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Evaluation of Gene Modification Strategies for the Development of Low-Alcohol-Wine Yeasts

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Saccharomyces cerevisiae **has evolved a highly efficient strategy for energy generation which maximizes ATP energy production from sugar. This adaptation enables efficient energy generation under anaerobic conditions and limits competition from other microorganisms by producing toxic metabolites, such as ethanol and CO_2. Yeast fermentative and flavor capacity forms the biotechnological basis of a wide range of alcohol-containing beverages. Largely as a result of consumer demand for improved flavor, the alcohol content of some beverages like wine has increased. However, a global trend has recently emerged toward lowering the ethanol content of alcoholic beverages. One option for decreasing ethanol concentration is to use yeast strains able to divert some carbon away from ethanol production. In the case of wine, we have generated and evaluated a large number of gene modifications that were predicted, or known, to impact ethanol formation. Using the same yeast genetic background, 41 modifications were assessed. Enhancing glycerol production by increasing expression of the glyceraldehyde-3-phosphate dehydrogenase gene, *GPD1*, was the most efficient strategy to lower ethanol concentration. However, additional modifications were needed to avoid negatively affecting wine quality. Two strains carrying several stable, chromosomally integrated modifications showed significantly lower ethanol production in fermenting grape juice. Strain AWRI2531 was able to decrease ethanol concentrations from 15.6% (vol/vol) to 13.2% (vol/vol), whereas AWRI2532 lowered ethanol content from 15.6% (vol/vol) to 12% (vol/vol) in both Chardonnay and Cabernet Sauvignon juices. Both strains, however, produced high concentrations of acetaldehyde and acetoin, which negatively affect wine flavor. Further modifications of these strains allowed reduction of these metabolites.**

Saccharomyces sensu stricto species, in particular *Saccharomyces cerevisiae*, have evolved a strategy for sugar utilization that maximizes ethanol production (39). This adaptation permits energy extraction under fermentation and, perhaps more importantly, leads to the production of a toxic metabolite, ethanol, which inhibits the growth of competing microorganisms. Production of other metabolites by yeast is also important, particularly in the context of alcoholic beverage industries, as these molecules shape the organoleptic properties of beer and wines.

Largely driven by consumer demand for rich and ripe fruit flavor profiles, the alcohol content of some beverages has increased in recent years (29). High alcohol content in wine, for example, has several important consequences: it can compromise product quality, including increasing the perception of mouthfeel parameters such as hotness and viscosity, and to a lesser extent, sweetness, acidity, aroma, flavor intensity, and textural properties can be negatively impacted (20–22); costs to the consumer are higher in countries where taxes are levied according to ethanol content; and excessive alcohol consumption has negative health impacts.

The combination of quality, economic, and health issues associated with high-alcohol wines has led to significant interest in the development of technologies to produce wines with reduced ethanol concentrations that retain balance, flavor profile, and other sensory characteristics (29).

Several genetic modification (GM) strategies are available to divert yeast metabolism away from ethanol formation by redirecting carbon to other endpoints (29, 41). However, maintaining yeast fermentation performance and wine quality in low-ethanol GM yeast strains remains a major challenge.

One GM approach to decrease ethanol formation involves increasing glycerol production. Several gene modifications can achieve this end, for example overexpression of *GPD1* and/or *GPD2* genes, which encode glycerol 3-phosphate dehydrogenase (Gpd) isozymes (6, 10, 15, 35, 46); deleting *PDC* genes encoding pyruvate decarboxylase (35); impairing alcohol dehydrogenase (*ADH*/Adh) expression and activity (13, 27); deleting *TPI1*, which encodes triose phosphate isomerase (7, 8); and modifying the glycerol transporter encoded by *FPS1* (56, 57).

Gpd catalyzes the conversion of dihydroxyacetone phosphate to glycerol 3-phosphate, which is subsequently dephosphorylated to glycerol by glycerol 3-phosphatase. Overexpression of *GPD1* or *GPD2* has been shown to increase glycerol yield by up to 548%, depending on the yeast strain, medium and fermentation conditions (29), and this is associated with lower ethanol production. However, production of other metabolites that might negatively impact wine quality is also observed; for instance, *GPD* overexpression leads to increased acetic acid production (10, 34, 46). At least some of these negative effects can be rectified by engineering further modifications. For example, the problem of increased acetic acid concentration can be ameliorated by deleting *ALD6*, which encodes aldehyde dehydrogenase (6, 15).

