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Glycerol Overproduction by Engineered *Saccharomyces cerevisiae* Wine Yeast Strains Leads to Substantial Changes in By-Product Formation and to a Stimulation of Fermentation Rate in Stationary Phase

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Six commercial wine yeast strains and three nonindustrial strains (two laboratory strains and one haploid strain derived from a wine yeast strain) were engineered to produce large amounts of glycerol with a lower ethanol yield. Overexpression of the *GPD1* gene, encoding a glycerol-3-phosphate dehydrogenase, resulted in a 1.5- to 2.5-fold increase in glycerol production and a slight decrease in ethanol formation under conditions simulating wine fermentation. All the strains overexpressing *GPD1* produced a larger amount of succinate and acetate, with marked differences in the level of these compounds between industrial and nonindustrial engineered strains. Acetoin and 2,3-butanediol formation was enhanced with significant variation between strains and in relation to the level of glycerol produced. Wine strains overproducing glycerol at moderate levels (12 to 18 g/liter) reduced acetoin almost completely to 2,3-butanediol. A lower biomass concentration was attained by *GPD1*-overexpressing strains, probably due to high acetaldehyde production during the growth phase. Despite the reduction in cell numbers, complete sugar exhaustion was achieved during fermentation in a sugar-rich medium. Surprisingly, the engineered wine yeast strains exhibited a significant increase in the fermentation rate in the stationary phase, which reduced the time of fermentation.

The usual glycerol concentration in wine ranges from 4 to 9 g/liter (30, 33, 34, 36). Although it has no direct impact on aromatic characteristics, glycerol has a favorable effect on wine quality. Sweetness is the main contribution of glycerol to sensory characteristics at levels commonly found in wines (16, 27, 36). *Saccharomyces cerevisiae* yeast strains producing large amounts of glycerol would therefore be of considerable value in improving wine quality. Moreover, the overproduction of glycerol at the expense of ethanol might represent an advantageous alternative for the development of beverages with low ethanol contents versus physical processes which alter the organoleptic properties of the final product.

Glycerol is quantitatively the most important fermentation product after ethanol and carbon dioxide. It is involved in osmotic cell regulation (5). During alcoholic fermentation, the main role of glycerol formation is to equilibrate the intracellular redox balance (13, 28, 31, 47) by converting the excess NADH generated during biomass formation to NAD^+. Its formation requires the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (G-3-P), a reaction catalyzed by G-3-P dehydrogenase (GPDH) and followed by the dephosphorylation of G-3-P to glycerol by glycerol-3-phosphatase.

Many growth and environmental factors have been reported to influence the amount of glycerol produced by yeast in wine, e.g., sulfite concentration, pH, fermentation temperature, aeration, inoculation level, grape variety and ripeness, and nitrogen composition (1, 14, 30, 33, 34). Under controlled conditions, it has been shown that the yeast strain strongly influences the amount of glycerol produced (34). This led to a distinction within the *S. cerevisiae* species between strains that are low and high glycerol producers. Significant interactions between strains, incubation temperature, and agitation time have also been reported (14).

A slight increase in glycerol production in wine can be achieved by using yeast strains selected for high glycerol production and by optimizing fermentation conditions (35). Increasing the level of glycerol even more has been attempted by the selective hybridization of wine yeast strains, leading to the construction of yeast producing 10 to 11 g of glycerol per liter (11). More recently, genetic engineering approaches have been successful in redirecting the carbon flux towards glycerol. GPDH, a limiting enzyme for glycerol formation, is encoded by *GPD1* and *GPD2* (1, 2, 10, 19). Overexpression of *GPD1* in a laboratory strain and in a haploid strain (V5) derived from a wine strain resulted in marked increases in glycerol production at the expense of ethanol (23, 26). Up to 28 g of glycerol per liter was formed by an engineered *S. cerevisiae* strain under conditions simulating wine fermentation. Larger amounts of by-products that are undesirable in wine, such as acetate and acetoin, were produced by this strain, and a marked decrease in the yeast population was reported (23).

