

Elimination of Glycerol Production in Anaerobic Cultures of a *Saccharomyces cerevisiae* Strain Engineered To Use Acetic Acid as an Electron Acceptor^{∇†}

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In anaerobic cultures of wild-type *Saccharomyces cerevisiae*, glycerol production is essential to reoxidize NADH produced in biosynthetic processes. Consequently, glycerol is a major by-product during anaerobic production of ethanol by *S. cerevisiae*, the single largest fermentation process in industrial biotechnology. The present study investigates the possibility of completely eliminating glycerol production by engineering *S. cerevisiae* such that it can reoxidize NADH by the reduction of acetic acid to ethanol via NADH-dependent reactions. Acetic acid is available at significant amounts in lignocellulosic hydrolysates of agricultural residues. Consistent with earlier studies, deletion of the two genes encoding NAD-dependent glycerol-3-phosphate dehydrogenase (*GPD1* and *GPD2*) led to elimination of glycerol production and an inability to grow anaerobically. However, when the *E. coli mhpF* gene, encoding the acetylating NAD-dependent acetaldehyde dehydrogenase (EC 1.2.1.10; acetaldehyde + NAD⁺ + coenzyme A ↔ acetyl coenzyme A + NADH + H⁺), was expressed in the *gpd1Δ gpd2Δ* strain, anaerobic growth was restored by supplementation with 2.0 g liter⁻¹ acetic acid. The stoichiometry of acetate consumption and growth was consistent with the complete replacement of glycerol formation by acetate reduction to ethanol as the mechanism for NADH reoxidation. This study provides a proof of principle for the potential of this metabolic engineering strategy to improve ethanol yields, eliminate glycerol production, and partially convert acetate, which is a well-known inhibitor of yeast performance in lignocellulosic hydrolysates, to ethanol. Further research should address the kinetic aspects of acetate reduction and the effect of the elimination of glycerol production on cellular robustness (e.g., osmotolerance).

Bioethanol production by *Saccharomyces cerevisiae* is currently, by volume, the single largest fermentation process in industrial biotechnology. A global research effort is under way to expand the substrate range of *S. cerevisiae* to include lignocellulosic hydrolysates of nonfood feedstocks (e.g., energy crops and agricultural residues) and to increase productivity, robustness, and product yield (for reviews see references 20 and 35). A major challenge relating to the stoichiometry of yeast-based ethanol production is that substantial amounts of glycerol are invariably formed as a by-product (24). It has been estimated that, in typical industrial ethanol processes, up to 4% of the sugar feedstock is converted into glycerol (24). Although glycerol also serves as a compatible solute at high extracellular osmolarity (10), glycerol production under anaerobic conditions is primarily linked to redox metabolism (34).

During anaerobic growth of *S. cerevisiae*, sugar dissimilation occurs via alcoholic fermentation. In this process, the NADH formed in the glycolytic glyceraldehyde-3-phosphate dehydrogenase reaction is reoxidized by converting acetaldehyde,

formed by decarboxylation of pyruvate to ethanol via NAD⁺-dependent alcohol dehydrogenase. The fixed stoichiometry of this redox-neutral dissimilatory pathway causes problems when a net reduction of NAD⁺ to NADH occurs elsewhere in the metabolism. Such a net production of NADH occurs in assimilation when yeast biomass is synthesized from glucose and ammonia (34). Under anaerobic conditions, NADH reoxidation in *S. cerevisiae* is strictly dependent on reduction of sugar to glycerol (34). Glycerol formation is initiated by reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol-3-phosphate, a reaction catalyzed by NAD⁺-dependent glycerol-3-phosphate dehydrogenase. Subsequently, the glycerol-3-phosphate formed in this reaction is hydrolyzed by glycerol-3-phosphatase to yield glycerol and inorganic phosphate.

