

Gpd1 and Gpd2 Fine-Tuning for Sustainable Reduction of Glycerol Formation in *Saccharomyces cerevisiae*[▽]

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Gpd1 and Gpd2 are the two isoforms of glycerol 3-phosphate dehydrogenase (GPDH), which is the rate-controlling enzyme of glycerol formation in *Saccharomyces cerevisiae*. The two isoenzymes play crucial roles in osmoregulation and redox balancing. Past approaches to increase ethanol yield at the cost of reduced glycerol yield have most often been based on deletion of either one or two isogenes (*GPD1* and *GPD2*). While single deletions of *GPD1* or *GPD2* reduced glycerol formation only slightly, the *gpd1Δ gpd2Δ* double deletion strain produced zero glycerol but showed an osmosensitive phenotype and abolished anaerobic growth. Our current approach has sought to generate “intermediate” phenotypes by reducing both isoenzyme activities without abolishing them. To this end, the *GPD1* promoter was replaced in a *gpd2Δ* background by two lower-strength *TEF1* promoter mutants. In the same manner, the activity of the *GPD2* promoter was reduced in a *gpd1Δ* background. The resulting strains were crossed to obtain different combinations of residual *GPD1* and *GPD2* expression levels. Among our engineered strains we identified four candidates showing improved ethanol yields compared to the wild type. In contrast to a *gpd1Δ gpd2Δ* double-deletion strain, these strains were able to completely ferment the sugars under quasi-anaerobic conditions in both minimal medium and during simultaneous saccharification and fermentation (SSF) of liquefied wheat mash (wheat liquefact). This result implies that our strains can tolerate the ethanol concentration at the end of the wheat liquefact SSF (up to 90 g liter⁻¹). Moreover, a few of these strains showed no significant reduction in osmotic stress tolerance compared to the wild type.

The major portion of total expenditure in today's bioethanol industry is allotted to feedstock costs (17). Therefore, it has to be ensured that all polysaccharides present in the raw material are efficiently converted into fermentable sugars and that all sugars are completely converted into ethanol with a high yield (ethanol per sugar consumed). We focus here on the question of how to improve the conversion yield (sugar to ethanol) in the yeast *Saccharomyces cerevisiae*.

The maximum theoretical ethanol yield of glucose fermentation by *S. cerevisiae* is 0.51 g (g of glucose)⁻¹. However, the ethanol yields in current industrial processes only reach 90 to 93% of this maximal theoretical value (6). In fact, some carbon is used for the formation of biomass and certain by-products, particularly glycerol. Since even small improvements in ethanol yield would have significant impacts on profits in large scale ethanol production, there is a great industrial interest to reduce by-product formation and increase ethanol yield.

A promising route toward an increase in ethanol yield has been the reduction of glycerol formation (13). Indeed, glycerol production in *S. cerevisiae* can be quite substantial. For exam-

ple, 2.0 to 3.6 g of glycerol per 100 g of consumed glucose have been already reported by Pasteur in 1858 (39). Glycerol formation depends on yeast strain, fermentation conditions (4, 8, 19), and medium composition, especially the type of nitrogen source (1). Therefore, glycerol yields (per glucose consumed) reported for different strains and conditions vary and can even significantly exceed the above-mentioned value reported by Pasteur (39).

Glycerol formation results primarily from balancing the net NADH surplus generated during yeast growth, particularly under anaerobic conditions (5, 7, 42, 46). In addition, intracellular glycerol is involved in osmo-adaptation (25), oxidative stress protection (38), and response to heat shock (3, 44).

A first group of past metabolic engineering approaches toward reduction of glycerol formation in yeast targeted either enzymes (9, 35, 47) and/or a plasma membrane transporter (14, 52), which are directly involved in the formation and intracellular accumulation of glycerol. A second type of approach attempted reducing the net NADH production during yeast growth (13, 36). Moreover, a number of studies have combined strategies of the first and second approaches (14, 29–31). Recently, Guadalupe Medina et al. (21) have opened up a third type of approach, namely, by providing an alternative route of NADH reoxidation. The additional value of this approach is that acetic acid, an unwanted compound in plant biomass hydrolysates, is converted into ethanol, thereby serv-

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ing as a redox sink and replacing glycerol formation. However, the approach of Guadalupe Medina et al. (21) also included a double deletion of *GPD1* and *GPD2* and was thus accompanied by osmoresistance sensitivity and a strongly reduced specific growth rate. A recently published route in order to overcome the osmoresistance sensitivity of mutants defective in *GPD1* has been the overexpression of the trehalose synthesis genes *TPS1* and *TPS2* (23).

The glycerol 3-phosphate dehydrogenase (GPDH) is the rate-controlling enzyme in the glycerol formation pathway of *S. cerevisiae* (15). Therefore, many previous attempts included deleting either one or both isogenes encoding GPDH. The phenotypes after deleting *GPD1* and/or *GPD2* have already been tested in different yeast strain backgrounds, media, and oxygenation conditions. Generally speaking, the single deletion of *GPD1* resulted in strains sensitive to osmotic stress (2), while the deletion of *GPD2* reduced growth under anaerobiosis (9, 35). However, neither the deletion of *GPD1* nor the deletion of *GPD2* resulted in a noticeable change in glycerol yield at least when prototrophic strains were studied (35). In contrast, the deletion of both isogenes led to the complete loss of glycerol formation under all tested conditions. However, the *gpd1Δ gpd2Δ* mutant is of no practical relevance since growth and ethanol productivity was abolished under anaerobic conditions and even strongly reduced under aerobic conditions (5, 9, 35).

In order to study *S. cerevisiae* strains that have a glycerol formation capacity ranging between that of the *gpd2Δ* single mutant (100%) and that of the *gpd1Δ gpd2Δ* double mutant (0%), we recently replaced the native *GPD1* promoter in a *gpd2Δ* background by two well-characterized *TEF1* promoter mutant versions (33, 37). The genetic modifications were accompanied by 61 and 88% reductions in glycerol yield on glucose and by 20 and 30% reductions in maximal aerobic growth rate compared to the wild type. Interestingly, our engineered ("intermediate") strains, referred to as TEFmut2 and TEFmut7, showed 2 and 5% increases, respectively, in ethanol yield and could well cope with process stress, which is in remarkable contrast to a *gpd1Δ gpd2Δ* double-deletion strain. These results were obtained in a very-high-ethanol-performance fed-batch process with aeration (37). It remained to be tested how these strains would perform under anaerobic conditions. This was the first motivation for the present study. A second motivation resulted from the fact that Gpd2 activity was completely abolished in the "intermediate" strains described by Pagliardini et al. (37), and the residual GPDH activity was solely based on the Gpd1 isoenzyme. As mentioned above, Gpd2 plays a crucial role in redox balancing under anaerobiosis. In fact, industrial bioethanol production usually occurs under anaerobic conditions. In the present study, two isogenic strains with abolished Gpd1 activity and two residual levels of Gpd2 activity were constructed. These novel strains have the opposite mating type compared to the strains described by Pagliardini et al. (37) and allowed us to eventually obtain all possible combinations of Gpd1 and Gpd2 residual activities by mating. Our set of 12 different strains formed a sound basis in order to study the impact of partial reduction of Gpd1 and Gpd2 activity on physiology under laboratory conditions, as well as conditions relevant in the bioethanol industry.