Wine yeast strains have an unusual genetic context (4) and display properties distinct from those of nonindustrial strains. There are marked differences in the formation of by-products which may have an impact on the organoleptic characteristics of wine. For example, wine yeast strains are initially selected for the low formation of undesirable compounds (e.g., acetate). Based on these strain differences, the consequences of glycerol overproduction might differ between industrial and nonindustrial strains. On the other hand, the secondary effects previously observed with the model V5 *GPD1* strain (23) coincided with a very large shift in the carbon flux towards glycerol. The objectives of this study were to increase the glycerol production of wine yeast strains to a moderate level, suitable for wine making, and to study the consequences of this over-

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production during alcoholic fermentation under conditions simulating wine fermentation. Six wine yeast strains overexpressing *GPD1* were constructed. The consequences of glycerol overproduction for by-product formation, growth, and fermentation kinetics were investigated.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* DH5α was used for cloning experiments. *E. coli* cultivation and media were as previously described (43). Yeast strains W303-1A (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 GAL SUC2 mal0*) and OL1 (*MAT***a** *leu2-3,112 his3-11,15 ura3-251,313*) were used as laboratory strains (L1 and L2). The wine yeast strains included in this study (W3, W6, W15, W18, W19, and R) are commercialized *S. cerevisiae* strains. The wine yeast strain R was used as a reference. The haploid model strain V5 (*MAT***a** *ura3*) derived from the industrial strain W6 was previously described (23). Yeast strains were maintained and grown on yeast-peptone-dextrose (YPD) medium (1% Bacto yeast extract, 2% Bacto yeast extract, 2% Bacto Peptone, 2% glucose).

Fermentation media and conditions. Batch fermentation experiments were carried out under previously defined conditions to simulate wine fermentation (3). The synthetic medium (MS) simulating standard grape juice containing 20% glucose was as previously described (3) but without proline. The total nitrogen concentration was 300 mg/liter (80 mg of ammoniacal nitrogen per liter). Cells were precultured on MS medium at 28°C in 50-ml flasks without agitation for 24 h with industrial strains and 36 h with V5, L1, and L2 strains. Fermentations were performed by the inoculation of precultured cells at a density of 10^6 per ml in fermentors (a working volume of 1.1 liters) equipped with fermentation locks and were carried out at 28°C with continuous stirring (500 rpm). CO_2 release was determined by automatic measurement of fermentor weight loss every 20 min (41).

DNA manipulation, cloning techniques, and transformation methods. Restriction and modification enzymes were used according to the manufacturer's instructions. *E. coli* plasmid DNA was prepared according to standard protocols (43). Purified oligonucleotides were synthesized by Eurogentec. *E. coli* transformation was carried out by the $CaCl_2$-$RbCl_2$ method (15). *S. cerevisiae* was transformed by the lithium acetate procedure (44). When required, 50 to 200 μg of phleomycin (Cayla) per ml was used for plasmid selection.

Plasmid construction. The multicopy plasmid pVT100-U-*GPD1* containing the gene *GPD1* cloned downstream of the *ADH1* promoter has been described previously (23). To construct the plasmids pVT100-U-ZEO and pVT100-U-ZEO-*GPD1*, the Tn*5 ble* gene, conferring phleomycin resistance under the control of the *S. cerevisiae* constitutive *TEF1* promoter, was PCR isolated from the plasmid pUT332 (Cayla) by using oligonucleotides bordering the expression cassette (GCGTTAACGACGGCCAGTGAAT and GCGTTAACAGCTATGA CCATGAT), into which *Hpa*I sites were introduced. The PCR fragment was digested by *Hpa*I and cloned into the *Hpa*I sites of the pVT100-U plasmid (49) and of the pVT100-U-*GPD1* plasmid to give the pVT100-U-ZEO and pVT100-U-ZEO-*GPD1* vectors, respectively. The pVT100-U-ZEO-*GPD1*-Δ*URA3* plasmid was obtained by the deletion of the 1.2-kb *Bgl*II fragment containing the *URA3* gene of the pVT100-U-ZEO-*GPD1* plasmid.

Analytical methods. Yeast cells were counted with an electronic particle counter (ZM; Coultronics). Glucose, glycerol, ethanol, pyruvate, acetic acid, and succinic acid were analyzed by high-pressure liquid chromatography, acetoin and 2,3-butanediol were analyzed by gas chromatography, and acetaldehyde was analyzed by an enzymatic method as previously described (23). Succinate concentrations in the media fermented with laboratory strains could not be determined by high-pressure liquid chromatography due to interference with an unknown compound, so they were measured with an enzymatic kit (Boehringer). Glucose was measured by a colorimetric method with 3,5-dinitrosalicylic acid (25).

