletion cassettes and for diagnostic PCR of the strains deleted in *NDE1, NDE2*, and *GUT2*, respectively, were described previously (31, 37). Sequences complementary to the *loxP-KanMX4-loxP* cassette (pUG6) are underlined.

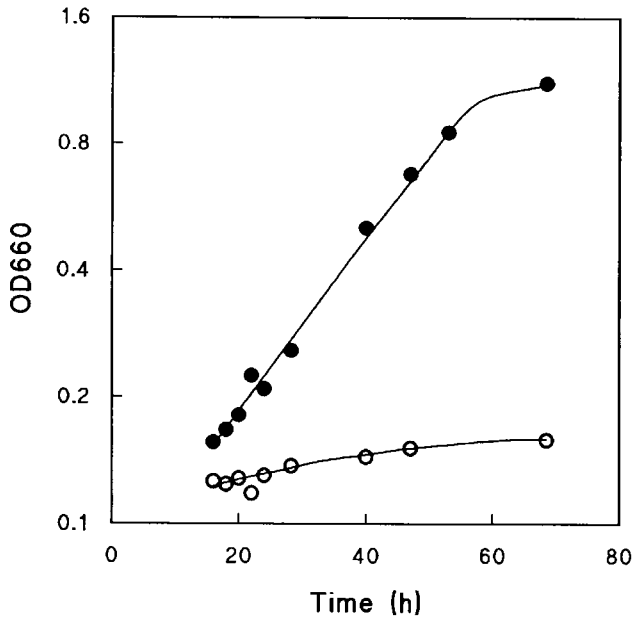

FIG. 2. Growth on glucose of *tpi1*Δ *S. cerevisiae* (○) and an isogenic *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain (●). Both strains were grown in shake flasks on MMU medium with 5 g of glucose · liter^{-1} as the sole carbon source. Three independent replicate cultures gave the same results.

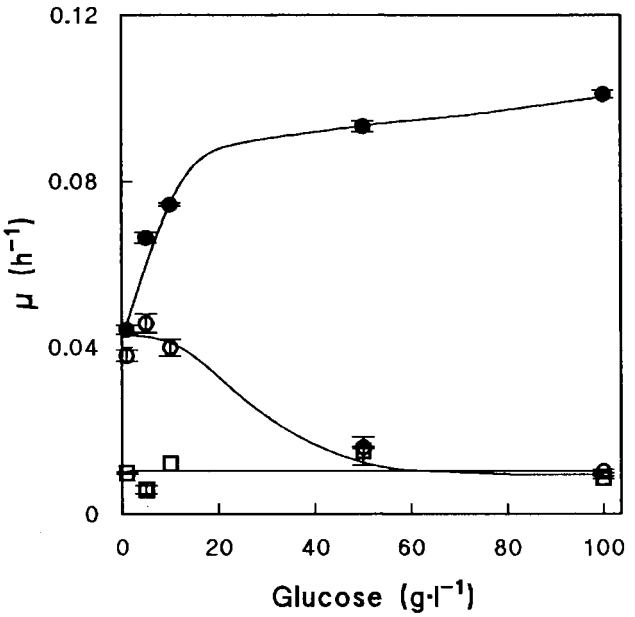

FIG. 3. Effect of glucose concentration on the specific growth rate of isogenic *S. cerevisiae tpi1*Δ (□) and *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ (○) strains and a *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ-AHG (adapted to high glucose) strain (●) in shake flask cultures. All cultures were grown on MMU medium supplemented with different initial glucose concentrations. Data are presented as the average ± mean deviation of at least two independent replicate cultures for each glucose concentration.

medium to remove residual substrates and metabolites. The washed cells were used to inoculate (initial OD_{660} of 0.1) fresh shake flasks containing MMU medium with 5 ml of ethanol · liter^{-1} and 1 g of glucose · liter^{-1}. During the exponential growth phase, cells were harvested as described above, washed, and resuspended in the medium used for the final shake flask cultivation, which was MMU medium with glucose as the sole carbon source at a concentration of 1, 5, 10, 50, or 100 g · liter^{-1}. Growth was monitored by regular OD_{660} measurements. When OD_{660} was above 0.3, samples were diluted in MMU medium containing the same glucose concentration as the culture, to avoid changes in OD_{660} due to osmotic effects.

Chemostat cultivation. Chemostat cultures were grown in 1-liter working-volume laboratory fermentors as described previously (31). This reference also describes procedures for gas analysis and determination of biomass dry weight. One-hundred-milliliter precultures in shake flasks, inoculated with 2 ml of a frozen stock culture, were grown to stationary phase on YPED medium. These precultures were used to inoculate a fermentor containing 1 liter of MMA medium [5.0 g of $(NH_4)_2SO_4$ · liter^{-1}, 3.0 g of KH_2PO_4 · liter^{-1}, 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter, vitamins, and trace elements as in the MMU medium described above] supplemented with 1 g of glucose · liter^{-1} and 10 ml of ethanol · liter^{-1}. The continuous medium supply was initiated when a steep increase of the dissolved-oxygen concentration indicated depletion of the carbon source. The synthetic medium used for continuous cultivation was MMA medium supplemented with 7.5 g of glucose · liter^{-1} (60% of total carbon) and 83 mM ethanol (40% of total carbon). The dilution rate was set at 0.05 h^{-1}. For growth on glucose as a sole carbon source, the feed of the chemostat culture growing on the glucose-ethanol mixture was switched to the same medium without ethanol.

