

Effects of *GPD1* Overexpression in *Saccharomyces cerevisiae* Commercial Wine Yeast Strains Lacking *ALD6* Genes

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The utilization of *Saccharomyces cerevisiae* strains overproducing glycerol and with a reduced ethanol yield is a potentially valuable strategy for producing wine with decreased ethanol content. However, glycerol overproduction is accompanied by acetate accumulation. In this study, we evaluated the effects of the overexpression of *GPD1*, coding for glycerol-3-phosphate dehydrogenase, in three commercial wine yeast strains in which the two copies of *ALD6* encoding the NADP⁺-dependent Mg²⁺-activated cytosolic acetaldehyde dehydrogenase have been deleted. Under wine fermentation conditions, the engineered industrial strains exhibit fermentation performance and growth properties similar to those of the wild type. Acetate was produced at concentrations similar to that of the wild-type strains, whereas sugar was efficiently diverted to glycerol. The ethanol yield of the *GPD1 ald6* industrial strains was 15 to 20% lower than that in the controls. However, these strains accumulated acetoin at considerable levels due to inefficient reduction to 2,3-butanediol. Due to the low taste and odor thresholds of acetoin and its negative sensorial impact on wine, novel engineering strategies will be required for a proper adjustment of the metabolites at the acetaldehyde branch point.

Glycerol is the most abundant by-product of alcoholic fermentation after ethanol and carbon dioxide (19, 22, 25) and contributes to the sensory characteristics of wine, particularly smoothness and overall body (8, 18, 19). Consequently, many attempts to increase the glycerol yield during fermentation have been made (34, 37). Furthermore, rerouting of the carbon flux towards glycerol leads to a decreased ethanol yield as a result of carbon diversion and decreased NADH availability. This is of great importance, since a large number of quality wines produced by modern winemaking practices, which favor harvesting fully ripened grapes, frequently contain an excessive ethanol content. Consequently, in recent years, there has been an increasing demand for wines with reduced ethanol content, which has been driven by both health and economic concerns. The utilization of strains with a low ethanol yield to produce wines with reduced alcohol content may be an alternative approach to physical methods, which are expensive and detrimental for aroma compounds.

In *Saccharomyces cerevisiae*, glycerol is synthesized by the reduction of dihydroxyacetone phosphate followed by a subsequent dephosphorylation catalyzed by glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, respectively. Glycerol overproduction has been obtained by overexpression of either *GPD1* or *GPD2*, encoding glycerol-3-phosphate dehydrogenase isoforms (5, 15, 16, 17, 24, 27). Wine yeast strains overexpressing *GPD1* produce between 12 and 18 g/liter of glycerol, and the final ethanol concentration is 1% to 1.5% (vol/vol) lower (23, 24). As a result of carbon shift and increased utilization of NADH through the glycerol pathway, *GPD1* strains exhibit major alterations in central metabolism,

such as increased formation of succinate, acetaldehyde, acetoin, 2,3-butanediol, and acetate.

Excessive production of acetate (above 1 g/liter) is a major side effect, since the maximum amount desirable in wine is around 0.6 g/liter. Acetate is produced mainly during fermentation by oxidation of acetaldehyde catalyzed by the cytosolic Mg²⁺-activated NADPH-dependent acetaldehyde dehydrogenase (ACDH) Ald6p (14, 26, 31). Since a deletion of *ALD6* results in a substantially lower acetate yield (26), this strategy was applied to laboratory- and wine-derived strains overexpressing *GPD1* (6, 7, 23). However, industrial strains exhibit major differences compared to laboratory strains (24). In addition, the environmental conditions for yeast cells during grape fermentation must significantly differ from those in laboratory media. Therefore, the consequences of *GPD1* overexpression and *ALD6* deletion on yeast physiology and technological performance need to be appraised in commercial wine yeast strains under enologically relevant conditions. We constructed three industrial strains from which the two copies of *ALD6* were deleted and in which *GPD1* is overexpressed. The potentialities of these strains for the wine industry were evaluated by analyzing the impact of these modifications on growth, fermentation performance, and metabolite profiles during fermentation under winemaking conditions. The results of this evaluation will serve as a strong basis for further improvements of these strains.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* DH5 α was used for cloning experiments. *E. coli* cultivation and media were described previously (32). All the yeast strains used in this study were *S. cerevisiae* strains and are described in Table 1. Yeast strains were maintained and grown in YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose). The multicopy vector pVT100U-*GPD1* carrying *GPD1* under the control of the *ADH1* promoter and terminator and its derivative, pVT100U-ZEO-*GPD1*, have been described previously (15, 24). Transformants were selected on SD minimal medium (6.7 g/liter yeast nitrogen base without amino acids, 2% glucose) or on YPD medium supplemented with 150 μ g/ml of geneticin or 100 μ g/ml of phleomycin.