The importance of glycerol production for fermentative growth of yeasts was already observed in the 1960s during studies of non-*Saccharomyces* yeasts that exhibit a so-called “Custers effect.” In such yeast species, which are naturally unable to produce glycerol, fermentative growth on glucose is possible only in the presence of an external electron acceptor that can be reduced via an NADH-dependent reaction (e.g., the reduction of acetoin to butanediol via NAD⁺-dependent butanediol dehydrogenase) (29). It was later shown that *gpd1Δ gpd2Δ* strains of *S. cerevisiae*, which are also unable to produce glycerol, are similarly unable to grow under anaerobic condi-

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TABLE 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source/reference
CEN.PK113-5D	<i>MATa ura3 GPD1 GPD2</i>	P. Kötter, Frankfurt
IME076	<i>MATa ura3 GPD1 GPD2 p426_GPD(URA3)</i>	This study
CEN.PK102-3A	<i>MATa ura3 leu2 GPD1 GPD2</i>	P. Kötter, Frankfurt
RWB0094	<i>MATa ura3 leu2 gpd1(-1,1133)::loxP-KanMX-loxP gpd2(-2,1281)::hphMX4</i>	BIRD Engineering, Rotterdam
IMZ008	<i>MATa ura3 leu2 gpd1(-1,1133)::loxP gpd2(-2,1281)::hphMX4 YEplac181(LEU2)</i>	This study
IMZ132	<i>MATa ura3 leu2 gpd1(-1,1133)::loxP gpd2(-2,1281)::hphMX4 YEplac181(LEU2)</i> <i>pUDE43(URA3 pTHD3::mhpF (E. coli)::CYC1t)</i>	This study
IMZ127	<i>MATa ura3 leu2 gpd1(-1,1133)::loxP gpd2Δ(-2,1281)::hphMX4</i> <i>YEplac181(LEU2) p426_GPD(URA3)</i>	This study

tions unless provided with acetoin as an external electron acceptor (8).

In view of its large economic significance, several metabolic engineering strategies have been explored to reduce or eliminate glycerol production in anaerobic cultures of *S. cerevisiae*. Nissen et al. (25) changed the cofactor specificity of glutamate dehydrogenase, the major ammonia-fixing enzyme of *S. cerevisiae*, thereby increasing NADH consumption in biosynthesis. This approach significantly reduced glycerol production in anaerobic cultures grown with ammonia as the nitrogen source. Attempts to further reduce glycerol production by expression of a heterologous transhydrogenase, with the aim to convert NADH and NADP⁺ into NAD⁺ and NADPH, were unsuccessful (24) because intracellular concentrations of these pyridine nucleotide cofactor couples favor the reverse reaction (23).

The goal of the present study was to investigate whether the engineering of a linear pathway for the NADH-dependent reduction of acetic acid to ethanol can replace glycerol formation as a redox sink in anaerobic, glucose-grown cultures of *S. cerevisiae* and thus provide a stoichiometric basis for elimination of glycerol production during industrial ethanol production. Significant amounts of acetic acid are released upon hydrolysis of lignocellulosic biomass, and, in fact, acetic acid is studied as an inhibitor of yeast metabolism in lignocellulosic hydrolysates (5, 7, 26). The *S. cerevisiae* genome already contains genes encoding acetyl coenzyme A (acetyl-CoA) synthetase (32) and NAD⁺-dependent alcohol dehydrogenases (ADH1-5 [12]). To complete the linear pathway for acetic acid reduction, we expressed an NAD⁺-dependent, acetylating acetaldehyde dehydrogenase (EC 1.2.1.10) from *Escherichia coli* into a *gpd1Δ gpd2Δ* strain of *S. cerevisiae*. This enzyme, encoded by the *E. coli mhpF* gene (15), catalyzes the reaction acetaldehyde + NAD⁺ + coenzyme A ↔ acetyl coenzyme A + NADH + H⁺. Growth and product formation of the engineered strain were then compared in the presence and absence of acetic acid and compared to those of a congenic reference strain.