MATERIALS AND METHODS

Microbial strains and cultivation conditions. *Escherichia coli* strain DH5α (Invitrogen Corp., Carlsbad, CA) was used for amplification of the plasmids. The strain was grown in Luria-Bertani (LB) medium (0.5% yeast extract, 1% peptone, 1% NaCl [pH 7]) at 37°C. *E. coli* transformation and isolation of plasmid DNA were carried out according to standard techniques (43). All engineered *S. cerevisiae* strains (Table 1) generated in the present study were derived from the prototrophic haploid wild-type strains CEN.PK113-7D (*MATa*) and CEN.PK113-1A (*MATα*). The medium used for yeast strain maintenance was YD, which contained 1% (wt/vol) yeast extract and 1% (wt/vol) glucose.

Yeast strain construction. Genetic modifications of *S. cerevisiae* carried out within the present study comprised the deletion of *GPD1* and the replacement of the native *GPD2* promoter by the two low-activity promoters (TEFmut2 and TEFmut7). Deletion of *GPD1* was carried out according to the method described by Güldener et al. (22). The primers for the amplification of the *GPD1* disruption and promoter replacement cassettes, as well as for the verification of the correct integration, are listed in Table 2. Gene disruption and promoter replacement cassettes were amplified by DNA polymerases with proofreading activity. The PCR conditions were adapted to the guidelines of the manufacturer. Transformation of *S. cerevisiae* was carried out according to the method of Gietz and Schiestl (20) using treatment with lithium acetate and polyethylene glycol. After the transformation, cells were incubated in YD for at least 4 h at 30°C to allow expression of the antibiotic resistance genes. In order to select positive yeast transformants, YD agar plates were supplemented with 7.5 μg of phleomycin/ml or 200 μg of Geneticin G418/ml.

The deletion of *GPD1* in the *gpd2Δ* background (strain Ab) was carried out using the *loxP-KanMX4-loxP* disruption cassette amplified from pUG6 plasmid (22). The resulting strain is referred to as the *gpd1Δ gpd2Δ* double-deletion strain (strain ab). The *loxP-ble^R-loxP* disruption cassette located on the pUG66 plasmid (22) was used to delete *GPD1* in *MATα* background in order to construct strain aB. The correct integration of the disruption cassettes into the *GPD1* gene locus was verified by PCR (Table 2).

In order to replace the native *GPD2* promoter by promoters of much lower activities, the *TEF1* promoter mutants 2 and 7 of our previously published promoter collection for fine-tuning gene expression in yeast (33) were used. Both promoters were located on CEN/ARS plasmids, which contained the *loxP-KanMX-loxP* cassette upstream of the *TEF1* promoter mutant. The replacement of the native *GPD2* promoter by the low-strength promoters in a *gpd1Δ* background (strain aB) was confirmed by PCR diagnosis using the primers listed in Table 2.

Mating, sporulation, and tetrad analysis. In order to obtain all combinations of promoter engineered *GPD1* and *GPD2* expression levels, the strains a₇b and a₂b (both *MATa*) described by Pagliardini et al. (37) were mated with the newly created strains ab₇ and ab₂ (both *MATα*). The diploids were grown overnight in YD medium, harvested, and transferred to sporulation plates containing 1.5% agar and 1% potassium acetate at pH 6. The plates were incubated at 25°C for 5 days. Asci were pretreated with 1,000 U of lyticase (Sigma)/ml to degrade the ascus walls before spore dissection. Dissected spores were grown on YD medium, and segregants afterward were replica plated onto YD medium containing either the antibiotic phleomycin or Geneticin (G418) at the above-mentioned concentrations in order to check the presence of the resistance markers *KanMX* and *ble^R*. The segregants which grew in the presence of both antibiotics were parental types (strains a₇b, a₂b, ab₇, and ab₂). Recombination types that had lost the Geneticin resistance but were resistant to phleomycin corresponded to the double deletion strain *gpd1Δ gpd2Δ* (strain ab). In recombination types with resistance to Geneticin and sensitivity against phleomycin both *GPD1* and *GPD2*, the promoters were replaced by the *TEF1* promoter mutant versions TEFmut2 or TEFmut7 (strains a₇b₇, a₇b₂, a₂b₇, and a₂b₂). The preselected segregants were checked by PCR for the presence of the promoter cassettes or gene deletions. PCR primers for *GPD1* promoter replacement and *GPD2* deletion have been described in Pagliardini et al. (37), while those for *GPD2* promoter replacement and *GPD1* deletion are given in Table 2.

Measurement of specific GPDH activity. To determine the specific GPDH activity, yeast strains were grown in minimal medium (49) containing 2% (wt/vol) glucose in shake flasks. The GPDH activity was measured in exponentially growing cells (i.e., an optical density at 600 nm [OD₆₀₀] of ca. 1) according to a previously described method (18, 34). One unit of enzyme activity corresponds to the conversion of 1 μmol of NADH to NAD⁺ per min (49).

Growth tests on solid medium. Cells were grown overnight in YD medium. The OD₆₀₀ was determined and adjusted with 0.85% NaCl solution to an OD₆₀₀ of 1. A 1:10 serial dilution of these cultures was prepared in triplicate using 0.85% NaCl solution, and 5 μl of each dilution was spotted onto different solid

TABLE 1. *S. cerevisiae* strains used

Group	Strain ^a	Code ^b	Genotype	Source or reference
I	CEN.PK113-7D	AB	<i>MATa</i>	48
	CEN.PK113-1A	AB	<i>MATα</i>	P. Kötter
	IA 1†	aB	<i>gpd1Δ::loxP-ble^R-loxP</i>	This study
	CE1*	Ab	<i>gpd2Δ::loxP-ble^R-loxP</i>	This study
	CE1.01*	ab	<i>gpd1Δ::loxP-KanMX4-loxP</i> <i>gpd2Δ::loxP-ble^R-loxP</i>	This study
II	CE1.71*	a ₇ b	<i>gpd2Δ::loxP-ble^R-loxP</i> <i>GPD1pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 7	37
	CE1.21*	a ₂ b	<i>gpd2Δ::loxP-ble^R-loxP</i> <i>GPD1pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 2	37
	IA1.743†	ab ₇	<i>gpd1Δ::loxP-ble^R-loxP</i> <i>GPD2pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 7	This study
	IA1.232†	ab ₂	<i>gpd1Δ::loxP-ble^R-loxP</i> <i>GPD2pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 2	This study
III	HGW12-P4‡	a ₇ b ₇	<i>GPD1pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 7 <i>GPD2pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 7	This study
	HGW15-F6‡	a ₇ b ₂	<i>GPD1pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 7 <i>GPD2pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 2	This study
	HGW14-J6‡	a ₂ b ₇	<i>GPD1pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 2 <i>GPD2pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 7	This study
	HGW17-H5‡	a ₂ b ₂	<i>GPD1pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 2 <i>GPD2pΔ::loxP-KanMX4-loxP-TEF1p</i> mutant 2	This study

^a *, strains derived from CEN.PK113-7D; †, strains derived from CEN.PK113-1A; ‡, strains isolated after mating of the “*” strains with the “†” strains of group II and sporulation.

^b Strain codes: A, *GPD1* controlled by the natural promoter; a, *gpd1Δ*; a₇, *GPD1* controlled by the TEFmut7 promoter; a₂, *GPD1* controlled by the TEFmut2 promoter; B, *GPD2* controlled by the natural promoter; b, *gpd2Δ*; b₇, *GPD2* controlled by the TEFmut7 promoter; b₂, *GPD2* controlled by the TEFmut2 promoter.

media. The minimal medium used for the spot test contained 1.7 g of yeast nitrogen base (Fisher Scientific) liter⁻¹, 5 g of NH₄SO₄ liter⁻¹, and 2% glucose (wt/vol). For testing osmotolerance, the glucose concentration was increased to 25% (wt/vol). Growth was monitored for 2 to 3 days at 30°C. Anaerobic incubation of agar plates was carried out in an anaerobic jar. The anaerobic environment was generated by using Anaerocult A (Merck, Darmstadt, Germany) and controlled with indicator stripes.