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TABLE 1. *S. cerevisiae* strains used and constructed in this study

Strain	Genotype	Source or reference
V5	<i>MATa ura3</i>	INRA UMR SPO, derivative from Champagne wine strain
V5 <i>GPD1</i>	<i>MATa ura3/pVT100U-GPD1</i>	24
V5 <i>ald6</i>	<i>MATa ura3 ald6::loxP</i>	31
V5 <i>ald6 GPD1</i>	<i>MATa ura3 ald6::loxP/pVT100U-GPD1</i>	This study
K1M (K1 marquée)		Commercial wine yeast, ICV-INRA, Montpellier
K1M <i>GPD1</i>	<i>pVT100U-ZEO-GPD1</i>	This study
K1M <i>ald6</i>	<i>ald6::loxP ald6::kanMX</i>	This study
K1M <i>ald6 GPD1</i>	<i>ald6::loxP ald6::kanMX/pVT100U-ZEO-GPD1</i>	This study
VL1 (Zymaflore VL1)		Commercial wine yeast, Institut d'Oenologie, Bordeaux
VL1 <i>GPD1</i>	<i>pVT100U-ZEO-GPD1</i>	This study
VL1 <i>ald6</i>	<i>ald6::loxP ald6::kanMX</i>	This study
VL1 <i>ald6 GPD1</i>	<i>ald6::loxP ald6::kanMX/pVT100U-ZEO-GPD1</i>	This study
BC (Uvaferm BC)		Commercial wine yeast, Davis University, Pasteur no. 1877
BC <i>GPD1</i>	<i>pVT100U-ZEO-GPD1</i>	This study
BC <i>ald6</i>	<i>ald6::loxP ald6::kanMX</i>	This study
BC <i>ald6 GPD1</i>	<i>ald6::loxP ald6::kanMX/pVT100U-ZEO-GPD1</i>	This study

Fermentation conditions. Batch fermentation experiments were carried out in MS synthetic medium that simulates standard grape juice as described previously (1). The MS medium contains 200 g/liter glucose, 6 g/liter malic acid, 6 g/liter citric acid, and a nitrogen source composed of 120 mg/liter nitrogen from ammonium and 340 mg/liter from amino acids. The medium was supplemented with uracil (50 mg/liter) and methionine (115 mg/liter) for growth of V5. During wine fermentation, yeast uses grape phytosterols and unsaturated fatty acids. To fulfill the lipid requirement of yeast cells during anaerobic growth, MS medium was supplemented with 7.5 mg/liter ergosterol, 0.21 g/liter Tween 80, and 2.5 mg/liter oleic acid. The pH of the medium is 3.3. Cells were precultured for 36 h at 28°C in 50-ml flasks filled with MS medium without agitation. The main fermentation culture was inoculated at a density of 1×10^6 cells per ml and carried out at 24°C or 28°C with continuous stirring (350 rpm) in fermentors of 1.1 liter (working volume) equipped with fermentation locks. The CO₂ release was determined by an automatic measurement of fermentor weight loss every 20 min. The CO₂ production rate dCO_2/dt is the first derivative of the amount of CO₂ produced with respect to time and was automatically calculated by polynomial smoothing of the CO₂ produced (30). Fermentation experiments were performed in triplicate. Since G418 is inefficient at low pHs, fermentations were realized in the absence of selection pressure. Consequently, plasmids were progressively lost during the culture as previously observed (24). Due to this rather uncontrolled process, independent cultures showed significant variation in the production of glycerol and consequently other metabolites, but the same evolution in metabolite profiles was verified in all the experiments.

Chromosomal mapping. Yeast chromosomal DNA was prepared in plugs as previously described (35), washed once in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) at 50°C for 30 min, and then washed three times in the same buffer at room temperature for 30 min. Each plug analyzed contained DNA isolated from about 5×10^7 cells. Electrophoresis was performed in a 1% (wt/vol) agarose (Seakem Gold) gel using a transverse alternating-field electrophoresis system (Geneline, Beckman) under the following conditions: a constant voltage of 250 V for a 6-h run time with a 35-s pulse time, followed by 20 h at 275 V with a 55-s pulse time at a constant temperature (14°C). DNA was transferred onto a nylon membrane (Hybond N; Amersham) as previously described (32). Chromosomes were hybridized with a ³²P-labeled *ALD6*-specific oligonucleotide probe (TCTCTGGGTCTTGGGTAGC) as previously described (4). Southern blots were analyzed with a PhosphorImager (Molecular Dynamics).