MATERIALS AND METHODS

Strain construction and maintenance. The *Saccharomyces cerevisiae* strains used in this study (Table 1) originate from the CEN.PK family, which was previously identified as a suitable background for combined genetic and physiological studies (33). Strain RWB0094, in which deleted portions of the open reading frames of the *GPD1* and *GPD2* genes of strain CEN.PK102-3A (*MATa ura3 leu2*) were replaced by the loxP-KanMX-loxP cassette from pUG6 (19) and the hphMX4 cassette from pAG32 (18), respectively, was acquired from BIRD Engineering, Rotterdam, the Netherlands. The KanMX marker of strain

RWB0094 was removed by expression of the Cre recombinase (19), and its leucine auxotrophy was complemented by transformation with the *LEU2*-bearing plasmid YEplac181 (17), yielding strain IMZ008. Transformation of strain IMZ008 with the *URA3*-bearing *mhpF* expression plasmid pUDE43 (see below) yielded the prototrophic, *mhpF*-expressing strain IMZ132, and transformation with the *URA3*-bearing “empty” vector p426_GPD yielded strain IMZ127. Finally, transformation of strain CEN.PK113-5D (*ura3*) with p426_GPD yielded the prototrophic *GPD1 GPD2* reference strain IME076. Cultures transformed with deletion cassettes were plated on YPD complex medium (11) containing G418 (200 mg liter⁻¹) or hygromycin (200 mg liter⁻¹). Successful integration of the deletion cassettes was confirmed by diagnostic PCR.

Stock cultures of all strains were grown in shake flasks containing 100 ml of synthetic medium (see below) with 20 g liter⁻¹ glucose as the carbon source. After 30% (vol/vol) glycerol was added, 1-ml aliquots of stationary phase cultures were stored at -80°C.

Plasmid construction. The *E. coli mhpF* gene (EMBL accession number Y09555.7) (15) was PCR amplified from *E. coli* K-12 strain JM109 genomic DNA using primer pairs *mhpF*-FW (5'-GGGGACAAGTTTGTACAAAAAAGCAG GCTATGAGTAAGCGTAAAGTCGCCATTATCGG-3') and *mhpF*-RV (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGTTTCATGCCGCTTCTCTCT GCCTTGC-3'), which contained attB1 and attB2 sequences, respectively. The PCR was performed using Phusion Hot Start high-fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland) according to manufacturer specifications and in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) with 25 cycles of 10-s denaturation at 98°C and 30-s annealing and extension at 72°C. The 1,011-bp PCR product was cloned using Gateway cloning technology (Invitrogen, Carlsbad, CA). Plasmid pDONR221, using the BP reaction, was used to create the entry clone, designated plasmid pUD64. From this entry clone and the multicopy plasmid pAG426GPD-ccdB (Addgene, Cambridge, MA), the yeast expression plasmid pUDE43 was constructed by using the LR reaction. Transformations of recombination reaction products into competent *E. coli* K-12 strain JM109 were performed with the Z-Competent *E. coli* transformation kit (Zymoresearch Corporation, Orange, CA) and plated on LB media containing either ampicillin (100 mg liter⁻¹) or kanamycin (50 mg liter⁻¹). Yeast transformations were performed by the method of Burke et al. (11). After transformations with the yeast expression plasmid, cells were plated on synthetic media. Successful insertion of multicopy plasmid pUDE43 was confirmed by diagnostic PCR using the primer pairs for cloning.

Cultivation and media. Shake flask cultivation was performed at 30°C in a synthetic medium (36). The pH of the medium was adjusted to 6.0 with 2 M KOH prior to sterilization. Precultures were prepared by inoculating 100 ml medium containing 20 g liter⁻¹ glucose in a 500-ml shake flask with 1 ml frozen stock culture. After 24 h of incubation at 30°C in an Innova incubator shaker (200 rpm; New Brunswick Scientific, NJ), cultures were transferred to bioreactors.