Yeasts engineered for *GPD* overexpression and *ALD6* deletion have been reported to produce elevated concentrations of acetoin, which has the aroma of rancid butter and has a low sensory threshold. However, this compound can be converted to innocuous 2,3-

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butanediol by increasing expression of *BDH1* which encodes 2,3-butanediol dehydrogenase (16).

Pyruvate decarboxylase catalyzes the decarboxylation of pyruvate to acetaldehyde and CO_2. There are three pyruvate decarboxylase genes in *Saccharomyces cerevisiae*: *PDC1*, *PDC5*, and *PDC6*, which are upregulated by the transcription factor Pdc2p; only Pdc1p and Pdc5p are known to be active in yeast during fermentation (18, 19, 26). Yeast strains lacking all three *PDC* genes are unable to grow in medium containing glucose as a sole carbon source, with excess NADH production inhibiting glycolytic flux (43). However, *PDC2* deletion reduced ethanol yield with associated increased glycerol production (35).

Alcohol dehydrogenases (encoded by *ADH1*, *AHD3*, *ADH4*, and *ADH5*) play an important role in yeast fermentation, catalyzing the reduction of acetaldehyde to ethanol (29). Yeast lacking the major fermentative isoform, *ADH1*, show decreased ethanol production and increased glycerol synthesis (27). In addition, deletion of *ADH1* causes a considerable decrease in growth rate (9, 12, 27). Yeast strains lacking additional *ADH* genes (*ADH3* and *ADH4*) exhibited a further decrease in ethanol production, greater glycerol formation, and significantly impaired growth (13).

Triose phosphate isomerase, encoded by *TPI1*, functions at the branch point of glycolysis, converting dihydroxyacetone phosphate to glyceraldehyde 3-phosphate. Yeast strains lacking *TPI1* produced elevated glycerol concentration with a concomitant substantial decrease in ethanol production (8). Growth on glucose as a sole carbon source was not possible for *TPI1* mutants; the addition of ethanol, however, restored growth, indicating an imbalance in NADH supply (7, 36).

Fps1p is a member of the major intrinsic protein (MIP) family of channel proteins and facilitates glycerol export and import in *S. cerevisiae*. Fps1p regulates intracellular glycerol concentrations, and its expression is controlled by the osmolarity of the surrounding medium (31, 57). Expression of a truncated form of Fps1p lacking the N-terminal domain in yeast resulted in continuous glycerol leakage from the cell, which was compensated for by increasing glycerol production (45). Yeast carrying the modified form of Fps1p, however, showed impaired growth on glucose and decreased biomass formation, suggesting that it may be of limited value in industrial yeast strains.

Another potential strategy to decrease ethanol production is to divert carbon toward the synthesis of organic acids, such as gluconic acid and acids involved in the tricarboxylic acid (TCA) cycle. Glucose oxidase catalyzes the conversion of glucose into gluconic acid and hydrogen peroxide. However, this enzyme is not encoded in the *S. cerevisiae* genome. Expression of the *Aspergillus niger GOX* gene in *S. cerevisiae*, and secretion of its product, led to reduced ethanol concentration during trial fermentations (33). However, the effect of hydrogen peroxide on the appearance of the resulting wine and the requirement for oxygen was not reported.

Deletions and overexpression of several genes involved in the TCA cycle have been shown to not only impact the formation of organic acids but also affect ethanol production (1, 37, 51). Although these modifications are promising, their real impact in a winemaking context has not been explored.

In this work, we evaluated several strategies aimed at decreasing ethanol production during wine fermentation. By performing stable chromosomal gene modifications in a wine yeast, we determined the most effective gene modifications for reducing ethanol concentration. Although the best low-ethanol strains carry no foreign DNA (i.e., they are self cloned), they are still considered genetically modified organisms (GMOs). The use of GMOs in food and beverages continues to be the subject of intense debate, particularly for winemakers and the wine sector (40, 42).

MATERIALS AND METHODS

Strains and molecular techniques. The wine yeast AWRI1631 was used as the parental strain for all genetically modified constructs. AWRI1631 is a stable haploid generated by sporulation of a wine yeast and deletion of the *HO* locus (4).

Genetic modifications of AWRI1631 performed for work described in this paper were chromosomally integrated and included (i) gene deletions, where the open reading frame (ORF) of the target gene was deleted; (ii) promoter replacement, where the native promoter of the target gene was replaced with a strong constitutive yeast promoter; (iii) gene cassette insertion, where a gene under the control of a strong constitutive yeast promoter was inserted into the chromosome; and (iv) discrete modifications, including nucleotide substitutions and deletions. Strains generated for this study and their genetic modifications are listed in Table 1.