Deletion of *ALD6* in industrial strains. The strain V5 *ald6::kanMX* was constructed as previously described (31) using the short flanking homology PCR-based gene disruption method and the Cre-*lox* recombination system (12). To delete *ALD6* from the industrial strains, a fragment carrying the *ald6::kanMX* allele of V5 *ald6* was amplified using primers CGAGGTC AAGCCTGGCG TGTT and GCAGTTGTTGTACTAGCTTA, corresponding to regions upstream (positions -169 to -147) and downstream (positions 2178 to 2200) from the *ALD6* open reading frame. The primers were designed in such a way as to generate large homologous flanking regions to increase integration efficiency in industrial strains. This fragment, carrying the KanMX marker, was used to transform the industrial strains K1M, VL1, and BC by using the LiAc procedure (33). The transformants that were resistant to G418 were analyzed by PCR with total DNA using primers upstream and downstream from the deleted region,

followed by digestion of the PCR product by XhoI, which cleaves the KanMX4 module but is absent from *ALD6*. Analysis of 10 transformants for each transformed strain led to the selection of several strains lacking one *ALD6* copy and carrying at least one wild-type *ALD6* gene. The similar band intensity for each copy (deleted or not) suggested that these strains were diploid for *ALD6*. To delete the remaining wild-type copy, the KanMX marker was excised using the Cre-*lox* system (12). To use the pSH47 vector carrying Cre recombinase (12) in industrial strains, the positive marker ZEO^R, conferring resistance to phleomycin, was introduced. The transcriptional unit carrying Tn5 *ble* under the control of *TEF1p* and *CYC1t* was amplified from pUT332 (CAYLA) using primers CGAGCTCGGTACCCG and CGGGTACCCTTGCAAATTAAGCCTTCG, which introduced a KpnI site (underlined sequence). This fragment was digested by KpnI and ligated to KpnI-digested pSH47 to produce pSH47-ZEO. The KanMX marker was excised by the transformation of pSH47-ZEO and the subsequent elimination of the plasmid (12), and the additional *ALD6* copy was deleted using the same strategy described above. The deletion of the additional copy was verified by PCR and restriction analysis and confirmed by Southern blot with chromosomes. The industrial strains lacking the two *ALD6* alleles were transformed with pVT100U-ZEO-*GPD1*.

Analytical methods. Glycerol, acetate, succinate, and ethanol concentrations were analyzed by high-pressure liquid chromatography, and acetoin and 2,3-butanediol levels were measured by gas chromatography as previously described (15). Acetaldehyde concentrations were determined enzymatically according to the method of Lundquist (13).

RESULTS

Decreasing acetate production in a model strain producing up to 30 g/liter glycerol. A 60% reduction in acetate production has been obtained by the deletion of *ALD6* from the yeast strain V5 (26). To study *ALD6* inactivation in a strain in which the flux has been strongly shifted towards glycerol production, the V5 (*ura3*) and V5 *ald6* (*ura3*) strains were transformed with the multicopy vector pVT100U-*GPD1*, producing V5 *GPD1* and V5 *ald6 GPD1*, respectively (Table 1). As previously described (24), V5 *GPD1* produced higher glycerol (28.4 g/liter) and acetate (1.5 g/liter) levels than the control strain (6.6 g/liter of glycerol and 0.6 g/liter of acetate) during wine fermentation. Strain V5 *ald6 GPD1* produced an even higher glycerol concentration (33 g/liter), but the acetate accumulation (0.6 g/liter) was no higher than the wild-type level. This indicates that the *ALD6* deletion effectively reduces acetate formation, even in strains exhibiting a large shift of carbon flux towards glycerol. We therefore investigated the consequences of the *ALD6* de-