Anaerobic batch fermentations were carried out at 30°C in 2-liter laboratory fermentors (Applikon, Schiedam, the Netherlands) with a working volume of 1 liter. Synthetic medium with 20 g liter⁻¹ glucose (36) was used for all fermentations and supplemented with 100 μl liter⁻¹ of silicone antifoam (Silcolapse 5020; Caldic Belgium, Bluestar Silicones) as well as with the anaerobic growth factors ergosterol (0.01 g liter⁻¹) and Tween 80 (0.42 g liter⁻¹) dissolved in ethanol. This resulted in 0.5 to 0.6 g liter⁻¹ ethanol in the medium. Where indicated, acetic acid was added at a concentration of 2 g liter⁻¹ and the pH was readjusted to 5.0 prior to inoculation. Culture pH was maintained at 5.0 by the automatic addition of 2 M KOH. Cultures were stirred at 800 rpm and sparged with 0.5 liter min⁻¹ nitrogen (<10 ppm oxygen). Dissolved oxygen was monitored with an autoclavable oxygen electrode (Applisens, Schiedam, the Netherlands). To minimize diffusion of oxygen, fermentors were equipped with Nor-

prene tubing (Cole-Parmer Instrument Company, Vernon Hills, IL). All fermentations were carried out at least in duplicate.

Determination of culture dry weight and optical density. Culture samples (10 ml) taken at selected time intervals were filtered over preweighed nitrocellulose filters (pore size, 0.45 μm ; Gelman Laboratory, Ann Arbor, MI). After removal of medium, the filters were washed with demineralized water and dried in a microwave oven (Bosch, Stuttgart, Germany) for 20 min at 350 W and weighed. Duplicate determinations varied by less than 1%. Culture growth was also monitored via optical density readings at a wavelength of 660 nm on a Novaspec II spectrophotometer.

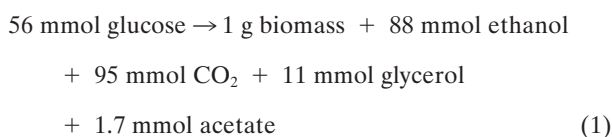
Gas analysis. Exhaust gas was cooled in a condenser (2°C) and dried with a Permapure type MD-110-48P-4 dryer (Permapure, Toms River, NJ). Oxygen and carbon dioxide concentrations were determined with an NGA 2000 analyzer (Rosemount Analytical, Orrville, OH). Exhaust gas flow rate and carbon dioxide production rates were determined as described previously (2). In calculating these biomass-specific rates, a correction was made for volume changes caused by withdrawing culture samples.

Metabolite analysis. Supernatant obtained by centrifugation of culture samples was analyzed for glucose, acetic acid, succinic acid, lactic acid, glycerol, and ethanol via high-performance liquid chromatograph (HPLC) analysis on a Waters Alliance 2690 HPLC (Waters, Milford, MA) containing a Bio-Rad HPX 87H column (Bio-Rad, Hercules, CA). The column was eluted at 60°C with 0.5 g liter⁻¹ H₂SO₄ at a flow rate of 0.6 ml min⁻¹. Detection was by means of a Waters 2410 refractive-index detector and a Waters 2487 UV detector. Initial and final glycerol concentrations were further determined using an enzymatic determination kit (R-Biopharm AG, Darmstadt, Germany). During cultivation in bioreactors that are sparged with nitrogen gas, a significant fraction of the ethanol is lost by evaporation through the off-gas (22). To correct for this, ethanol evaporation kinetics were analyzed in bioreactors operated under identical conditions at different working volumes with sterile synthetic medium. The resulting volume-dependent ethanol evaporation constants (for this setup, equal to 0.0080 divided by the volume in liters, expressed in h⁻¹) were used to correct HPLC measurements of ethanol concentrations in culture supernatants, taking into account changes in volume that were caused by sampling.

Enzyme activity assays. Cell extracts for activity assays of NAD⁺-dependent acetaldehyde dehydrogenase (acetylating) were prepared from exponentially growing anaerobic batch cultures and analyzed for protein content as described previously (1). NAD⁺-dependent acetaldehyde dehydrogenase (acetylating) activity was measured at 30°C by monitoring the oxidation of NADH at 340 nm. The reaction mixture (total volume, 1 ml) contained 50 mM potassium phosphate buffer (pH 7.5), 0.15 mM NADH, and cell extract. The reaction was started by the addition of 0.5 mM acetyl coenzyme A. For glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activity determination, cell extracts were prepared as described above except that the phosphate buffer was replaced by triethanolamine buffer (10 mM; pH 5) (4, 16). Glycerol-3-phosphate dehydrogenase activities were assayed in cell extracts at 30°C as described previously (9). Reaction rates were proportional to the amounts of cell extract added.