Single gene deletions in AWRI1631 were obtained from the AWRI Wine Yeast Deletion Library (WYDL) collection. Deletions in this collection were carried out by replacing the ORFs with a KanMX cassette encoding G418 resistance. Cassettes (kindly provided by Charlie Boone, University of Toronto) were PCR amplified using primers containing 50-bp flanking regions corresponding to up- and downstream regions outside the ORF.

Gene deletions in strains intended for multiple modifications were conducted using a selection-counterselection system described previously (53), with some modifications. A counterselection (CORE) cassette was cloned in the plasmid pAG25 (EURSCARF collection). Briefly, the gene *GIN11*, which is lethal when expressed in *S. cerevisiae*, was cloned in pAG25 behind the galactose-inducible promoter *GAL1*, along with the gene *natMX*, which encodes resistance to the antibiotic ClonNAT. In a first step, the CORE cassette was amplified by PCR from plasmid pAG25-*GIN11* using primers with 40-bp flanking regions complementary to up- and downstream sequences outside the targeted region. This PCR product was then transformed into yeast, where it was integrated into the targeted site.

In the counterselection step, the CORE cassette was replaced using oligonucleotides or PCR products depending on the gene modification. For gene deletions, 100-bp double-strand oligonucleotides, containing 50 bp complementary to the upstream and 50 bp complementary to the downstream regions, were used to remove the CORE cassette (53). For promoter replacement, a PCR product containing the strong constitutive yeast promoter *FBA1* and 40 bp flanking each side of the CORE cassette was employed. For gene cassette insertions, a PCR product containing either the native *GPD1* gene or the *GOX* gene from *Aspergillus niger*, both under the control of the *FBA1* promoter and carrying 40-bp flanking sequences, complementary to regions to the CORE cassette was introduced in yeast. Nucleotide substitution and deletions were conducted similarly to gene deletions, except that the oligonucleotides carried base substitutions.

All yeast transformations were carried out using the lithium acetate-polyethylene glycol method (2). Transformed strains were selected on plates containing galactose as a sole carbon source and subsequently tested for ClonNAT sensitivity. ClonNAT-sensitive strains containing the desired genetic modifications were confirmed by sequencing.

Cloning of GOX gene. The *GOX* gene, which encodes glucose oxidase, was amplified by PCR from genomic DNA of *Aspergillus niger* and cloned into plasmid pCV1-FBA1pMFα (AWRI plasmid collection). This plasmid encodes the secretion signal from the mating pheromone factor MFα immediately downstream of the *FBA1* promoter. In the resulting plasmid (pCV1-MFαGOX), the *GOX* gene was cloned in frame with the MFα secretion signal to enable secretion of glucose oxidase to the extracellular medium.

TABLE 1 Genetic modifications of constructed strains and ethanol production compared to parental strain AWRI1631