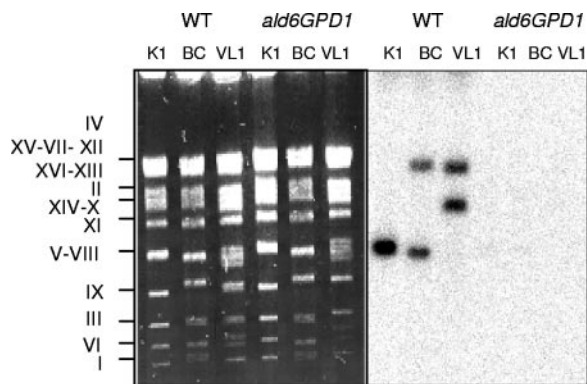


FIG. 1. Southern blot analysis of chromosomes of wild-type (WT) and *ald6 GPD1* VL1, K1M, and BC strains. Chromosomes were separated by pulsed-field electrophoresis. Membranes were hybridized with an *ALD6*-specific oligonucleotide probe.

letion in combination with *GPD1* overexpression in true commercial wine yeast strains during wine fermentation.

Deletion of *ALD6* from three commercial wine strains overexpressing *GPD1*. Wine yeast strains are considered to be genetically complex. Many strains are diploids, and some are aneuploids or polyploids (19, 20). In addition, they exhibit chromosome rearrangements and show a high level of chromosome size polymorphism (2, 21). To determine the number of *ALD6* copies present in the three wine yeast strains K1M, VL1, and BC and their localization, we performed Southern blot analysis of chromosomes. *ALD6* maps on chromosome XVI in the laboratory reference strain S288C. Chromosomal mapping analysis (Fig. 1) identified at least two copies of *ALD6*, each carried on a different chromosome in strains VL1 and BC. The hybridization signal for the large *ALD6* copy is consistent with its mapping on chromosome XVI, whereas the other copy was detected on a smaller chromosome, corresponding to a region where chromosomes II, XIV, and X comigrate (strain VL1) or to chromosomes V or VIII (strain BC). Only one hybridization signal was observed for strain K1M, which also corresponded to chromosomes V or VIII; however, the intensity of the signal suggested that strain K1M also carried two copies of *ALD6*. The data obtained for VL1 and BC are consistent with those of previous studies showing that although a majority of wine yeast strains have an approximately diploid DNA content (as VL1) (3), these strains may be aneuploids and frequently carry several homologous chromosomes of different sizes (2, 20).

To delete *ALD6* from the three strains, we used a modification of the short flanking homology PCR-based gene disruption method: the integrative fragments were flanked by large homologous regions to increase the recombination efficiency. Extensions of 154 bp and 239 bp were used, allowing the selection of several transformants with correct integration for each strain. Analysis of the transformants obtained after one round of transformation was consistent with the presence of two *ALD6* genes in each strain, with only one being deleted (data not shown). After excision of the KanMX module, a second transformation step was used to generate the double deletion mutants. The absence of any *ALD6* open reading

frame in the mutants was confirmed by Southern blotting with chromosomes (Fig. 1).

Growth and fermentation of the engineered industrial strains. The *ald6 GPD1* strains were obtained by transformation of the *ald6* null mutants with pVT100U-ZEO-*GPD1*. Growth and fermentation kinetics of the three series of four strains (each series being wild-type, *GPD1*, *ald6*, and *ald6 GPD1* strains) (Table 1) were monitored during standard wine fermentation on MS medium.

During wine fermentation, growth typically becomes rapidly limited by the low nitrogen concentration in grape must. Therefore, wine yeast strains exhibit a short exponential growth phase followed by stationary phase, during which most of the sugars are consumed (Fig. 2). Overexpression of *GPD1* may alter the cell number, depending on the level of acetaldehyde accumulated during the growth phase, with this effect being directly proportional to the level of glycerol formed (24). Indeed, the numbers of VL1 *GPD1* and K1M *GPD1* cells were lower than control strain numbers. Deletion of *ALD6* did not affect growth, confirming previous data for the wine yeast-derived strain V5 (26). In contrast, there were substantial differences in fermentation rates between *ald6 GPD1* and *GPD1* strains. Strains overexpressing *GPD1* had higher fermentation rates during stationary phase, resulting in a shorter fermentation. In contrast, the duration of fermentation was longer than that of the wild type for *ald6* strains. Interestingly, the combination of *GPD1* overexpression and *ALD6* deletion resulted in an intermediate profile, with a duration of fermentation similar to that of the wild-type strains, despite a lower maximal fermentation rate.