RESULTS

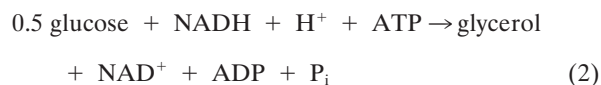
Theoretical analysis of the introduction of a linear acetate reduction pathway. For a theoretical prediction of the impact of the introduction of a linear acetate reduction pathway into a *gpd1Δ gpd2Δ* strain of *S. cerevisiae*, we used published data on the yields of biomass, ethanol, carbon dioxide, and acetate in glucose-grown anaerobic batch cultures of the reference strain *S. cerevisiae* CEN.PK113-7D (22). According to these data, the anaerobic growth stoichiometry of this strain is described by equation 1:



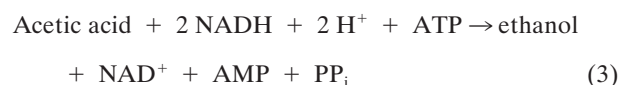
In this scenario, 10 C-mol% of the glucose is converted to glycerol and 52 C-mol% to ethanol. These yields are higher and lower, respectively, than predicted values for large-scale anaerobic ethanol production (25). A number of factors may

contribute to this difference, including the often-lower biomass yield under industrial conditions, the identity of the nitrogen source (3), a redox-sparing effect of acetic acid in hydrolysates (31), and the presence of alternative electron acceptors, such as furfural, in hydrolysates (21).

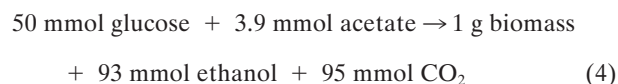
Production of glycerol for redox balancing occurs according to equation 2:



The metabolic engineering strategy in the present study is based on the replacement of this reaction by the reaction described by equation 3:



If it is assumed that the formation of AMP and pyrophosphate in the acetyl-CoA synthetase reaction is equivalent to the hydrolysis of 2 ATP to ADP and inorganic phosphate due to hydrolysis of pyrophosphate to orthophosphate (27), the formation of 1 mol of glycerol from glucose is equivalent to the reduction of 0.5 mol of acetate to ethanol, in terms of NADH oxidation as well as in terms of ATP hydrolysis. If it is furthermore assumed that the acetate produced by the reference strain is reconsumed (equivalent to not being formed), the growth stoichiometry can be rewritten as in equation 4:



In this new situation, the glycerol yield has decreased to zero, while the apparent ethanol yield on glucose has increased to 62 C-mol%, which represents a theoretical 18% increase relative to the ethanol yield of the reference strain grown on glucose as the sole carbon source.

Growth and product formation in anaerobic batch cultures. When cultures of the prototrophic reference strain *S. cerevisiae* IME076 (*GPD1 GPD2*) were supplemented with 2.0 g liter⁻¹ acetic acid, the specific growth rate (0.32 h⁻¹) was identical to that reported for cultures grown in the absence of acetic acid (0.34 h⁻¹ [22]). Consistent with results from a recent study, the addition of acetic acid led to a slight decrease of the biomass yield and, consequently, a decrease of the glycerol yield on glucose relative to cultures grown in the absence of acetic acid (Fig. 1 and Table 2). This effect has been attributed to the higher rate of glucose dissimilation for intracellular pH homeostasis due to diffusion of acetic acid into the cell, which in turn results in a lower biomass yield on glucose (7). Under the same conditions, an isogenic *gpd1Δ gpd2Δ* strain, in which an absence of NAD⁺-dependent glycerol-3-phosphate dehydrogenase activity was confirmed in cell extracts (Table 2), was completely unable to grow anaerobically (data not shown), consistent with the notion that glycerol production via Gpd1 and Gpd2 is essential for NADH reoxidation in anaerobic cultures of *S. cerevisiae* (8).