Batch fermentations under quasi-anaerobic conditions in both minimal and industrial media (wheat liquefact). The fermentations were carried out in 250-ml Erlenmeyer flasks containing 150 ml of medium inoculated with yeast cells adjusted to an initial OD₆₀₀ of 1. The inoculum was prepared in minimal medium containing 2% (wt/vol) glucose. Flasks were equipped with air locks, ensuring the exclusion of oxygen but allowing the release of CO₂. This setup was chosen to imitate industrial bioethanol production and can be considered to provide quasi-anaerobic conditions for the major part of the fermentation, i.e., after a short initial phase during which cells consumed residual oxygen. The fermentations were performed at 30°C, and cultures were continuously mixed at 200 rpm by using a magnetic stirrer.

The synthetic minimal medium used for the fermentation experiments was composed as follows: (NH₄)₂SO₄, 3 g liter⁻¹; KH₂PO₄, 3.51 g liter⁻¹; K₂HPO₄ · 3H₂O, 2.1 g liter⁻¹; MgSO₄ · 7H₂O, 0.74 g liter⁻¹; EDTA (disodium salt), 1 mg liter⁻¹; CaCl₂ · 2H₂O, 6 mg liter⁻¹; ZnSO₄ · 7H₂O, 9 mg liter⁻¹; FeSO₄ · 7H₂O, 6 mg liter⁻¹; H₃BO₃, 2 mg liter⁻¹; MnCl₂ · 4H₂O, 1.23 mg liter⁻¹; Na₂MoO₄ · 2H₂O, 0.6 mg liter⁻¹; CoCl₂ · 6H₂O, 0.8 mg liter⁻¹; CuSO₄ · 5H₂O, 0.5 mg liter⁻¹; KI, 0.2 mg liter⁻¹; D-biotin, 0.05 mg liter⁻¹; *p*-aminobenzoic acid, 1 mg liter⁻¹; nicotinic acid, 1 mg liter⁻¹; calcium pantothenate, 8 mg liter⁻¹; pyridoxine HCl, 5 mg liter⁻¹; thiamine HCl, 5 mg liter⁻¹; *m*-inositol, 25 mg liter⁻¹; and D-glucose, 50 g liter⁻¹.

The wheat liquefact (24.5% dry mass content) used in the simultaneous saccharification and fermentation (SSF) experiments was kindly provided by a local ethanol producer. The pH was adjusted to 4.5 with sulfuric acid. In order to obtain wheat hydrolysate from wheat liquefact, enzymatic starch hydrolysis was performed using Spirizyme (Novozyme, Denmark). In order to perform the SSF analysis of the wheat liquefact, Spirizyme was added up to 0.05% (wt/wt) of the wheat liquefact dry mass. At the same time, wheat liquefact was inoculated with a yeast OD₆₀₀ of 1. We added 5 mg liter⁻¹ of the antibiotic chloramphenicol to inhibit bacterial growth in the first hours of the fermentation. All Erlenmeyer flasks were weighed during fermentation to monitor CO₂ production as readout for the volumetric productivity.

In order to obtain the glucose concentration after the complete hydrolysis of the liquefact (for product yield calculations), an aliquot was incubated at 60°C for 24 h with the Spirizyme (without yeast).

Determination of biomass in minimal medium fermentations. The OD₆₀₀ was determined at the beginning and at the end of the fermentation. In addition, the yeast dry mass was determined at the end of fermentation by filtering 50 ml of culture through preweighed nitrocellulose filters with a pore size of 0.45 μm. The filters were washed once with distilled water and kept at 80°C for 2 days. Afterward, they were weighed again. The biomass at the start of the minimal medium fermentation was estimated from the OD₆₀₀ by using the dry mass OD₆₀₀/ratio obtained from samples taken at the end of fermentation.

Determination of ethanol, glycerol, and glucose. Glucose, glycerol, and ethanol concentrations in the fermentation supernatants were determined by high-pressure liquid chromatography (Waters Isocratic Breeze HPLC, ion-exchange column WAT010290) and refractive index detection (Waters 2414 RI detector) under the following conditions: 75°C column temperature, 5 mM H₂SO₄ used as the eluent, and a 1-ml min⁻¹ flow rate.

Yield calculations. The product yields in the minimal medium fermentations were calculated from the final product concentration (grams per liter) and the difference in glucose concentrations at the start and end of a fermentation (consumed glucose in grams per liter). The product yields in the SSF were based on the final product concentrations and the equivalent initial glucose concentration (the latter was measured in a completely hydrolyzed sample of wheat liquefact as described above).

Maximal volumetric production rate. The fermentation tubes were weighed throughout the fermentation process. The loss of the medium mass was recorded and related to the initial medium mass. The maximal volumetric ethanol production rate r_{\max} (g liter⁻¹ h⁻¹) was calculated from the monitoring of the mass loss during anaerobic fermentation as described by Zwietering et al. (53).

RESULTS

Genetic modifications of *GPD1* and *GPD2*. The goal of the present study was to study the effect of partly reduced Gpd1p and Gpd2p cellular levels (separately and in combinations) on the physiology of *S. cerevisiae*. The CEN.PK background was used as a model genetic background, since strains of the

TABLE 2. PCR primers and plasmids used as templates for amplification of *GPD1* disruption and *GPD2* promoter replacement cassettes and for verification of their correct integration in the *S. cerevisiae* genome

PCR application	Reference	Primer code	Sequence ^a
Amplification of <i>GPD1</i> deletion cassette; template pUG6 or pUG66	22	P56	CATCAAATCTATCCAACCTAATTTCGCACGTAGACTGGCTTGGTAT
		P57	cagctgaagcttcgtacgc CGACGTCCTTGCCCTCGCCTCTGAAATCCTTTGGAATGTGGTAAG gcataggccactagtggatctg
Verification of <i>GPD1</i> deletion		P58	CCGCACAACAAGTATCAGA
		P59	AAGTAAGGTCTGTGGAACAA
<i>GPD2</i> promoter replaced byTEFmut7; template p416-loxP-KmR-TEFmut7-yECitrine	33	A2631	TAGCTTACGGACCTATTGCCATTGTTATTCCGATTAATCTATTGTc
		A2633	agctgaagcttcgtacgc TGCCTTCGCTTAAGGAATGTGTATCTTGTTAATCTTCTGACAGCA AGCATttttctagaaaactag
<i>GPD2</i> promoter replaced byTEFmut2; template p416-loxP-KmR-TEFmut2-yECitrine	33	A2631	See above
		A2632	TGCCTTCGCTTAAGGAATGTGTATCTTGTTAATCTTCTGACAGCA AGCATttttctagaaaactgg
Verification of <i>GPD2</i> promoter replacement		A2766	ACGACGATGGCTCTGCCATTG
		A2767	AGGATCGGCCACTAGATTATGG

^a Nucleotides in capital letters were derived from the *S. cerevisiae* genomic sequence for homologous recombinations at the *GPD1* and *GPD2* loci. Sequences in lowercase letters served as primers for amplification of the deletion or promoter cassettes from the respective plasmids (22, 33).