RESULTS

Glycerol and ethanol production of engineered yeast strains under conditions simulating wine fermentation. The plasmids pVT100-U-ZEO-*GPD1* and pVT100-U-ZEO (control) were introduced into six industrial strains (R, W18, W6, W3, W15, and W19). All of them were commercial *S. cerevisiae* wine strains and produced 6.8 to 9.6 g of glycerol per liter and moderate acetate levels (250 to 500 mg/liter) during alcoholic fermentation on MS medium (35). The amount of glycerol formed by the engineered and control strains (empty vector reference strains) during fermentation on MS medium was determined after complete sugar exhaustion.

Significantly higher glycerol concentrations (a 1.5- to 2.5-fold increase) were formed by the recombinant wine yeast strains than by control strains (Fig. 1A). No relation was found between the amount of glycerol formed by the engineered strains and the basic level produced by the corresponding control strains. A reduction of 5 to 10 g of ethanol per liter, depending on the strain, was observed (Fig. 1C). The amount of glycerol formed by engineered industrial strains (12 to 18 g/liter) was lower than that formed by the haploid V5 strain previously transformed by the pVT100-U-*GPD1* plasmid (23) (Fig. 1B). This difference was probably due to a higher plasmid stability in the latter strain, since the pVT100-U-*GPD1* plasmid was efficiently maintained by complementation of the V5 uracil auxotrophy. In contrast, phleomycin, required to maintain the pVT100-U-ZEO-*GPD1* plasmid in the industrial strains, was not added in the growth medium, because this antibiotic was shown to be relatively ineffective in combination with acidic media (data not shown). Despite the low number of cell generations (six to seven) under these growth conditions, a high plasmid loss was observed at the end of fermentation (50 to 80% of cells without a plasmid). To verify this hypothesis, the *URA3* gene of the pVT100-U-ZEO-*GPD1* plasmid was deleted, and the plasmid obtained, named pVT100-U-ZEO-*GPD1*-Δ*URA3*, was used to transform the V5 strain. The transformed strain (V5 *GPD1L*) produced amounts of glycerol (13 g/liter [Fig. 1B]) similar to those from the industrial strains, suggesting that the difference in glycerol yield was related to the plasmid copy number rather than the genetic background of the strain.

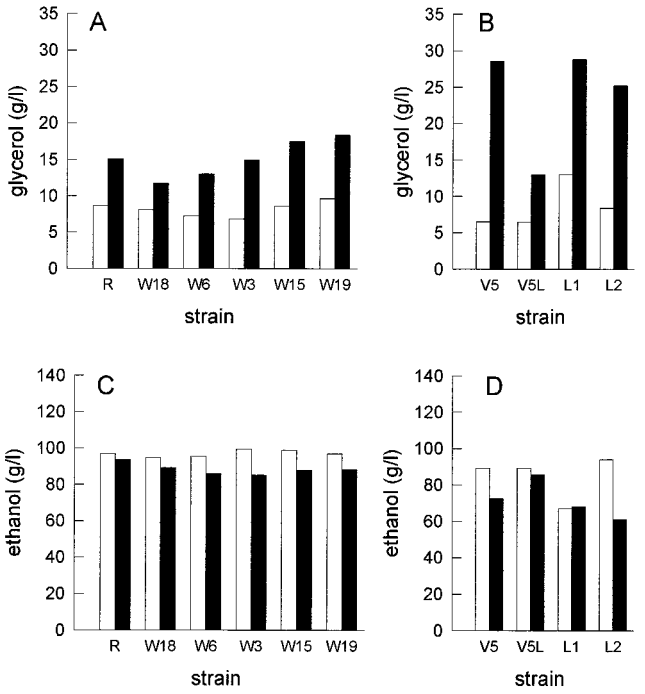

FIG. 1. Glycerol and ethanol production by *S. cerevisiae* strains overexpressing *GPD1* on MS medium. The fermentation of wild-type (□) and engineered strains (■) was performed on MS medium in 1.2-liter fermentors at 28°C with agitation with commercial wine yeast strains (A and C) and nonindustrial yeast strains (B and D). Wine yeast strains and laboratory strains (L1, L2) were transformed by the pVT100-U-ZEO-*GPD1* plasmid. The V5 *GPD1* strain contains the pVT100-U-*GPD1* plasmid (23). The strain V5 *GPD1L* (V5L) contains the pVT100-U-ZEO-*GPD1* Δ*URA3* plasmid. Glycerol and ethanol were determined after complete sugar exhaustion, except for L1 and L2 strains, which were unable to complete fermentation. The residual sugar content in the corresponding fermented medium was 35 and 9 g/liter (L1 and L1 *GPD1*) and 0.6 and 30 g/liter (L2 and L2 *GPD1*).