Strain	Modified gene(s)	Genetic modification	Ethanol (%)[a]	Significance[b]
AWRI1631	None	None (parental strain)	100 ± 1.4	NS
AWRI1631 ΔACO1	*ACO1*	ORF deletion	99 ± 0.6	NS
AWRI1631 ΔACO2	*ACO2*	ORF deletion	99 ± 0.2	NS
AWRI1631 ΔADH1	*ADH1*	ORF deletion	100 ± 0.6	NS
AWRI1631 ΔADH3	*ADH3*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔADH1ΔADH3	*ADH1*, *ADH3*	ORF deletion	99 ± 0.6	NS
AWRI1631 ΔFRDS1	*FRD1*	ORF deletion	100 ± 0.9	NS
AWRI1631 ΔGPH1	*GPH1*	ORF deletion	99 ± 0.3	NS
AWRI1631 ΔGRR1	*GRR1*	ORF deletion	100 ± 1.5	NS
AWRI1631 ΔHXK2	*HXK2*	ORF deletion	97 ± 0.4	S
AWRI1631 ΔIDH1	*IDH1*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔIDP2	*IDP2*	ORF deletion	99 ± 0.6	NS
AWRI1631 ΔKGD1	*KGD1*	ORF deletion	100 ± 0.1	NS
AWRI1631 ΔMDH1	*MDH1*	ORF deletion	99 ± 1.4	NS
AWRI1631 ΔMIG1	*MIG1*	ORF deletion	97 ± 0.8	S
AWRI1631 ΔMIG2	*MIG2*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔOSM1	*OSM1*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔPDC1	*PDC1*	ORF deletion	100 ± 2.1	NS
AWRI1631 ΔPDC5	*PDC5*	ORF deletion	97 ± 0.3	S
AWRI1631 ΔPYC1	*PYC1*	ORF deletion	99 ± 0.8	NS
AWRI1631 ΔPYC2	*PYC2*	ORF deletion	99 ± 1.7	NS
AWRI1631 ΔTPI1	*TPI1*	ORF deletion	Stuck ferment[c]	NA
AWRI1631 FPS1Δ11	*FPS1*	Truncated Fps1p	92 ± 0.4	S
AWRI1631 gcrTPI1	*TPI1*	Point mutation in TPI1 promoter at GCR1 binding site	98 ± 0.9	S
AWRI1631 rapTPI1	*TPI1*	Point mutation in TPI1 promoter at RAP1 binding site	100 ± 0.7	NS
AWRI1631 aspTPI1	*TPI1*	Point mutation in TPI1 to change Glu_{165} to Asp in Tpi1p active site	94 ± 0.3	S
AWRI1631 GOX	*GOX*	Expression of glucose oxidase from *Aspergillus niger*	95 ± 2.1	S
AWRI1631 PYC1	*PYC1*	Promoter replacement	99 ± 0.4	NS
AWRI1631 MDH2	*MDH2*	Promoter replacement	98 ± 0.3	S
AWRI1631 FUM1	*FUM1*	Promoter replacement	100 ± 0.5	NS
AWRI1631 FRDS1	*FRD1*	Promoter replacement	98 ± 0.5	NS
AWRI1631 ICL1 MLS1	*ICL1*, *MLS1*	Promoter replacement	98 ± 0.7	NS
AWRI1631 ADH2	*ADH2*	Promoter replacement	100 ± 0.6	NS
AWRI1631 ZWF1	*ZWF1*	Promoter replacement	99 ± 0.8	NS
AWRI1631 GND1	*GND1*	Promoter replacement	99 ± 0.9	NS
AWRI1631 GPD1	*GPD1*	Promoter replacement	89 ± 0.3	S
AWRI1631 2GPD1	*GPD1*	Two copies of the *FBA1*p-*GPD1* cassette	81 ± 0.4	S
AWRI1631 3GPD1	*GPD1*	Three copies of the *FBA1*p-*GPD1* cassette	71 ± 0.3	S
AWRI1631 GPD1 FPS1Δ11	*FPS1*, *GPD1*	*GPD1* promoter replacement and truncated Fps1p	87 ± 0.5	S
AWRI1631 GPD1 ΔTPI1	*GPD1*, *TPI1*	*GPD1* promoter replacement and *TPI1* deletion	Stuck ferment	NA
AWRI1631 2GPD1 ACS1	*GPD1*, *ACS1*	Two copies of the *FBA1*p-*GPD1* cassette, and *ACS1* promoter replacement	84 ± 0.9	S
AWRI 2531	*GPD1*, *ALD6*	Two copies of the *FBA1*p-*GPD1* cassette and *ALD6* deletion	71 ± 0.4	S
AWRI 2532	*GPD1*, *ALD6*	Three copies of the *FBA1*p-*GPD1* cassette and *ALD6* deletion	65 ± 1.2	S

[a] Values are averages and standard deviations from nine replicates for the parental strain and three replicates for all other strains.
[b] S, $P < 0.05$ (strain produced a significantly lower ethanol yield); NS, not significantly different; NA, not applicable.
[c] Unfinished fermentation with high residual sugar.

Determination of glucose oxidase activity. Yeast colonies transformed with the PCR product containing the MFα secretion signal-GOX gene construct were screened for their ability to secrete active glucose oxidase using a plate assay as described previously (33). Briefly, colonies on plates were overlaid with 10 ml of 0.1 M K_2HPO_4 buffer (pH 7.0) containing 10 g/liter glucose, 1% (wt/vol) agarose, 100 mg/liter *o*-dianisidine dihydrochloride (Sigma-Aldrich, Australia), and 15 U/ml peroxidase (Sigma-Aldrich, Australia). Plates were incubated at 37°C for 1 h once the overlay agar was set. Colonies secreting active glucose oxidase produced a brown halo.