CEN.PK family have been previously proposed as “acceptable references for quantitative yeast research” (48). We exclusively used prototrophic strains in order to avoid any potential artifacts based on auxotrophic markers (40). The strains with several residual activities of Gpd1p and Gpd2p in different combinations were obtained as described in Materials and Methods and depicted in Fig. 1. The resulting strains listed in Table 1 were classified into three groups. Group I contains the

wild-type (strain AB), *gpd1Δ* (strain aB), and *gpd2Δ* (strain Ab) single-deletion strains, as well as the *gpd1Δ gpd2Δ* double-deletion strain (strain ab). These strains served as references in our study since these genetic modifications correspond to those that have already been characterized by other authors though in a different genetic background (2, 5, 9, 23, 35, 41, 47). Strains of group II each have one deleted isogene and the other isogene under the control of either the TEFmut2 or the TEFmut7

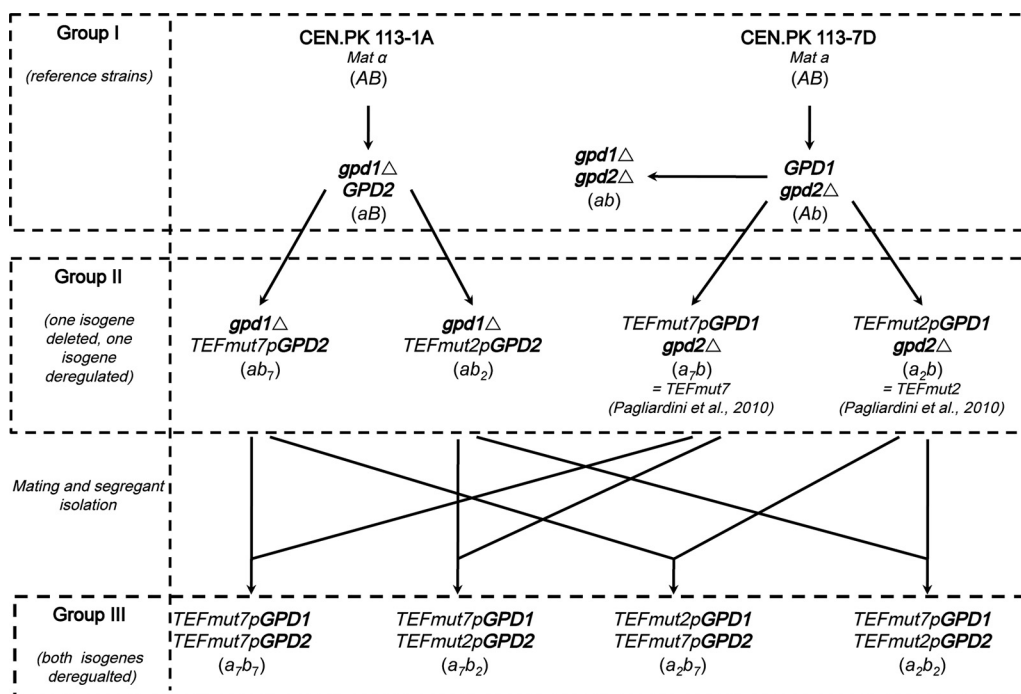


FIG. 1. Schematic overview showing the generation of 11 *S. cerevisiae* strains with reduced levels of Gpd1 and Gpd2 by genetic engineering and mating. The isogenic haploid and prototrophic wild-type strains CEN.PK113-7D and CEN.PK113-1A were used as starting strains.

promoter. These promoters are mutated versions of the *S. cerevisiae* *TEF1* promoter (33), i.e., not regulated by osmotic stress or anaerobiosis and weaker than the native promoters of *GPD1* and *GPD2*. Group III comprises strains having residual levels of both isogenes, i.e., the expression of both *GPD1* and *GPD2* gene is de- and downregulated by the *TEFmut2* or the *TEFmut7* promoter.

The specific GPDH activities of all engineered strains and the wild type (CEN.PK113-7D) were measured in exponentially growing cells from shake flask experiments (see Fig. 3). The deletion of the *GPD1* (strain aB) reduced GPDH activity to 26%, whereas the deletion of *GPD2* (strain Ab) led to an increase of GPDH activity by 12% compared to the wild type. The GPDH activity measured in the double-deletion *gpd1Δ gpd2Δ* strain (strain ab) was close to zero. The relative GPDH activities (expressed as a percentage of the wild-type activity) measured for group I reference strains more or less matched the findings obtained by other authors when studying the GPDH gene deletions in different genetic backgrounds (35, 46). The increased GPDH activity in the *gpd2Δ* deletion strain has been traced back to a kind of autoregulation, i.e., an increased transcription level of *GPD1* (46). This autoregulation should be abolished in all engineered strains that do not have the native *GPD1* promoter.

The main objective of the present study was to generate “intermediate” strains, i.e., specific GPDH activities between the level of the wild type (strain AB) and the *gpd1Δ* single-deletion strain (strain aB). When looking at the GPDH activities of the group II strains (see Fig. 3A), it becomes obvious that only strain a₇b represents such an “intermediate” strain, while the levels of the other three strains are equal to or lower than that of strain aB. In contrast, strains of group III show finely graded residual activities between those of strains AB and aB.

Growth during osmotic stress or anaerobic conditions. As mentioned in the introduction, the formation of glycerol has several important biological functions in *S. cerevisiae*. It enables growth under osmotic stress and its formation maintains cytosolic NADH/NAD⁺ balance. Surplus cytosolic NADH is generated during biomass formation, particularly when yeast grows in minimal medium and has to synthesize all amino acids *de novo* (1). Moreover, maintaining cytosolic redox balance is particularly important under anaerobiosis when cells cannot reoxidize the surplus NADH by the external NADH dehydrogenases coupled to the respiratory chain (7). Therefore, we tested our set of strains under conditions that particularly challenge glycerol formation, such as minimal medium in the presence or absence of oxygen, as well as minimal medium with 25% (wt/vol) glucose (to test osmotolerance). In the presence of 25% glucose, growth on solid medium was unaffected as long as *GPD1* expression was driven by the native *GPD1* promoter. Interestingly, 25% glucose also did not negatively influence the growth if the *GPD1* expression was controlled by the *TEFmut7* promoter, which has been shown to confer 16% of native *TEF1* promoter activity (33). The *TEFmut7* promoter is assumed to be much less active than the native *GPD1* promoter, particularly during osmotic stress, where the native *GPD1* promoter is strongly induced. Growth in 25% glucose was, however, severely decreased if *GPD1* expression was either under the control of the weak *TEFmut2* promoter (7% of the

native *TEF1* promoter) or completely abolished by gene deletion.

Without osmotic stress, virtually all strains grew fairly well on minimal medium in the presence of oxygen; however, the combination of minimal medium with anaerobiosis negatively influenced the growth of the strains with reduced Gpd1 and/or Gpd2 activities (Fig. 2). It seems that the impact of a reduced GPDH activity on growth under anaerobic conditions in minimal medium is independent of whether Gpd1 or Gpd2 activity was reduced. For example, there was no significant difference in growth between the strains a₇b and ab₇ or between the strains a₇b₂ and a₂b₇ under anaerobic conditions (Fig. 2). Remarkably, these pairs of strains showed clear differences in growth under osmotic stress, respectively (Fig. 2).