TABLE 1. Pyruvate and acetaldehyde formed by wild-type and engineered industrial strains[a]

Strain	Yield (mg/liter) of:	
	Pyruvate	Acetaldehyde
Wild type		
R	120	11
W18	80	13
W6	80	7
W3	65	6
W15	75	16
W19	75	11
GPD1 strain		
R	210	44
W18	105	19
W6	135	24
W3	150	38
W15	270	35
W19	270	144

[a] Fermentation conditions were as described in the legend to Fig. 1.

Finally, the glycerol and ethanol production of two laboratory strains, L1 and L2, transformed by pVT100-U-ZEO-*GPD1* (maintained by the complementation of the *ura3* mutation) was investigated (Fig. 1B and D). These strains displayed enhanced glycerol production compared to control strains. A reduction in ethanol concentration was observed for L2 *GPD1* but not for L1 *GPD1*. However, since these strains exhibited a sluggish and stuck fermentation on MS medium, a large amount of residual glucose was found in particular for the strains L1 (control) and L2 *GPD1* (Fig. 1). When the yields of glycerol and ethanol produced from glucose, instead of the amounts formed, were compared (data not shown), variations similar to those found for V5 *GPD1* were observed.

By-product formation. It was previously shown that the increased utilization of NADH through glycerol formation led to a transient accumulation of pyruvate and acetaldehyde (23). Concentrations of these compounds were also enhanced with the engineered industrial strains, compared to controls, but they subsequently decreased during the stationary phase (data not shown). Pyruvate and acetaldehyde concentrations in the fermented medium remained slightly higher than those for the control strain (Table 1). The increased formation of acetate, succinate, 2,3-butanediol, and acetoin was also observed (Fig. 2). Acetate formation was significantly increased (a final concentration of around 1 g/liter) compared to that by control wine yeast strains. Succinate reached a final concentration of 1 to 1.4 g/liter, representing a 1.5- to 2.5-fold increase relative to the control strains. Acetoin was completely reduced to 2,3-butanediol by three engineered wine yeast strains and almost completely reduced by the other three strains (40 to 390 mg of residual acetoin per liter was detected). Final 2,3-butanediol production was therefore high (1 to 3.3 g/liter), with large variation between the strains.

The pattern of by-products formed by engineered wine yeast strains differed significantly from that observed with V5 *GPD1* (23) (Fig. 2). The latter strain exhibited higher acetate production (1.6 g/liter) and lower succinate formation (0.5 g/liter). Similar 2,3-butanediol production and considerably higher acetoin production (6.1 g/liter) than the trace amounts obtained with the wine strains were observed. To examine whether these differences depended on the strain or were related to the difference in the amount of glycerol produced (28 g/liter for V5 *GPD1* and 12 to 18 g/liter for wine yeast strains), these data were compared to the level of by-products formed by V5 *GPD1L*, which produced 13 g of glycerol per liter (Fig. 2). Comparisons of the amounts of metabolites formed by engineered wine yeast strains and V5 *GPD1L* indicated that the level of acetate and succinate formed depended on the genetic background of the yeast. In contrast, acetoin and 2,3-butanediol production seemed to be closely linked to the level of glycerol produced: the reduction of acetoin to 2,3-butanediol was limited in the high-glycerol-producing (28 g/liter) V5 *GPD1* strain, resulting in an extracellular accumulation of acetoin up to 6.1 g/liter, whereas this reduction was quasicomplete for V5 *GPD1L* and wine yeast strains producing 12 to 18 g of glycerol per liter. These results reflect a competition of GPDH and acetoin reductase for NADH utilization.