Batch cultivation in fermentors. One milliliter of frozen stock culture of the glucose-adapted CEN.PK546-12B strain (CEN.PK546-AHG, where AHG is a designation for adapted to high glucose; see Results) was inoculated in shake flasks with double-strength MMU medium supplemented with 400 g of glucose · liter^{-1}. After 4 days of incubation, 150 ml of this culture was transferred to a 2-liter laboratory fermentor (Applikon, Schiedam, The Netherlands). The fermentor was previously autoclaved (20 min at 110°C) while containing 1.2 liters of demineralized water with 600 g of glucose and 100 μl of silicone antifoam (BDH). Together with the inoculum, 45 ml of a solution containing 9 g of urea and 100 ml of 30-fold concentrated MM medium (MMU without urea) was added. This yielded an initial culture volume of 1.5 liters consisting of double-strength MMU medium and an initial glucose concentration of 400 g·liter^{-1}. The pH was kept at 5.0 by an Applikon ADI 1030 biocontroller, via the automatic addition of 4 M KOH or 4 M H_2SO_4. The fermentor was aerated (0.75 liter · min^{-1}) and stirred at 900 rpm. Additional antifoam was added manually when foaming occurred. Biomass growth was monitored by OD_{660} measurements (when necessary, diluted in a 400-g · liter^{-1} glucose solution) and via biomass dry-weight measurements (31). Metabolite concentrations were determined by high-performance liquid chromatography (30). High-biomass-density cultures were performed in the same way, but were inoculated with 200 ml of a stationary-phase fermentor culture grown as described above.

RESULTS

Growth on glucose in shake flask cultures. Previously published reports state that *tpi1*Δ mutants of *S. cerevisiae* are unable to grow on glucose as the sole carbon source, both in shake flasks (9, 38) and in chemostat cultures (11). This was confirmed in shake flask cultures containing 5 g of glucose · liter^{-1}, in which the specific growth rate was below 0.01 h^{-1} (Fig. 2). To investigate whether this inability to grow on glucose was due to mitochondrial reoxidation of cytosolic NADH and/or G3P (Fig. 1), thus preventing the complete reduction of dihydroxyacetone phosphate, a *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ mutant was constructed. Indeed, this quadruple deletion mutant was able to grow on glucose with a specific growth rate of 0.045 h^{-1} (Fig. 2). Yet, this specific growth rate is still substantially lower than that of the isogenic wild-type strain CEN.PK113-7D, which under these conditions exhibits a ca. eightfold-higher specific growth rate (data not shown).

Specific growth rates of the *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain on glucose strongly depended on the glucose concentration in the medium (Fig. 3). At glucose concentrations of 50 g · liter^{-1} and higher, the specific growth rate of the quadruple deletion

TABLE 4. Growth of the reference strain *S. cerevisiae* CEN.PK113-7D and the isogenic *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ mutant CEN.PK546-12B.[a]

Strain (relevant genotype)	Biomass yield ($g \cdot g$ glucose^{-1})	Glycerol yield ($mol \cdot mol$ glucose^{-1})	qO_2	qCO_2	Carbon recovery (%)
CEN PK113-7D (*TPI1 NDE1 NDE2 GUT2*)	0.47 ± 0.01	<0.01	1.3 ± 0.1	1.4 ± 0.1	95
CEN.PK546-12B (*tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ)	0.20 ± 0.02	0.83 ± 0.01	2.2 ± 0.2	2.7 ± 0.3	98

[a] Strains were grown in aerobic, glucose-limited chemostat cultures (30°C, pH 5.0, dilution rate = 0.05 h^{-1}, 7.5 g of glucose·liter^{-1} in feed). Data are presented as the average ± mean deviation of two independent chemostat experiments for each strain. Values for qO_2 and qCO_2 are in millimoles per gram per hour.

strain was very low and similar to that of the *tpi1*Δ single mutant. Conversion of glucose into equimolar amounts of glycerol and pyruvate is neutral in terms of ATP and redox metabolism. Therefore, growth of the *tpi1*Δ mutants on glucose critically depends on respiration for ATP production. The synthesis of many enzymes involved in the respiratory dissimilation of pyruvate is subject to glucose catabolite repression (16, 50), which may explain the glucose sensitivity of the *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain.