In contrast to several previous studies which showed that mutants lacking *GPD2* showed poor growth under anaerobic conditions (9, 35), our *gpd2Δ* deletion strain (Ab) did not show any remarkable growth problems under anoxic conditions in minimal medium (Fig. 2 and Table 3). Moreover, the expression of *GPD2* has been shown to be stimulated by cytosolic NADH accumulation as it occurs, e.g., during anoxic conditions in minimal medium (5). In fact, this upregulation was impossible in strains where *GPD2* was under the control of the *TEFmut* promoters. Our results put an isoform-specific role of Gpd2 under anaerobic conditions somewhat into question. It should, however, be emphasized that a growth test on solid medium does not allow a deduction of specific growth rates and is less sensitive than liquid medium when it comes to the detection of only slight differences in specific growth rates.

Fermentations in defined minimal medium. We first chose defined minimal medium in order to investigate the fermentation performance of our strains with different *GPD1/2* expression levels, particularly their glycerol and ethanol yields, as well as their maximal volumetric ethanol production rates. One advantage of using synthetic minimal medium is a generally high demand in NAD⁺ regeneration, particularly under anaerobic conditions, as explained above. In fact, slight differences in glycerol production capacity between the 12 strains were expected to be more pronounced in minimal medium than in industrially used media which are complex and contain amino acids (see below). Another major advantage of using defined minimal medium is the possibility to calculate precise carbon balances.

The small-scale batch fermentations in synthetic minimal medium were carried out under quasi-anaerobic conditions (see Materials and Methods). The glucose concentration used for this experiment was 50 g liter⁻¹, which was not expected to cause osmotic stress (50). Apart from the double-deletion *gpd1Δ gpd2Δ* strain, all strains were able to completely consume the sugar even though the required time was different (Table 3). Product yields are also shown in Table 3.

Although obtained under different conditions, we plotted the glycerol yield (obtained under quasi-anaerobic conditions) against the corresponding GPDH activity measured in shake flasks. The decision to measure all GPDH activities under aerobic conditions was due to comparable growth characteristics that was not achievable under anaerobic conditions. A fairly good correlation can be seen in Fig. 3B, when disregarding the wild type and the *gpd1Δ* deletion strain. In these two

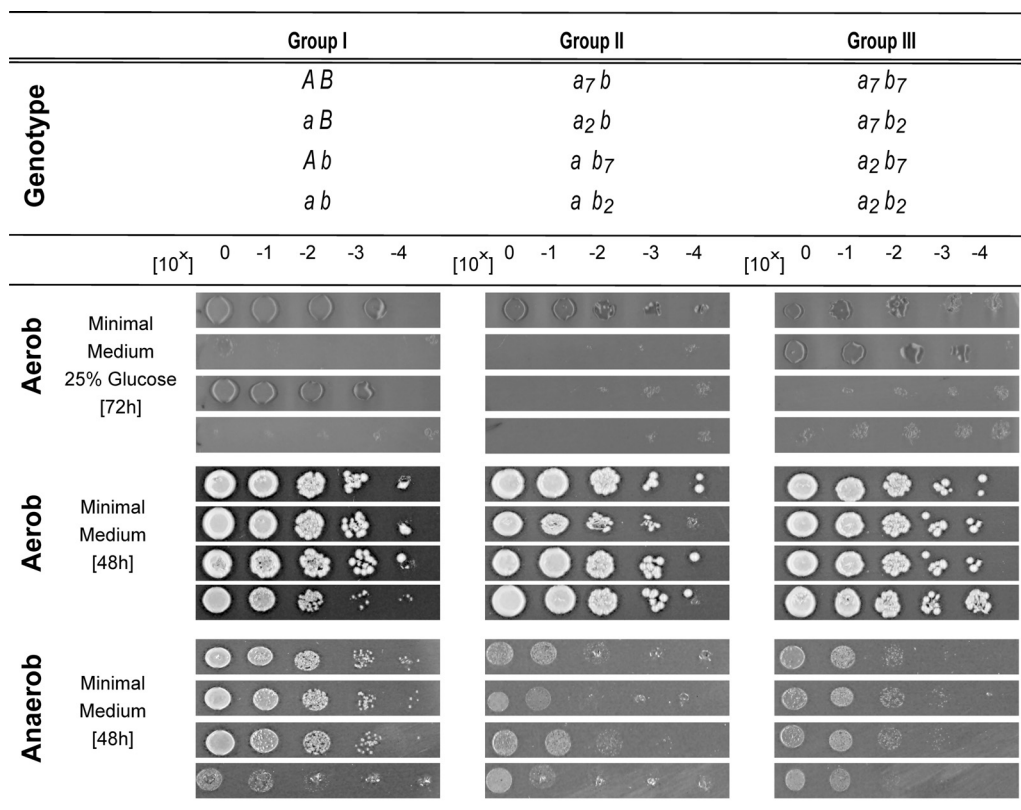


FIG. 2. Growth of *S. cerevisiae* wild-type and engineered strains with reduced levels of Gpd1 and Gpd2 under conditions that challenge glycerol formation, i.e., osmotic stress (synthetic minimal medium plus 25% glucose) and NADH accumulation (synthetic minimal medium with 2% glucose under anaerobiosis). Minimal medium under aerobiosis was used as a reference condition. Growth tests were performed on solid medium. Cells were pregrown in YD. The OD₆₀₀ values of all cultures were adjusted to 1, serial 1:10 dilutions were prepared, and 5 μ l of each dilution was spotted.

strains, *GPD2* expression is under the control of the native *GPD2* promoter. This promoter has been shown to be induced by NADH accumulation under quasi-anaerobic conditions (5, 46), and the GPDH activity measured under aerobic condi-

tions clearly underestimated the actual value under quasi-anaerobic conditions. Since all other strains have a deregulated or no *GPD2* gene, we do not assume major differences between anaerobic and aerobic GPDH activity.

TABLE 3. Fermentation time, product yields, and carbon balances of engineered strains of *S. cerevisiae* with reduced levels of Gpd1 and Gpd2, as well as the isogenic wild type, calculated at the end of fermentations in synthetic minimal medium under quasi-anaerobic conditions

Group	Strain	Time (h)	Yields of fermentation products (C-mol ratio product per glucose consumed)					Carbon recovery (%)
			Ethanol	Carbon dioxide ^a	Glycerol	Acetate	Biomass	
I	AB	25.8	0.586	0.275	0.058	0.008	0.064	99.1
	aB	28.5	0.595	0.276	0.050	0.006	0.075	100.2
	Ab	27.1	0.595	0.279	0.046	0.005	0.070	99.4
	ab	ND ^b	0.630	0.309	0.005	0.000	0.044	98.8
II	a ₇ b	68.2	0.623	0.295	0.021	0.001	0.065	100.6
	a ₂ b	152.3	0.632	0.324	0.009	0.000	0.050	101.6
	ab ₇	75.3	0.615	0.288	0.022	0.001	0.064	99.0
	ab ₂	140.5	0.627	0.311	0.017	0.001	0.054	101.0
III	a ₇ b ₇	51.0	0.613	0.288	0.032	0.002	0.060	99.3
	a ₇ b ₂	48.0	0.610	0.295	0.025	0.001	0.070	100.1
	a ₂ b ₇	54.2	0.621	0.287	0.023	0.001	0.067	99.9
	a ₂ b ₂	110.0	0.622	0.301	0.019	0.000	0.062	100.5

^a Carbon dioxide production was calculated from the total weight loss of the medium during fermentation.

^b ND, not determined. The *gpd1Δ gpd2Δ* double deletion was not able to completely consume the sugar.