A distinct pattern of by-product formation was observed for the laboratory strains (L1 and L2) compared with that of V5 or the industrial strains (Fig. 2). The final acetaldehyde concentration formed by the engineered L1 and L2 strains was extremely high (2 and 2.9 g/liter, respectively). The concentration of acetate, which is directly produced from acetaldehyde by means of acetaldehyde dehydrogenase (21, 24), was also markedly increased (up to 3 g/liter). However, since L1 and L2 naturally produced very large amounts of acetate (more than 1 g/liter) under these experimental conditions, the increase in acetate production relative to that of control strains was similar to that of other engineered strains. Succinate production by engineered laboratory strains was also increased, although to a lesser extent than that by other engineered strains. These data confirm the influence of the genetic background on the pro-

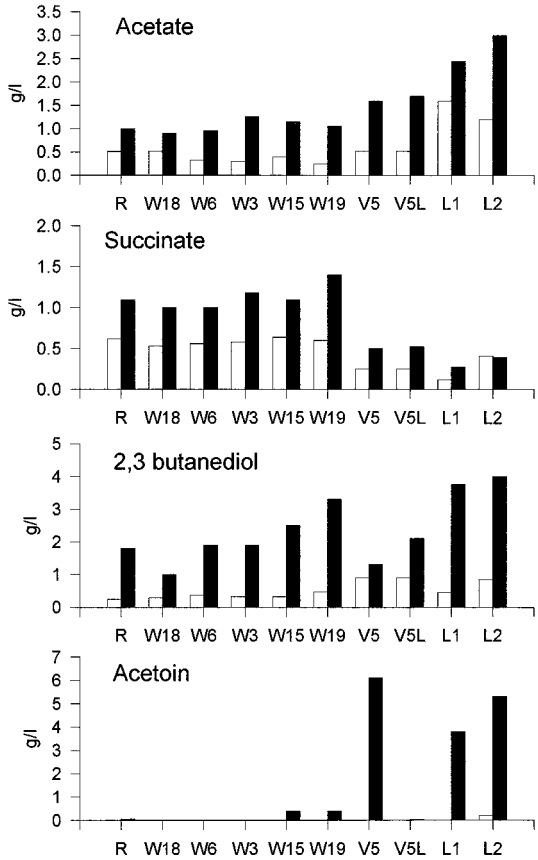

FIG. 2. By-products formed by engineered V5, industrial, and laboratory strains. Strains and growth conditions are as described in the legend to Fig. 1. The detectable level for acetoin was 40 mg/liter. V5L, V5 *GPD1L*.

duction of acetate and succinate. Finally, acetoin and 2,3-butanediol concentrations were markedly increased. The reduction of acetoin to 2,3-butanediol by L1 *GPD1* and L2 *GPD1* was more efficient than with V5 *GPD1*, despite the similar amounts of glycerol produced. This suggests that the balance between acetoin and 2,3-butanediol is influenced both by the level of glycerol produced and by the genetic background of the strain. Strain-dependent production of these compounds has been previously shown for wine yeasts during wine fermentation (37, 38), and an inverse correlation between acetoin and 2,3-butanediol was reported (39).

The effects of glycerol overproduction on carbon and redox balances were characterized in detail for the R and R *GPD1* strains. As shown for one experiment (Table 2), redox and carbon levels were balanced for both strains. Ethanol and CO_2 production was reduced, as a result of the diversion of carbon flux towards glycerol. The ethanol yield was 85.5% (expressed as a percentage of the theoretical molar yield) for the R *GPD1* strain, compared to 92.3% for the control strain. A limited decrease in the CO_2 yield was observed (89.9% for R *GPD1* compared to 91.8% for the control strain). Similar variations were found in a replicate experiment (data not shown). Acetate, 2,3-butanediol, and succinate were the main by-products which increased in concentration. The concentration of malate in the fermented medium was reduced with the engineered R strain. To examine if this effect was specific to the R strain, final malate concentrations were measured in the media fermented with the other industrial engineered strains. Wines obtained from engineered strains contained 0.4 to 0.5 g of malate per liter less than those from control strains. Finally, the biomass formation of the engineered R strain was slightly lower (10%) than that of the control strain.