Media and growth conditions. All yeast strains were evaluated for decreased ethanol production in YPD10 medium (yeast extract, 10 g/liter; peptone, 20 g/liter; glucose, 100 g/liter) (30). Briefly, a yeast starter culture was made in 20 ml of YPD medium (yeast extract, 10 g/liter; peptone, 20 g/liter; glucose, 20 g/liter) and incubated at 28°C with shaking (180 rpm). The starter culture was then used to inoculate fermentation flasks at a cell density of 5×10^6 cells/ml. Fermentations were carried out in triplicate in 250-ml flasks equipped with fermentation locks and sampling ports closed with Suba-seals, and contained 100 ml of YPD10 medium. Aerobic conditions were attained by growing cultures in flasks covered with aluminum foil that allowed free gas exchange with the environment. Cultures were fermented at 28°C for 48 h. At the end of fermentation, samples were collected and centrifuged for 5 min at 15,000 × *g*, and the cell-free supernatants were kept at 4°C for high-performance liquid chromatography (HPLC) analysis.

Strains showing a considerable reduction in ethanol concentration after fermentation were assessed for their ability to ferment chemically defined grape juice medium (CDGJM) (48) and/or grape juice. Chardonnay juice was prepared commercially from grapes mechanically harvested from Adelaide Hills (South Australia), and Cabernet Sauvignon grapes

TABLE 2 Chemical parameters for juices fermented with modified strains[a]

Juice	Sugar concn (g/liter)	YAN (mg N/liter)
CDGJM	200	300
Chardonnay	246[b]	360
Cabernet Sauvignon	246	300

[a] The pH was adjusted to 3.5 for each juice.
[b] Increased from 210 g/liter to match Cabernet Sauvignon composition.

were collected from Langhorne Creek (South Australia). CDGJM contained 200 g/liter of sugar and yeast assimilable nitrogen (YAN) at 300 mg N/liter. Sugar concentration in Chardonnay juice was increased from 210 g/liter and to 246 g/liter to match Cabernet Sauvignon. YAN concentrations in Chardonnay and Cabernet Sauvignon were 360 mg N/liter and 300 mg N/liter, respectively (Table 2). CDGJM and Chardonnay juice were filter sterilized (0.2 μm; Millipore) immediately after preparation. Cabernet Sauvignon grapes were aliquoted for individual fermentations and crushed prior to inoculation. CDGJM, Chardonnay, and Cabernet Sauvignon pH was adjusted to 3.5. CDGJM and Chardonnay fermentations were carried out in triplicate in 250-ml fermentation flasks (with fermentation locks and sampling ports), each containing 200 ml medium. Cabernet Sauvignon fermentations were conducted in 1-liter Schott bottles with fermentation locks and containing 400 ml juice and skins. CDGJM and grape juice fermentations were inoculated at a cell density of 5×10^6 cells/ml and incubated at 20°C with shaking, with progress monitored by measuring refractive index of culture supernatants. At the end of fermentation, cultures were cold settled and racked. Samples for HPLC analysis were centrifuged for 5 min at 15,000 × *g*, and the cell-free supernatants were stored at 4°C. Samples for volatile compound analysis were centrifuged in glass test tubes, poured into glass ampoules under nitrogen gas, and kept at 4°C. Glass was used to avoid stripping of volatile compounds.

Analytical methods. Yeast growth was monitored spectrophotometrically by measuring absorbance at 600 nm. Concentrations of residual sugar, ethanol, glycerol, malic acid, succinic acid, gluconic acid, and acetic acid were determined by HPLC using a Bio-Rad HPX-87H column as described previously (58). Ammonia concentration was determined using the glutamate dehydrogenase enzymatic bioanalysis UV method test (Roche, Mannheim, Germany). Free α-amino acid nitrogen (FAN) was determined using the o-phthaldehyde–*N*-acetyl-L-cysteine spectrophotometric assay (NOPA) (14). YAN was calculated by adding the nitrogen present in ammonium to the FAN concentration.