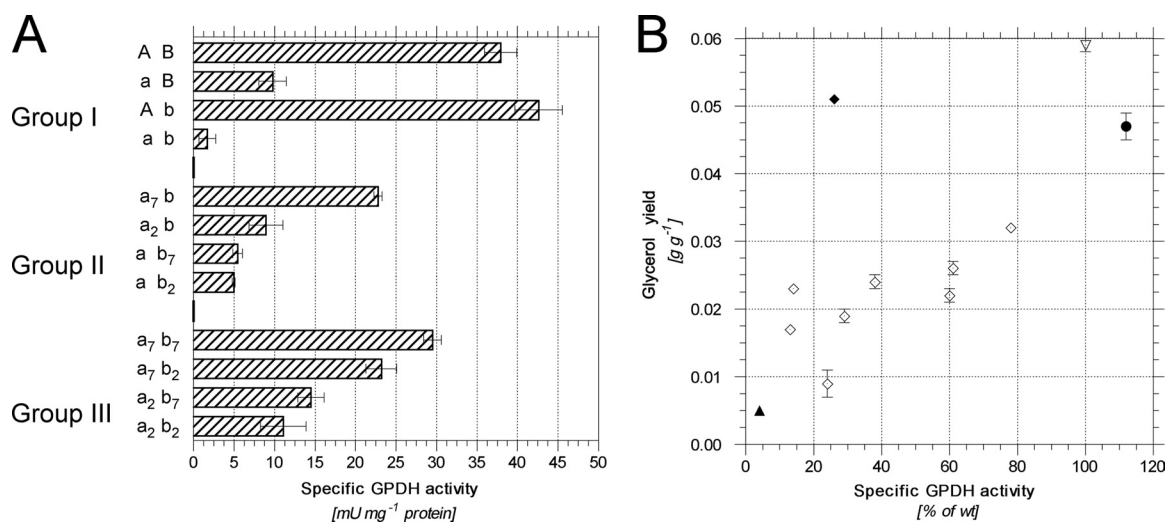


FIG. 3. Specific GPDH activities and glycerol yields of *S. cerevisiae* wild-type and engineered strains with reduced levels of Gpd1 and Gpd2. (A) GPDH activity measured in yeast cell extracts of aerobically grown cells harvested during the exponential growth phase. (B) Glycerol yield obtained with the strains in synthetic minimal medium under quasi-anaerobic conditions are plotted against the specific GPDH activity shown in panel A (for an explanation, see the text). Strains: ∇ , wild-type CEN.PK113-7D (strain AB); \blacklozenge , *gpd1Δ* (strain aB); \bullet , *gpd2Δ* (strain Ab); \blacktriangle , *gpd1Δ gpd2Δ* (strain ab); and \diamond , all intermediate strains (strains a₂b₂, a₇b₂, a₂b₇, a₇b₇, a₂b₇, a₇b₇, a₂b₂, and a₇b₇). All results shown are mean values including the standard deviations from three biological replicates.

To evaluate the effect of reduced glycerol formation capacity on other fermentation parameters, we decided to plot the relevant fermentation product yields against the glycerol yield. Figure 4A shows that any reduction in glycerol yield leads to a corresponding increase in the ethanol yield. This inverse correlation can be seen over the entire range of glycerol yields. However, the metabolic shift from glycerol to ethanol is accompanied by a reduction in the maximal volumetric ethanol production rate (expressed as g h⁻¹ liter⁻¹; for its calculation, see Materials and Methods) as shown in Fig. 4B. We can conclude that even a slight reduction in glycerol formation capacity resulted in a decrease in growth rate and/or specific ethanol production rate. This is in contrast to the biomass yield, where we can only see a reduction at glycerol yields lower than 50% of the wild-type level (Fig. 4C). Interestingly, acetate yields exhibited a nice direct correlation with glycerol yield (Fig. 4D), particularly in the range between 50 and 100% of wild-type glycerol yield. Carbon balances were nicely closed for virtually all strains (Table 3).

SSF in wheat liquefact. It was of interest to investigate our set of strains in media and/or conditions relevant to industrial practice. Hydrolyzed starch from corn and other small grains is a common carbon source in fuel ethanol production. For our study, wheat liquefact was provided by a local bioethanol producer. Wheat liquefact contains mainly dextrins and results from milling, cooking, and α -amylase treatment of wheat kernels (27). In order to further convert the dextrins into fermentable sugars, a treatment with glucoamylase is required. Two different scenarios are common in industry (27): separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In SHF, the glucoamylase treatment of wheat liquefact is performed before the hydrolysate is inoculated with yeast. The sugar concentration at the end of the mashing process was 200 g liter⁻¹ and thus represents an osmotic stress for the yeast cells. In the SSF scenario,

the hydrolytic enzymes plus the yeast are added to the liquefact at the same time, i.e., saccharification and fermentation occur simultaneously. This SSF process, which is now more frequently used in industrial practice, does not allow an accumulation of sugars released from starch and thus avoids osmotic stress. Based on our plate growth assays (Fig. 2), we already knew that osmotic stress was deleterious for the strains with very low *GPD1* expression levels. Therefore, we decided to investigate our strains in the SSF process. The SSF experiments in wheat liquefact revealed that not all strains were able to completely ferment the available sugars in an acceptable time. In fact, the wild type required 53 h to finish fermentation and, apart from the single deletion strains (strains aB and Ab), only four further strains (strains a₇b, a₇b₇, a₇b₂, and a₂b₇) completed the fermentation within a period no longer than 150 h (Table 4).

The glycerol yield obtained with the wild type in the SSF was only half of the glycerol yield in minimal medium (Fig. 4 and Table 3) because of two reasons. First, wheat mash (hydrolysate) is rich in nutrients and thus also contains amino acids, even though assimilable nitrogen in wheat hydrolysate is not sufficient to support fermentation at the fastest rate (45). Second, it is well known that the major part of glycerol formation is coupled to growth (10). Glycerol yield per sugar consumed becomes lower if there is a longer production phase after finishing cell growth. This was the case in wheat liquefact since the total amount of glucose (180 g liter⁻¹) was much higher compared to the experiments in minimal medium (50 g liter⁻¹). Due to the generally lower glycerol yields in wheat liquefact, the differences between strains were, as expected, less pronounced in the SSF compared to minimal medium. For example, the reduction of the glycerol yield in the a₇b strain was only 33% (Table 4) compared to the wild type, while the same strain showed a reduction by 63% (Table 3) in the fermentations in minimal medium.