Effect on growth: relation between acetaldehyde and cell numbers. V5 *GPD1* strains exhibited a 60% decrease in final biomass in comparison with the control strain. This might be explained by a net ATP consumption resulting from the diversion of carbon towards glycerol or by a toxic effect of acetaldehyde, which was produced at a high level during the growth phase and reached 0.6 g/liter when the cells entered the stationary phase (23). To assess this hypothesis, the production of acetaldehyde and the growth of the six engineered industrial strains were monitored during fermentation. The acetaldehyde level was transiently increased to a maximum amount when cells entered the stationary phase (data not shown). A close relation was observed between the maximum level of acetaldehyde reached and the final population (Table 3). The transformed strains W3, W6, R, and W18 were not affected (R and W18) or were only slightly affected (W6 and W3) in terms of growth, and the maximum amount of acetaldehyde produced did not exceed 300 mg/liter. Large amounts of acetaldehyde were formed by W19 *GPD1* and W15 *GPD1* (400 to 500 mg/liter), while these strains exhibited a marked growth defect. These strains were also the highest glycerol producers (18.4 and 17.5 g/liter). A marked decrease in final biomass was observed in the V5 *GPD1* strain (60%), which produced 600 mg of acetaldehyde per liter (23) (Table 3). The maximum level of acetaldehyde formed by the engineered laboratory strains was dramatically high (up to 6 g/liter), but the basic level of the control strains was also very high (up to 400 mg/liter) in comparison with that of the wine yeast strains (a maximum of 40 mg/liter) or that of the V5 strain (140 mg/liter). The growth of the engineered L2 strain was dramatically affected in comparison with that of the control strain. A less marked effect was observed for the L1 *GPD1* strain, but the basic growth of the wild-type L1 strain was very poor. Overall, these results show a direct relation between the amount of acetaldehyde present in the medium at the end of the growth phase and the cell population level. Although it is possible that the net ATP loss resulting from diversion of carbon towards glycerol participates in the decreased biomass formation, these results indicate that the reduction in cell numbers might result from acetaldehyde toxicity. This is consistent with the fact that acetaldehyde is a potent inhibitor of cellular functions if it is allowed to accumulate to levels above 500 μM (220 mg/liter) (17). Acetaldehyde at concentrations above 0.3 g/liter inhibits yeast growth (46). Levels higher than this value were reached for all of the engineered strains exhibiting a significant reduction in cell numbers.

TABLE 2. Glucose and malate consumed and products formed by transformed R strains during fermentation on MS medium[a]

Activity	Concn obtained for R transformed with:			
	pVT100-U-ZEO		pVT100-U-ZEO-*GPD1*	
	g/liter	mM	g/liter	mM
Consumption of:				
Glucose	187.5	1,041.7	196	1,088.9
Malate	0	0	0.65	4.85
Production of:				
Ethanol	88.4	1,921.7	85.7	1,863.0
CO_2	84.2	1,913.6	86.2	1,959.1
Glycerol	7.4	80.4	16.5	179.3
Pyruvic acid	0.12	1.4	0.21	2.4
Acetaldehyde	0.01	0.23	0.04	1
Acetate	0.42	7.1	1.18	19.7
Acetoin	0	0.0	0.06	0.7
2,3-Butanediol	0.24	2.7	1.92	21.3
Succinate	0.4	3.4	1.11	9.4
Biomass formation[b]	6.5	64.56	5.8	57.61
Carbon recovery	100.8%		101.1%	
Redox balance[c]	101.8%		101.7%	

[a] Fermentation conditions were as described in the legend to Fig. 1.
[b] The carbon-molar mass of biomass was estimated with the elemental composition ($C_4H_{7.32}O_{2.24}N_{0.68}S_{0.006}$) previously used (47).
[c] The redox balance represents the ratio between the reductance degree of fermentation products (including biomass) and the reductance degree of glucose, expressed as a percentage.

Stimulation of the fermentation rate in the stationary phase. To study the effects of glycerol overproduction on the fermentation rate, on-line monitoring of CO_2 release was performed during fermentation. The determination of the CO_2 production rate is an accurate method for monitoring alcoholic fermentation kinetics in wine making. As shown for the R strain (Fig. 3), control and *GPD1* strains exhibited similar CO_2 production rates during the growth phase and reached similar maximum rates at the end of this phase. Surprisingly, marked differences were shown during the stationary phase, which is characterized by a decline in the CO_2 production rate. This decline was much slower for the engineered strain than for the control. The *GPD1* strain exhibited a higher CO_2 production rate than the control strain up to the point of complete sugar exhaustion. A linear correlation has been established between ethanol and sugar concentrations and the volume of CO_2 released (9). This relation applies to the control strain but not to *GPD1* strains, since carbon metabolism was redirected into glycerol and other by-products. The glucose consumption rate for *GPD1* strains is therefore underestimated. However, the theoretical loss in CO_2 release due to glycerol overproduction (the main by-product whose formation is increased) represents less than 5% of the total CO_2 formed, which is negligible

TABLE 3. Relationships between maximum acetaldehyde concentration and cell numbers[a]