Expression of the *E. coli mhpF* gene in a *gpd1Δ gpd2Δ* strain, resulting in acetyl-CoA-dependent rates of NADH reduction in cell extracts of 0.020 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ (Table 2),

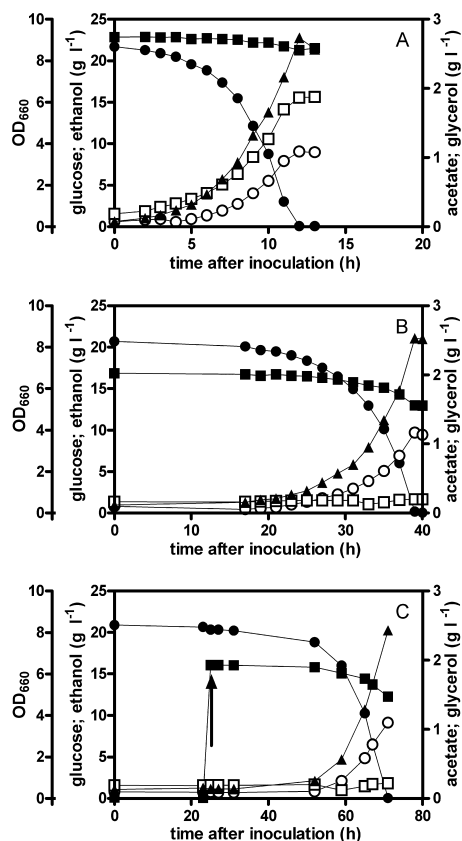


FIG. 1. Concentrations of biomass and products in anaerobic batch cultures of different *S. cerevisiae* strains on glucose (20 g liter⁻¹). Acetic acid (2.0 g liter⁻¹) was present from the start of the fermentation (A and B) or added at the time point indicated by the arrow (C). Growth conditions: temperature = 30°C; pH 5.0. Symbols: ▲, optical density at 660 nm; ●, glucose; ○, ethanol; ■, acetate; □, glycerol. Each graph represents values from one of two independent replicates, yielding data that differed by less than 5%. (A) *S. cerevisiae* IME076 (*GPD1 GPD2*). (B) *S. cerevisiae* IMZ132 (*gpd1Δ gpd2Δ* overexpressing the *E. coli mhpF* gene). (C) *S. cerevisiae* IMZ132 (*gpd1Δ gpd2Δ* overexpressing the *E. coli mhpF* gene).

did not enable anaerobic growth when glucose was the sole carbon source. However, when the medium was supplemented with 2.0 g liter⁻¹ acetic acid, either before or after inoculation, exponential growth was observed at a specific growth rate of 0.14 h⁻¹. No formation of glycerol occurred during cultivation (Fig. 1 and Table 2). The trace amounts (<0.1 g liter⁻¹) of

glycerol present in cultures of *gpd1Δ gpd2Δ* strains originate from the inoculum cultures, which were started from frozen glycerol stocks. Ethanol was the major organic product, and the small amounts of succinate and lactate produced were similar to those observed in cultures of the reference strain grown under the same conditions (data not shown). Acetate addition did not rescue the anaerobic growth defect of a congenic *gpd1Δ gpd2Δ S. cerevisiae* reference strain that did not express the *E. coli mhpF* gene (data not shown).

In contrast to the theoretical predictions described above, only a slight difference in ethanol yield on glucose was observed based on HPLC data (Table 2). However, since the anaerobic IMZ132 fermentations (40 h) lasted longer than the anaerobic cultures of the wild-type strain (15 h) and the anaerobic batch cultures were sparged with nitrogen gas, the fraction of ethanol lost through evaporation was higher for strain IMZ132. After determination of the kinetics of ethanol evaporation in sterile control experiments and correction of the ethanol yields, a 13% higher apparent ethanol yield on glucose was shown for the engineered strain using the linear pathway for NADH-dependent reduction of acetic acid to ethanol (Table 2).