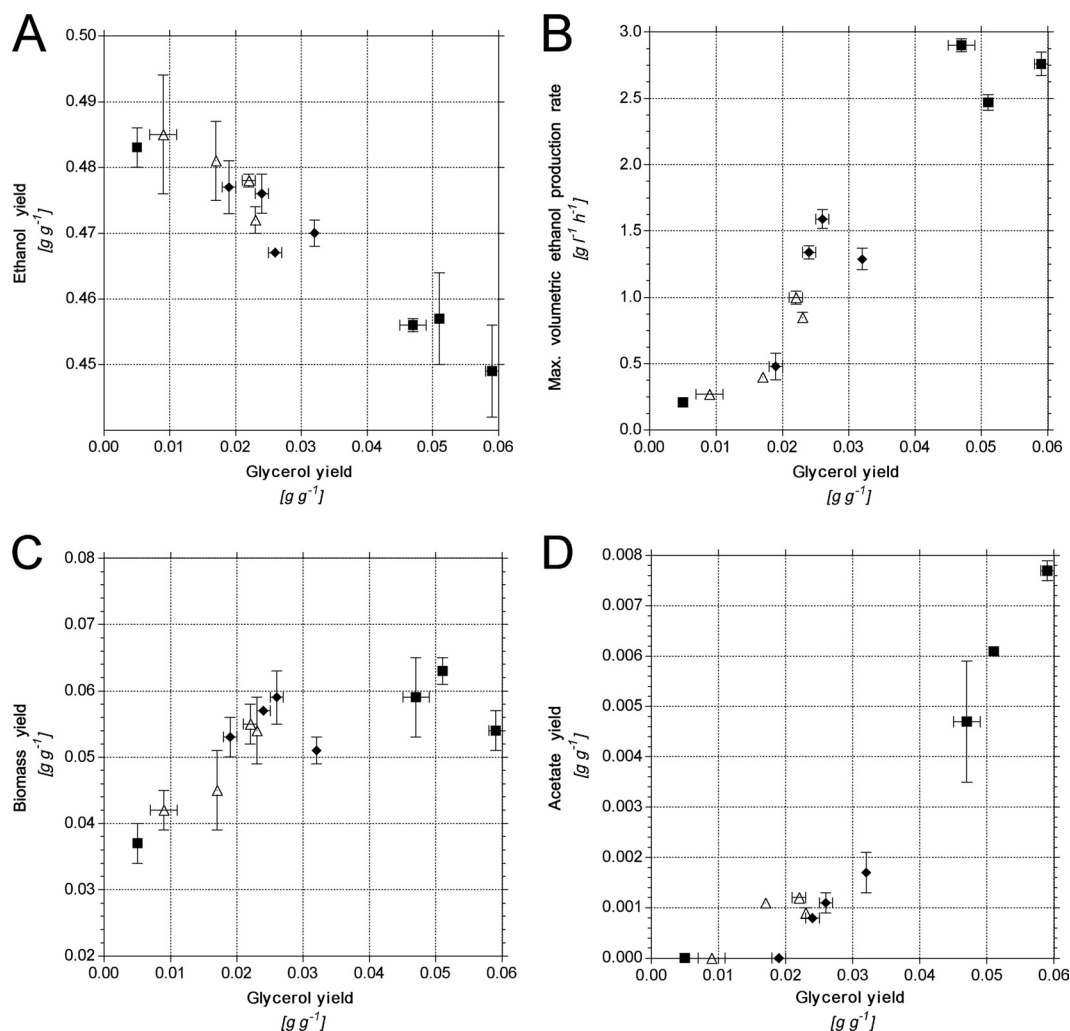


FIG. 4. Ethanol yields (A), maximal volumetric ethanol production rates (B), biomass yields (C), and acetate yields (D) of *S. cerevisiae* wild-type and engineered strains with reduced levels of Gpd1 and Gpd2 obtained in synthetic minimal medium under quasi-anaerobic conditions were plotted against the corresponding glycerol yield, respectively. Symbols: ■, group I; △, group II; ♦, group III. All results shown are mean values including the standard deviations from two biological replicates. Yields were calculated from the final product concentration divided by the consumed glucose. To calculate the maximal volumetric ethanol production rate, the flasks were weighed throughout the fermentation process. The loss of the medium mass was recorded. It was assumed that the mass loss of the medium was solely due to production of CO_2 . Moreover, our assumption included the finding that ethanol and CO_2 are produced in equimolar amounts. The maximal volumetric ethanol production rate r_{max} (expressed as $\text{g liter}^{-1} \text{h}^{-1}$) was determined by applying the mass balance on the fermentation system (see Materials and Methods).

Regarding the four “intermediate” strains which were able to completely ferment the available sugars, the reductions in glycerol yield compared to the wild type were 33.3% in strain a_7b , 25.9% in strain a_7b_7 , and 29.6% in strains a_7b_2 and a_2b_7 . Consistent with this, these strains also showed an increase in ethanol yield that was 1.0% in strain a_7b , 0.6% in strain a_7b_7 , 1.0% in strain a_7b_2 , and 0.8% in strain a_2b_7 . Although the increases in ethanol yield do not seem to be significant (when looking at the standard deviations), we still believe that the slight increases are true due to the following considerations. First, we measured a slight increase in the ethanol yield in all four strains considered here (i.e., strains a_7b , a_7b_7 , a_7b_2 , and a_2b_7). Second, a reduction in glycerol yield by 30% compared to the wild type (i.e., from 0.027 to 0.019 g g^{-1}) should be, in theory, accompanied by an increase in the ethanol yield by 0.84% (from 0.487 to 0.491 g g^{-1}) provided that all carbon is

redirected from glycerol to ethanol. However, we previously showed that the increase in the ethanol yield with strains a_7b and a_2b observed in aerobic ethanolic fermentation was due to redirection of the carbon from not only glycerol but also from biomass to ethanol (37).

It has to be emphasized once more that even slight increases in the ethanol yield are not irrelevant and could have major impacts on profits of a bioethanol company. However, the increases in ethanol yield in SSF also come along with a decrease in maximal volumetric ethanol production rates accompanied by prolonged fermentation times (Table 4). For example, the two strains which show a 1% increase in ethanol yield (strains a_7b and a_7b_2) show a maximal volumetric production rate that is decreased by ca. 56 or 44% compared to the wild type, which means that the fermentation time was roughly doubled for these strains (Table 4).

TABLE 4. Performance of engineered strains of *S. cerevisiae* with reduced levels of Gpd1 and Gpd2, as well as the isogenic wild-type SSF of wheat liquefact, under quasi-anaerobic conditions^a

Group	Strain	Fermentation time (h)	Mean yield on total substrate (g g ⁻¹) ± SD		Mean r_{\max} (g liter ⁻¹ h ⁻¹) ± SD
			Glycerol	Ethanol	
I	AB	53	0.027 ± 0.002	0.487 ± 0.004	4.8 ± 0.1
	aB	59	0.028 ± 0.001	0.486 ± 0.001	4.5 ± 0.7
	Ab	64	0.024 ± 0.002	0.489 ± 0.001	3.9 ± 0.2
	ab	>150	ND	ND	ND
II	a ₇ b	107	0.018 ± 0.000	0.492 ± 0.003	2.1 ± 0.1
	a ₂ b	>150	ND	ND	ND
	ab ₇	>150	ND	ND	ND
	ab ₂	>150	ND	ND	ND
III	a ₇ b ₇	87	0.020 ± 0.001	0.490 ± 0.006	2.7 ± 0.2
	a ₇ b ₂	99	0.019 ± 0.001	0.492 ± 0.003	2.5 ± 0.1
	a ₂ b ₇	150	0.019 ± 0.001	0.491 ± 0.004	1.6 ± 0.1
	a ₂ b ₂	>150	ND	ND	ND

^a The results shown are mean values of two independent experiments ± the standard deviation. The ethanol yield, glycerol yield, and the maximum volumetric ethanol production rate (r_{\max}) were only calculated if fermentation was completed within 150 h. Fermentation was considered to be completed when the mass of the medium did not change anymore. ND, not determined.