Impact on the main fermentation by-products. The concentration of the main fermentation by-products was measured after complete sugar exhaustion (Table 2). The biosynthesis of acetate by the three *ald6 GPD1* strains was low, similar to that of wild-type strains. The production of succinate by *ald6 GPD1* strains was slightly higher than that by *GPD1* strains, indicating that the flux towards the tricarboxylic acid cycle was not greatly increased in these strains. Surprisingly, the *ald6 GPD1* strains produced significantly more glycerol than did the *GPD1* strains. Ethanol production by *ald6 GPD1* strains was much lower than that in *GPD1* strains, consistent with the greater glycerol production. The production of acetaldehyde and derived metabolites (Table 2) was also monitored throughout fermentation for the set of strains constructed in the K1M background (Fig. 3). Acetaldehyde production was markedly higher in the *GPD1* strains than in the control strains and was even higher in the *ald6 GPD1* strains, consistent with the limitation of ACDH. This was accompanied by higher levels of acetoin and 2,3-butanediol production. The *ald6 GPD1* strains accumulated higher levels of acetoin than the *GPD1* strains but were unable to convert this surplus into 2,3-butanediol.

To further understand the relationships between the production of glycerol, acetoin, and 2,3-butanediol, a large range of glycerol production levels was generated by cultivating the strains K1M, K1M *GPD1*, and K1M *ald6 GPD1* on MS medium with initial glucose contents of 140, 170, 200, and 250 g/liter (Fig. 4). V5 *ald6 GPD1* clearly produced higher levels of acetoin and lower levels of 2,3-butanediol than did V5 *GPD1*. Interestingly, the production of these