Strain	Acetaldehyde maximum concn (mg/liter)[b]		Cell count (10^6)[c]		% of *GPD1* over wild type
	Control	*GPD1* strain	Control	*GPD1* strain	
W18	40	181	168	172	102
R	35	150	210	195	93
W6	42	255	160	140	88
W3	30	260	170	140	82
W15	25	470	150	110	73
W19	66	394	215	130	60
V5	136	600	210	90	43
L1	428	3,970	58	42	72
L2	380	6,390	200	60	30

[a] Fermentation conditions were as described in the legend to Fig. 1. Acetaldehyde production and growth were monitored during fermentation.
[b] Maximum concentration of acetaldehyde produced at the end of the growth phase.
[c] Number of cells counted during the stationary phase.

compared to the differences in the CO_2 production rate observed between the control and *GPD1* strains. All commercial strains overexpressing *GPD1* exhibited a faster rate of CO_2 production during the stationary phase (data not shown). As a consequence of this stimulated CO_2 production rate, complete glucose exhaustion for strains overproducing glycerol was achieved 10 to 22 h earlier than for the control strains (Fig. 3 and Table 4).

DISCUSSION

In this study, we report the effect of glycerol overproduction in industrial and nonindustrial yeast strains. Except for small differences in the levels of acetate and succinate produced, the effects of engineered industrial strains on by-product formation were similar to those observed with the engineered haploid model strain V5 for the same amount of glycerol formed. In contrast, marked differences were observed with laboratory strains, which produced, in particular, very large amounts of acetaldehyde and acetate relative to the industrial or V5 strains. These differences are consistent with the fact that wine yeast strains are initially selected for low acetate production, among other characteristics. In addition, the low acetaldehyde production of industrial strains may be a consequence of selection based on good fermentation performance, since it has been suggested that the ability to avoid acetaldehyde accumulation is a prerequisite for ethanol tolerance (17).

The response to glycerol overproduction involves three main pathways (leading to the formation of acetate, 2,3-butanediol, and succinate) that play a role in maintaining the redox balance. The increase in acetate production is certainly a way to provide additional NAD(P)H, since 1 mol of acetate from glucose leads to the production of 2 mol of NAD(P)H. Acetoin, formed by pyruvate decarboxylase and the condensation of active acetaldehyde with free acetaldehyde (6, 18), is reduced to 2,3-butanediol via acetoin reductase. Although the secretion of acetoin and butanediol from glucose releases 2 and 1 mol of NADH, respectively, the reduction of acetoin to 2,3-butanediol via acetoin reductase consumes 1 mol of NADH and therefore appears to be unfavorable in the context of NADH shortage. The stimulation of the acetoin-butanediol pathway could reflect a need to eliminate acetaldehyde, whose toxicity and pleiotropic effects have been fully described (17). This hypothesis is supported by the fact that acetoin is a much weaker inhibitor than acetaldehyde (data not shown). Moreover, such a detoxication mechanism has been observed in higher eucaryotes (29). The contribution of succinate to the redox balance is more difficult to assess. Succinate formation may be carried out via the oxidative branch of the tricarboxylic acid cycle [5 mol of NAD(P)H formed per mol of excreted succinate] or via a reductive pathway (1 mol of NAD formed per mol of succinate) (32). However, the observation of a decrease in malate concentration together with enhanced succinate formation suggests that some malate is converted to succinate via a reductive pathway. Further work is required to assess the contribution of each pathway to succinate formation during wine fermentation. The mechanisms that would explain the formation of succinate via a reductive pathway in the engineered strains are unknown.

The engineered wine yeast strains exhibited a higher fermentation rate in the stationary phase than the control strains. A higher rate of glucose consumption has been recently reported for a *GPD1* multicopy transformant during growth on YPD medium (26). As suggested by the authors of that study, this effect may be due to the enhanced release of inorganic phosphate or to the net ATP loss resulting from the redirecting of carbon flux towards glycerol. A strong negative correlation between intracellular ATP content and the rate of glycolysis has been recently shown (20). In addition to glycerol overproduction, enhanced acetate production may also contribute to a decrease in ATP, since acetate dissipates the pH gradient across the plasma membrane (48). However, the latter mechanism seems unlikely since the fermentation rate was stimulated at the beginning of the stationary phase when the acetate concentration was very low. Alternatively, the increased fer-

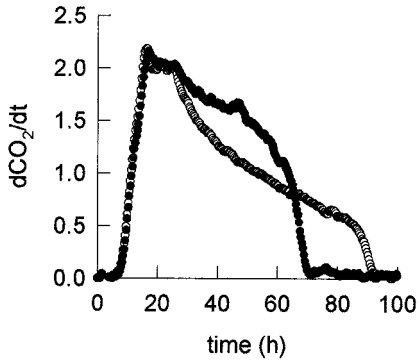

FIG. 3. Fermentation kinetics of the engineered (●) and control R strains (○) on MS medium. dCO_2/dt, CO_2 production rate (grams of CO_2 produced per liter per hour).