Glucose-limited chemostat cultivation. Analysis of fermentation products in glucose-grown shake flask cultures suggested that the *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain produced large amounts of glycerol. For a quantitative analysis of biomass and product yields, the *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain and the reference strain CEN.PK113-7D were grown in aerobic, glucose-limited chemostat cultures. In such cultures, glucose catabolite repression can be alleviated by the low-residual-glucose concentrations. The biomass yield of the *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain was more than twofold lower than the reference strain during growth at a dilution rate of 0.05 h^{-1} (Table 4). This was partly due to the conversion of glucose into glycerol. The molar yield of glycerol on glucose was 0.83 $mol \cdot mol^{-1}$. Under the same cultivation conditions, the isogenic reference strain CEN.PK113-7D did not produce detectable amounts of glycerol. The extensive production of glycerol is consistent with the metabolic scheme proposed in Fig. 1. This scheme predicts a glycerol yield of 1 $mol \cdot mol^{-1}$. The lower glycerol yield in the glucose-limited chemostat cultures can be explained from the assimilation of glucose-6-phosphate via metabolic pathways other than glycolysis, such as the pentose phosphate pathway, cell wall biosynthesis, and storage carbohydrate biosynthesis.

Selection of a strain adapted to high-glucose concentrations. The glycerol yield (0.83 $mol \cdot mol$ glucose^{-1}, i.e., 0.42 $g \cdot g$ of glucose^{-1}) in glucose-limited chemostat cultures of the *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain is among the highest reported for a growing, metabolically engineered *S. cerevisiae* strain, although it is matched by a bioconversion process with the nongrowing *tpi1*Δ strain (Table 1). However, the glucose sensitivity of the quadruple mutant is a drawback for any large-scale application in glycerol production. The *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain (CEN.PK 546-12B) was adapted to growth at high-glucose concentrations by serial transfer. This was started by transferring 10 ml of a steady-state glucose-limited chemostat culture to a shake flask containing MMA medium with 100 g of glucose $\cdot$ liter^{-1} (555 mM). This culture showed extremely slow growth accompanied by acetate production and a decrease of the culture pH to ca. 3. After 2 days, growth and acetate production ceased. After another 4 days, acetate consumption occurred and growth resumed. Eventually the low pH (ca. 2.5) caused by ammonia consumption abolished growth altogether, despite the presence of approximately 350 mM residual glucose. From this culture, 1 ml was transferred to a new shake flask with the same medium. No acetate production was observed, but acidification again prevented complete consumption of glucose. Use of urea instead of ammonium salts as the sole nitrogen source can sometimes prevent acidification of yeast cultures (24). Therefore, 1 ml of culture was transferred to a shake flask with MMU medium containing 100 g of glucose $\cdot$ liter^{-1}. This indeed led to complete consumption of glucose. A total of 0.5 ml of culture was transferred to the next flask with the same medium. After two further transfers, the culture was streaked onto agar plates with MMU medium containing 50 g of glucose $\cdot$ liter^{-1}. The culture was purified by three subsequent transfers of a single colony to a new plate. Finally, a single colony was transferred to a shake flask with MMU medium containing 100 g of glucose $\cdot$ liter^{-1}. This glucose-adapted culture of the *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain was called CEN.PK546-AHG. Especially at high-glucose concentrations, the selected mutant exhibited much higher specific growth rates than the original, nonadapted *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ strain (Fig. 3).

Production of glycerol by engineered *S. cerevisiae*. Glycerol production by *S. cerevisiae* CEN.PK546-AHG was investigated in aerated batch cultures with 400 g of glucose $\cdot$ liter^{-1}, which were inoculated from glucose-grown shake flasks at 0.3 g (dry weight) $\cdot$ liter^{-1}. Despite the high initial glucose concentration, these batch cultures exhibited exponential growth (μ = 0.027 h^{-1}). The slow growth was accompanied by the accumulation of glycerol to a final concentration of over 200 g $\cdot$ liter^{-1} (data not shown). Glycerol was the major fermentation product. Concentrations of acetate and ethanol remained below 1.5 and 4 g $\cdot$ liter^{-1}, respectively, and were completely consumed towards the end of fermentation (data not shown). Throughout the fermentation process, the glycerol yield on glucose was 1.0 $mol \cdot mol^{-1}$. Due to the low initial biomass concentration and the long start-up period, the overall glycerol production rate was 1.0 g glycerol $\cdot$ liter$^{-1} \cdot h^{-1}$. When the experiment was repeated with an initial biomass dry weight of 3.9 g $\cdot$ liter^{-1} (Fig. 4A), all glucose was consumed in 80 h and the final concentration of glycerol was 219 g $\cdot$ liter^{-1}. The molar ratio of glycerol produced per glucose consumed was 0.99 $mol \cdot mol^{-1}$ (Fig. 4B), and the overall glycerol production rate was 2.4 g glycerol $\cdot$ liter$^{-1} \cdot h^{-1}$. Glycerol consumption did not occur, even after glucose depletion. Most wild-type *S. cerevisiae* strains can consume glycerol, although it is a very poor carbon and energy source (43, 49). However, in the quadruple mutant, glycerol dissimilation is not possible as the deletion of the *GUT2* gene and the absence of triose phosphate isomerase block the oxidation pathway.

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