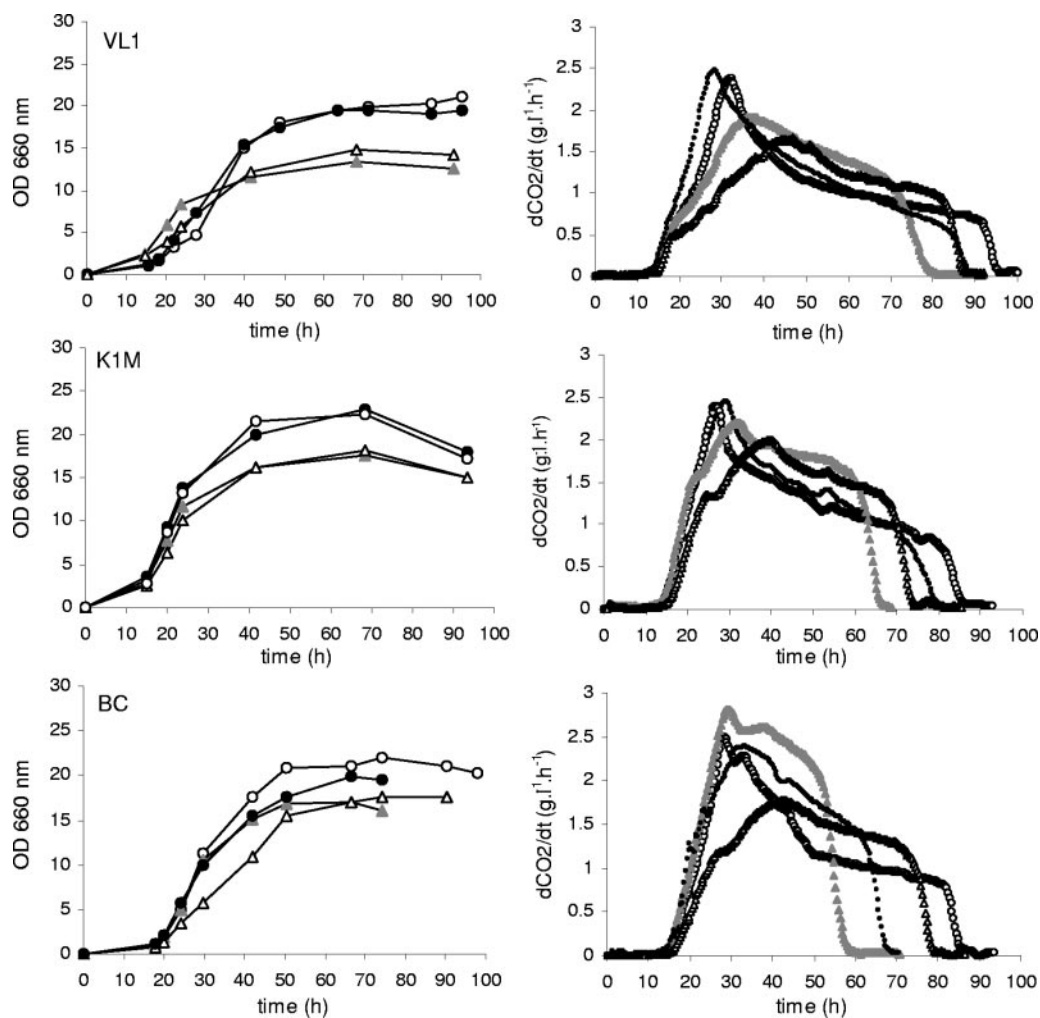


FIG. 2. Growth and fermentation rate of the wine yeast strains VL1, K1M, and BC (●) and the corresponding engineered *ald6* (○), *GPD1* (▲), and *ald6 GPD1* (△) strains. Fermentation experiments were performed on MS medium at a 1.1-liter scale under conditions simulating wine fermentation. One representative of three independent fermentation experiments is shown. OD, optical density.

TABLE 2. Main fermentation by-products formed by engineered wine yeast strains during fermentation on MS medium

Strain	Concn of:						
	Glycerol (g liter ⁻¹)	Ethanol (g liter ⁻¹)	Acetate (g liter ⁻¹)	Succinate (g liter ⁻¹)	Acetaldehyde (mg liter ⁻¹)	Acetoin (g liter ⁻¹) ^a	Butanediol (g liter ⁻¹)
VL1	6.5	97.2	0.57	0.31	16	ND	1.14
<i>ald6</i>	7.4	96.6	0.19	0.29	8	ND	1.30
<i>GPD1</i>	24.4	80.2	2.64	0.48	105	5.4	4.01
<i>GPD1 ald6</i>	26.8	74.7	0.62	0.62	182	6.2	6.05
K1M	5.8	93.3	0.38	0.31	39	ND	0.61
<i>ald6</i>	6.8	93.6	0.10	0.43	18	ND	0.52
<i>GPD1</i>	18.2	82.6	0.98	0.78	183	4.2	4.85
<i>GPD1 ald6</i>	21.9	78.8	0.43	0.88	228	5.8	3.93
BC	7.2	92.4	0.38	0.37	9	ND	1.45
<i>ald6</i>	7.1	90.4	0.05	0.64	10	ND	0.99
<i>GPD1</i>	16.5	82.7	1.36	0.58	95	2.9	3.9
<i>GPD1 ald6</i>	26.9	75.5	0.50	0.74	320	9.5	5.09

^a ND, not detected.