TABLE 4. Fermentation duration by control and engineered strains

Strain	Fermentation duration[a] (h)	
	Control	*GPD1* strain
W18	90	72
R	92	70
W6	93	80
W3	93	84
W15	92	80
W19	90	70

[a] Time required for complete sugar exhaustion in fermentation conditions as described in the legend to Fig. 1.

mentation rate might result from the redox imbalance. We recently observed that the addition of exogenous electron acceptors such as acetaldehyde (40) stimulates the fermentation rate in the stationary phase. Whether this stimulation results from a direct effect of acetaldehyde or an indirect effect like redox unbalancing remains to be clarified. In contrast to results previously obtained with YPD medium (26), the fermentation rate of *GPD1* strains was stimulated only during the stationary phase. During wine fermentation most of the sugar (50 to 80%) is fermented during the stationary phase (in a nitrogen-depleted medium), while the fermentation rate continuously declines. Although all the factors involved in the decrease in the glycolytic rate are still unknown, the catabolic inactivation of the hexose transporters is thought to play a major role in the fermentation rate decrease (42). It cannot be excluded that the metabolic changes induced by glycerol overproduction [the levels of by-products, ATP-ADP, and NAD(P)-NAD(P)H)] may affect, by unknown mechanisms, some of these factors (i.e., by increasing the stability of transporters). Finally, it should be noted that engineered strains overproducing glycerol contained more glycerol than the control strains, since intra- and extracellular glycerol concentrations were the same at the end of fermentation for both the wild-type and engineered strains (data not shown). Because of glycerol's role as a compatible solute, it cannot be ruled out that a higher glycerol content may be of some help to the cell in maintaining the level of fermentation performance during the stationary phase.

Since similar effects of *GPD1* overexpression were observed for an engineered wine yeast during fermentation on MS medium and on a grape must (data not shown), the present results are of considerable importance for evaluating the technological advantages of strains overproducing glycerol for wine making. The ranges of metabolites usually found in unspoiled wines are 10 to 250 mg of acetaldehyde per liter (22), 0.2 to 0.8 g of acetate per liter (12, 36), 2 to 80 mg of acetoin per liter, 0.3 to 1.3 g of 2,3-butanediol per liter (36), and 0 to 2 g of succinate per liter (32, 36). Most wine yeast strains engineered for glycerol formation produced acetaldehyde, acetoin, and succinate amounts within the concentration ranges commonly found in wine. Although up to 2.9 g of 2,3-butanediol per liter is found in some wines (45), the usual concentration in wine is below that formed by the engineered wine strains. This compound may contribute to the body of wine in very large amounts because of its viscosity, but it probably has no impact on the sensory qualities of wine (39). In contrast, the level of acetate produced (1 to 1.4 g/liter) was far above the concentration acceptable to ensure wine quality. Depending on the amount of glycerol desired, it would therefore be necessary to limit acetate formation or even to redirect the carbon flux toward the formation of compounds favorable for the organoleptic balance of wine. Succinate, for example, could fulfil these requirements, since it is regarded as favorable for wine quality because of its salt-bitter acidic taste. To reduce acetate formation, we are currently constructing strains with modified expression levels of pyruvate decarboxylase, acetaldehyde dehydrogenase, or acetyl coenzyme A synthetase.

A significant decrease in ethanol content, albeit limited, could be achieved with a strain overproducing 12 to 18 g of glycerol per liter. We have previously shown that it was possible to decrease ethanol yields by redirecting the carbon flux toward lactate (7, 8). However, the utilization of strains producing lactate (at yields up to 5 g/liter) for wine making is limited to grape musts lacking acidity. Significantly decreasing the ethanol yield would require the formation of larger lactate amounts, which is not desirable in wine. From this point of view, the utilization of strains with high glycerol yields combined with the formation of desirable metabolites could offer new prospects for the elaboration of fermented products with a slightly reduced ethanol content.

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