DISCUSSION

Even slight reductions in glycerol production (which result in ethanol yield increases without impacting volumetric productivity) would be of great interest for the bioethanol industry due to the fact that ethanol is a bulk product and feedstock is a major cost factor. GPDH is known to be the rate-controlling enzyme of glycerol formation, and the isogenes *GPD1* and *GPD2* encode for it. On one hand, many approaches to reduce glycerol formation have included the deletion of one or both isogenes. On the other hand, the two gene products have been shown to fulfill different important biological functions in the cell. Thus, the complete deletion of one gene might have negative impacts in particular when it comes to industrial bioethanol production subjecting the yeast to several stress conditions (28). We wondered whether it is more sustainable for the cell if isoenzyme activities are not completely shut down to zero but rather reduced to a certain residual level. Here, several prototrophic strains were constructed which have different expression levels of both isogenes (i.e., gene expression is driven by either the native promoter, the TEFmut7 promoter, or the TEFmut2 promoter or completely abolished by gene deletion). Different combinations of *GPD1* and *GPD2* expression levels were generated. The resulting set of strains represents a comprehensive tool for in-depth quantitative studies about the impact of reduced Gpd1 and/or Gpd2 levels on the physiology of *S. cerevisiae*.

The use of prototrophic strains is particularly important in studying glycerol formation since the flux through this pathway is strongly dependent on whether amino acids have to be *de novo* synthesized or taken up from the medium (see below). In addition, medium composition strongly influences glycerol formation. We therefore believe that it is crucial to test engineered strains in real industrial media which, in terms of free amino acid availability, strongly differ from media used in fundamental yeast research such as synthetic minimal medium or YPD complex medium. To our knowledge, this is the first study of strains with reduced GPDH activity in a medium relevant in industrial bioethanol production.

S. cerevisiae strains with *GPD1* deleted have been proven to be osmosensitive (2). Although osmotic stress can be virtually avoided during bioethanol production by using the SSF process, we studied the behavior of our strains when subjected to osmotic stress. This was particularly interesting because the replacement of the native promoters of *GPD1* and *GPD2* by *TEF1* promoter versions must be accompanied by a loss of native gene regulation by osmotic stress and NADH accumulation, respectively. Interestingly, the three “intermediate” strains a₇b, a₇b₇, and a₇b₂ did not show any significant loss of osmotolerance (Fig. 2) despite the replacement of the native *GPD1* promoter by a constitutive and weaker promoter. The residual GPDH activity in these strains was between 60 and 78% of the wild type (Fig. 3A) under non-osmotic stress conditions, and the activity of the wild type is known to even increase significantly by osmotic stress (2). Our result showing that *GPD1* expression driven by the weaker and non-osmosensitive TEFmut7 promoter is sufficient to cope with osmotic stress such as the wild type is particularly interesting in the context of recent findings published by other authors suggesting that *GPD1* upregulation only plays a minor (if any) role in the survival of osmotic shock (12, 32, 51). Instead, metabolic changes seem to be far more important for increased glycerol formation and counteracting osmotic stress.

Another interesting result of our study was the striking difference regarding how one *GPD* isogene was able to replace the other one for its major biological function, such as osmotic stress tolerance and NADH balancing under anaerobiosis, respectively. Obviously, the major function of *GPD2*, i.e., redox balancing, can be more easily complemented by *GPD1*, whereas *GPD2* is less efficient in taking over the role of *GPD1* in osmotic stress response. In fact, no significant differences in growth between strains a₇b and ab₇ or between strains a₇b₂ and a₂b₇ in minimal medium under anaerobic conditions could be detected, whereas these pairs of strains showed clear differences under osmotic stress, respectively (Fig. 2). It rather seems that the role of GPDH in NADH balancing can be virtually equally fulfilled by both isoenzymes. This even seems

to be confirmed by the nice correlation of GPDH activities and glycerol yields in Fig. 3B (provided that the GPDH activities measured in the present study are the sum of residual Gpd1 and Gpd2 activities).

In contrast to redox balancing, osmostress tolerance was rather dependent on the residual level of only one isoenzyme which is *GPD1*. Probably, the action of the metabolic regulation on Gpd1 for glycerol formation during osmostress recently proposed by Bouwman et al. (12) is stronger than on Gpd2. The detailed mechanism of this regulation is not yet known. However, it has been assumed by Jung et al. (26) that Gpd1 relocates to the nucleus during osmostress. If this relocation solely occurs on Gpd1 and not on Gpd2, it could explain the inability of Gpd2 to act in the same way as Gpd1 after osmostress. We have to emphasize that the expression levels of *GPD1* and *GPD2* are driven by opposite promoter strengths in the strains a_7b_2 and a_2b_7 . Thus, we can expect that the levels of transcription for *GPD1* in strain a_7b_2 should be comparable to the level for *GPD2* in strain a_2b_7 and *vice versa*. Nevertheless, we cannot completely exclude that a different efficiency of translation was the reason for the difference in phenotype.

The metabolic shift from glycerol to ethanol was accompanied by a reduction in metabolic rates, as shown here by the maximal volumetric ethanol production rates (see the discussion below). However, the biomass yield (per glucose consumed) was not reduced as long as glycerol yield was >50% of the wild-type level. The question arises which route of NADH reoxidation was used then instead of glycerol formation. The reduction of NADH production during acetate production in strains with reduced GPDH activity observed by us and other authors (24, 46) can also not serve as a quantitative explanation since acetate production was extremely low. We rather believe that cells with reduced glycerol formation capacity were to a certain part (i.e., up to 50% reduction of glycerol yield) able to adjust their metabolism for maximizing biomass yield. In this regard, the reader is referred to the discussion of Valadi et al. (46), who proposed that NADP^+ could probably substitute for NAD^+ as a cofactor in biosynthetic pathways to a larger extent than expected.

A major industrially relevant question of the present study concerns the level that Gpd1 and Gpd2 expression can be reduced without a negative impact on the maximal volumetric ethanol production rate. Based on our results, we can conclude that even a small reduction in glycerol yield leads to a corresponding negative impact on maximal volumetric ethanol production rate in CEN.PK, which means that this strain does not produce more glycerol than absolutely necessary for fulfilling the crucial biological functions. It has to be however mentioned that three industrial *S. cerevisiae* strains have been demonstrated to show a 60 to 80% higher glycerol yield compared to the model strain CEN.PK used in the present study (16). Therefore, it remains to be studied whether these commercial strains differ from CEN.PK in that they produce more glycerol than absolutely necessary for maximal growth.

The work presented here shows that higher yields in ethanol seem to be feasible if longer fermentation times were acceptable. However, a higher risk of contamination impairs such an approach. In fact, industrial ethanol production is carried out under nonsterile conditions, and quick ethanol production by

yeast is required in order to efficiently inhibit the growth of lactic acid bacteria and compete for the available sugars.

In summary, strains with intermediate activities of Gpd1 and Gpd2 showed an improved ethanol yield combined with the ability to completely ferment the sugars in both minimal medium and wheat liquefact (SSF). Our result implies that these strains were able to tolerate the high ethanol concentration at the end of the wheat liquefact SSF (up to 90 g liter⁻¹), i.e., fermentation did not stick due to an elevated sensitivity against high ethanol caused by reduced GPDH activity. In fact, the *gpd1Δ gpd2Δ* double-deletion strain has been shown to be severely affected in ethanol tolerance (11). Moreover, three of our strains were comparable to the wild type in terms of osmotolerance. Thus, compared to the *gpd1Δ gpd2Δ* double-deletion strain which virtually cannot grow and ferment at all under anaerobic conditions, our “intermediate” strains represent a clear improvement, even though the maximal volumetric ethanol production rate was only 50% in these strains compared to wild type. Although strains solely based on partly reduced levels of both Gpd1 and Gpd2 might not be directly applicable in bioethanol production, they seem to be a good starting point for further metabolic engineering approaches which provide alternative pathways for NADH reoxidation such as the strategy recently presented by Guandalupe et al. (21).

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