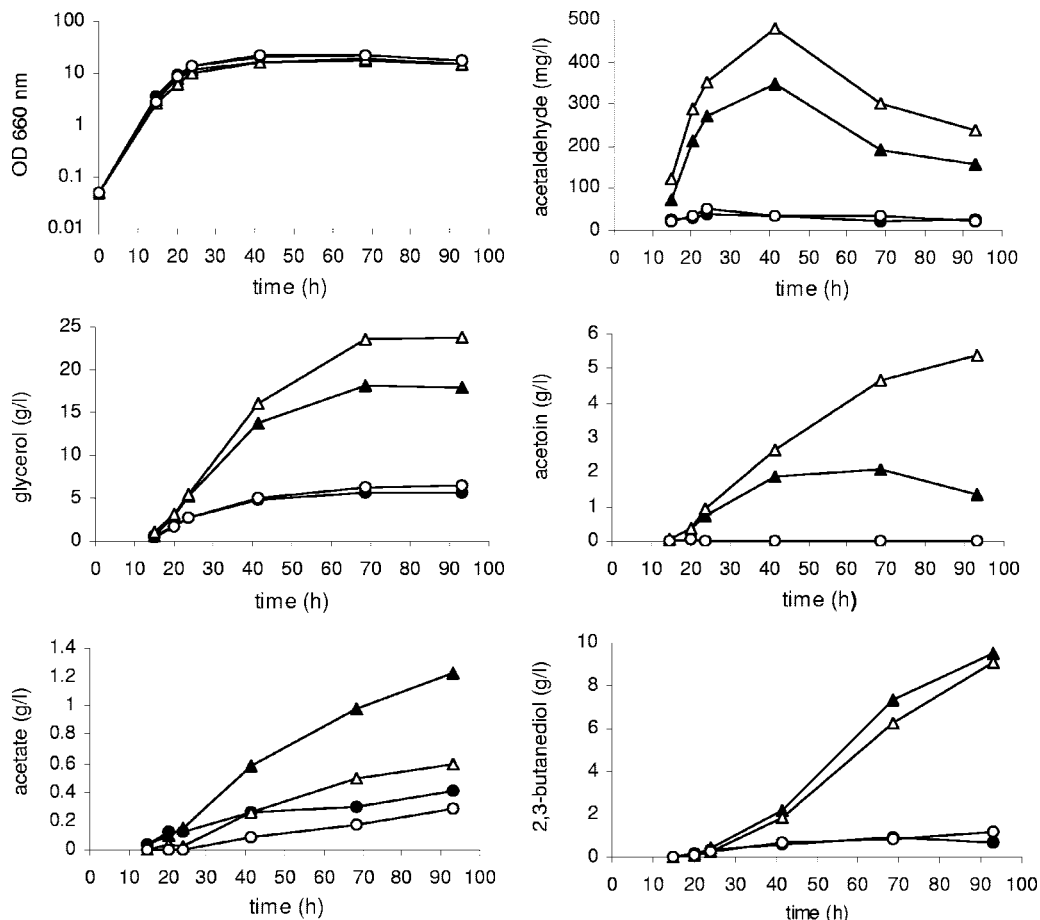


FIG. 3. By-product concentrations in fermentations with the strains K1M (●), K1M *GPD1* (▲), K1M *ald6* (○), and K1M *ald6 GPD1* (△) at various time points. Fermentation conditions are described in the legend of Fig. 2. OD, optical density.

two compounds was related to the level of glycerol produced. The production of acetoin increased progressively as a function of glycerol concentration and then sharply increased for glycerol production to higher than 16 g/liter,

while the 2,3-butanediol concentration markedly decreased. Although factors other than the level of glycerol might contribute to this effect, since different strains were used in this experiment, these data strongly suggest that the 2,3-butanediol dehydrogenase reaction becomes suddenly limited above a certain level of production of glycerol.

DISCUSSION

We show that the deletion of *ALD6* efficiently reduces acetate production both by wild-type industrial wine yeasts and by engineered strains in which the carbon flux has been strongly shifted towards glycerol. Interestingly, the production of glycerol was increased as a result of the *ALD6* deletion, and this effect was particularly clear when *ALD6* was deleted from *GPD1* strains. The high glycerol production, which in turn results in the production of NAD⁺, is surprising, since Ald6p preferably uses NADPH (11, 36); the deletion of *ALD6* was therefore expected to result in an NADPH shortage. We show here that the redistribution of carbon in the *GPD1 ald6* strains involves mainly acetaldehyde accumulation and increased flux through the acetoin-butanediol pathway (Fig. 5), consistent with the limitation of both alcohol dehydrogenase and ACDH reactions. Since the synthesis of acetaldehyde and acetoin from

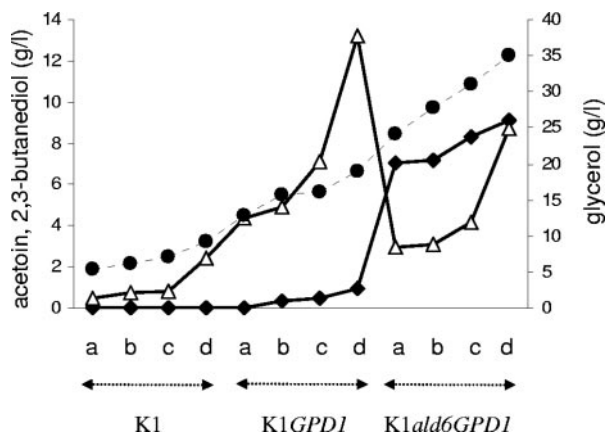


FIG. 4. Glycerol (●), acetoin (◆), and 2,3-butanediol (△) production by the strains K1M, K1M *GPD1*, and K1M *ald6 GPD1* grown on MS medium containing 140 (a), 170 (b), 200 (c), and 250 (d) g/liter glucose.