DISCUSSION

The present study provides a proof of principle that, stoichiometrically, the role of glycerol as a redox sink for anaerobic growth of *S. cerevisiae* can be fully replaced by a linear pathway for NADH-dependent reduction of acetate to ethanol. This offers interesting perspectives for large-scale ethanol production from feedstocks that contain acetic acid, such as lignocellulosic hydrolysates. The “bacterial” pathway from acetyl-CoA to ethanol has previously been integrated into *S. cerevisiae* as part of a strategy to ferment xylose via a phosphoketolase pathway (30). While this approach indeed resulted in the conversion of xylose, the impact on glycerol production was not evaluated and the pathway was not tested with a *gpd1Δ gpd2Δ* strain.

In addition to reducing the organic carbon content of spent media and increasing the ethanol yield, the reduction of acetic acid to ethanol may at least partially alleviate acetate inhibition of yeast growth and metabolism, which is especially problematic at low pH and during the consumption of pentose sugars by engineered yeast strains (7). However, before industrial implementation can be contemplated, several issues remain to be addressed. First, growth and product formation in the en-

TABLE 2. Physiology of the engineered *S. cerevisiae* strain IMZ132 and the empty-vector reference strain IME076 during anaerobic batch cultivation on synthetic medium (pH 5) with glucose-acetate mixtures^a

Yeast strain	Relevant genotype	Glycerol-3-phosphate dehydrogenase (μmol mg protein ⁻¹ min ⁻¹)	Acetaldehyde dehydrogenase (acetylating) (μmol mg protein ⁻¹ min ⁻¹)	Specific growth rate (h ⁻¹)	Yield on glucose			
					Biomass (g mol ⁻¹)	Glycerol (mol mol ⁻¹)	Ethanol (mol mol ⁻¹)	
							Not corrected for evaporation	Corrected for evaporation
IME076	<i>GPD1 GPD2</i>	0.034 ± 0.003	<0.002	0.32 ± 0.01	15.0 ± 0.1	0.14 ± 0.01	1.54 ± 0.02	1.61 ± 0.03
IMZ132	<i>gpd1Δ gpd2Δ</i> + <i>mhpF</i>	<0.002	0.020 ± 0.004	0.14 ± 0.01	14.8 ± 1.6	<0.002	1.67 ± 0.04	1.82 ± 0.05

^a Results are represented as average and mean deviations of data from two independent batch cultures. Glycerol yields on glucose were calculated using glycerol concentrations obtained by enzymatic determination.

gineered strain were significantly slower than in the reference strain. Several factors may contribute to this. First, the *in vivo* kinetics of acetyl coenzyme A synthetase may limit the rate of acetate reduction. In anaerobic, glucose-grown batch cultures, the high-affinity Acs1 isoenzyme is not expressed due to a combination of transcriptional repression and glucose catabolite inactivation (13, 32). The constitutively expressed Acs2 enzyme has a low affinity for acetate ($K_m = \text{ca. } 20 \text{ mM}$) (32), which may limit *in vivo* rates of acetate activation. Despite its uncoupling effect, the intracellular acetic acid concentration can be low due to active export of acetic acid from yeast cells (28). Obviously, the *in vivo* activity of the heterologous acetaldehyde dehydrogenase may also control the rate of acetate reduction.

A further factor that may affect growth kinetics is that glycerol-3-phosphate is a key precursor of glycerolipids. Although it has been demonstrated that, in the absence of Gpd1 and Gpd2, glycerolipids can be produced by reduction of the corresponding dihydroxyacetone esters (6), the kinetics of this process have not been studied with anaerobic cultures. Analysis and optimization of the kinetics of acetate reduction and growth by further metabolic engineering and/or evolutionary approaches are essential to make the rates of ethanol production of *gpd1Δ gpd2Δ* strains expressing the *E. coli mhpF* gene compatible with industrial production.

In addition to the kinetics of growth and ethanol formation, another important aspect of the physiology of the engineered strains requires further investigation. The role of glycerol as a compatible solute, which protects yeast cells at high extracellular osmolarity, is likely to be relevant in industrial fermentations with high initial sugar concentrations. The metabolic engineering strategy described in this paper provides an excellent experimental platform to analyze osmotic stress in anaerobic cultures unable to produce glycerol. Such research should, ultimately, address the question whether robust industrial yeast strains that do not produce glycerol can be constructed.

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