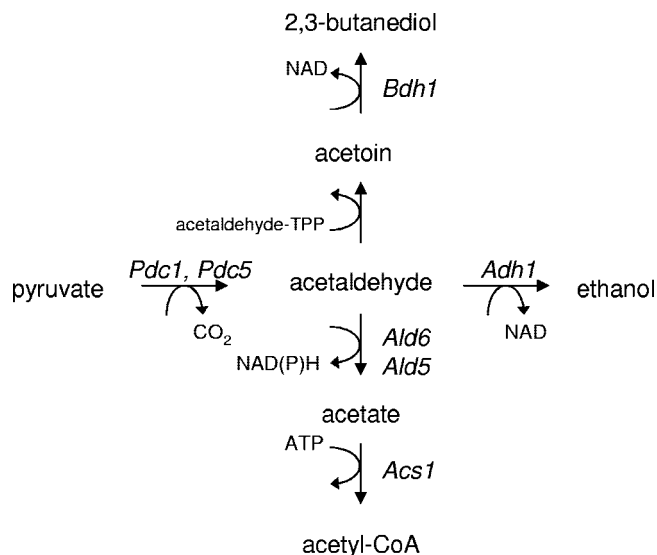


FIG. 5. Pathways for acetaldehyde metabolism in *Saccharomyces cerevisiae*. The main isoforms active during glucose fermentation are indicated. Pdc1 and Pdc5, pyruvate decarboxylase; Adh1, alcohol dehydrogenase; Ald6, Ald5, acetaldehyde dehydrogenase; Acs1, acetyl coenzyme A (CoA) synthase; Bdh1, butanediol dehydrogenase; TPP, thiamine pyrophosphate.

glucose results in a net production of 2 mol NADH and the synthesis of 2,3-butanediol generates 1 mol NADH, glycerol production might be increased to balance this NADH surplus. Indeed, the amount of NADH generated by the surplus of acetaldehyde, acetoin, and 2,3-butanediol in *GPD1 ald6* compared to *GPD1* strains is similar to the amount of NAD⁺ generated by the excess of glycerol (data not shown).

An inverse correlation was clearly shown between the ethanol and glycerol yields of the strains (Fig. 6). The ethanol yield of the *ald6 GPD1* strains was the lowest, 15 to 20% lower than that of the wild type.

While *GPD1* overexpression per se largely affects central metabolism, in particular at the acetaldehyde node (15, 16, 17, 24), additional changes are triggered by the *ALD6* deletion. The production of acetaldehyde of *ald6 GPD1* strains was higher than that of *GPD1* strains (up to 300 mg/liter instead of 50 to 150 mg/liter) but remained in the upper range of the concentrations found in wine (28). The concentration of 2,3-butanediol can reach 1.3 g/liter in some wines and is not expected to appreciably affect the sensory qualities of wine. In contrast, acetoin levels higher than the detection threshold (150 mg/liter) are undesirable in table wines (29). The reduction of acetoin to 2,3-butanediol is catalyzed by the butanediol dehydrogenase Bdh1p, which uses NADH as a preferred cofactor (9, 10). We show in this study that this reaction was less efficient in *ald6 GPD1* strains than in *GPD1* strains, resulting in the accumulation of considerable amounts of acetoin. Furthermore, the efficiency of this reaction seems to be directly correlated to the amount of glycerol produced. Since the production of 2,3-butanediol from acetoin consumes 1 mol of NADH, acetoin accumulation may therefore be due to the low availability of NADH in *ald6 GPD1* strains, as they produce more glycerol than *GPD1* strains. Alternatively, it is possible that

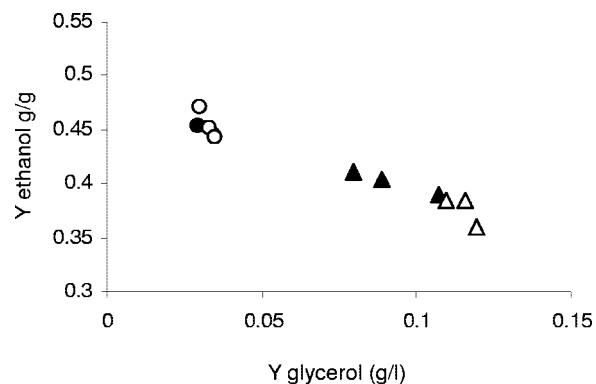


FIG. 6. Ethanol yield as a function of glycerol yield. Strains K1M (●), K1M *GPD1* (▲), K1M *ald6* (○), and K1M *ald6GPD1* (△) were cultivated under fermentation conditions described in the legend of Fig. 2.

butanediol dehydrogenase becomes rate limiting in *ald6 GPD1* strains.

In summary, these results highlight the great potential of yeast strains overexpressing *GPD1* and producing *ALD6* for producing low-alcohol wines. An alcohol content of at least 2% (vol/vol) lower might be expected in wines produced using a yeast strain overproducing around 20 g/liter glycerol. However, accumulation of acetoin limits the extent to which carbon flux can be diverted to glycerol. Further improvement of these strains requires new efforts to minimize the formation of undesirable compounds, in particular at the acetaldehyde branch point.

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