Analysis of volatile compounds. Concentrations of acetaldehyde, acetoin, and 2,3-butanediol in wine were determined using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME/GCMS), with polydeuterated internal standards for stable isotope dilution analysis (SIDA) as follows. Briefly, an Agilent 6890 gas chromatograph was equipped with a Gerstel MPS2 multipurpose sampler and coupled to an Agilent 5973N mass selective detector. The instrument was controlled with Agilent GC ChemStation software (rev. D.02.00.275) and Maestro software (integrated version 1.3.3.51/3.3), and the data were analyzed with Agilent MassHunter quantitative analysis software, version B.04.00. The gas chromatograph was fitted with a 60-m by 0.250-mm J&W DB-wax fused silica capillary column, with a 0.25-μm film thickness and a 0.5-m by 0.250-mm Restek Siltek-deactivated retention gap. Helium (BOC ultrahigh purity) with a linear velocity of 30 cm/s and a flow rate of 1.4 ml/min was used as carrier gas in constant flow mode. The oven temperature was started at 40°C, held for 4 min, increased to 160°C at 5°C/min, increased to 240°C at 40°C/min, and held for 5 min. The inlet was fitted with a borosilicate glass SPME inlet liner (0.75-mm inside diameter; Supelco) and was held at 220°C. Samples were diluted 1:10 or 1:5 with MilliQ water in a 20-ml headspace vial. Vials were immediately sealed (magnetic, polytetrafluoroethylene [PTFE] septum). Subsequently, 100 μl of combined deuterated internal standards solution was injected through the septum and the vial was thoroughly shaken. The concentrations of each deuterated internal standard in the vial were 29.75 mg/liter for d_4-acetaldehyde, 36.33 mg/liter for d_7-acetoin, and 29.92 mg/liter for d_8-2,3-butanediol. A Supelco polydimethylsiloxane-divinylbenzene (PDMS/DVB, blue) 65-μm fiber was exposed to the headspace of the sample vials for 10 min at 35°C and desorbed in the GC inlet in splitless mode for 10 min.

Polydeuterated standards. d_4-Acetaldehyde was purchased from Sigma-Aldrich (Castle Hill, Australia). d_7-Acetoin and d_8-2,3-butanediol were synthesized from d_6-2,3-butanedione, which was prepared from 2,3-butanedione (Alfa Aesar, Australia), deuterium sulfate (D_2SO_4) and D_2O (Sigma-Aldrich, Australia) as previously described (59). d_7-Acetoin was prepared from d_6-2,3-butanedione as described previously (59). Briefly, d_6-2,3-butanedione (1.0 g, 11 mmol) and Zn powder (1.5 g, 22 mmol) were stirred under an N_2 atmosphere in 12.8 g D_2SO_4 (20% in D_2O) for 15 min; the mixture was then filtered, saturated with NaCl, and extracted with CH_2Cl_2 (seven times, 30 ml each time). The organic layer was dried, concentrated and purified by Kugelrohr distillation to give the product as a colorless liquid (341 mg, 32%, 91.2% purity by GC-MS). d_8-2,3-Butanediol was prepared by reducing d_6-2,3-butanedione using a variation of the method described by Maier et al. (32). A solution of d_6-2,3-butanedione (310 mg in 5 ml diethyl ether) was slowly added to a cooled suspension of $LiAlD_4$ (Sigma-Aldrich, Australia) (110 mg in 5 ml diethyl ether) under an N_2 atmosphere. After stirring for 2.5 h, the suspension was hydrolyzed with KOH solution (50% wt/vol, 5 ml). The product was extracted with diethyl ether (10 times, 5 ml each time), dried with $MgSO_4$, and concentrated to give a pale yellow oil. The aqueous layer was then taken up with Na_2SO_4, washed with diethyl ether (five times, 5 ml each time), dried, and concentrated *in vacuo*. The resultant oil was purified by column chromatography (2:3 ethyl acetate-petroleum ether) to give deuterated 2,3-butanediol (270 mg, 82%, 98.9% purity by GC-MS).

RESULTS

Screening gene modifications that impact ethanol production. A collection of mutant *S. cerevisiae* wine yeast strains was generated from the same parent, AWRI1631, to identify the best candidate genes for constructing low-ethanol strains. Expression of genes encoding enzymes that divert carbon away from ethanol production was increased, whereas genes that contribute to ethanol formation were deleted or downregulated. Target genes included some involved in glycolysis, pentose phosphate pathway, and TCA cycle; some regulating glucose repression; and some with the potential to catabolize glucose in the medium (Fig. 1).

Only 15 of the 41 strains that were constructed showed significantly lower ethanol production than the parental strain AWRI1631 when grown in YPD10 medium (Table 1). Increased expression of genes involved in the reductive branch of the TCA cycle (*PYC1*, *MDH2*, *FUM1*, and *FRD1*) affected the formation of organic acids (data not shown), but only *MDH2* and *FRD1* showed a marginal (2%) decrease in ethanol production. Deletion of *PDC5*, *HXK2*, and *MIG1*, the last two involved in glucose repression, produced a minor (3%) decrease in ethanol concentration. Under aerobic conditions, deletion of genes involved in glucose repression did not show any further decrease in ethanol formation (data not shown). Expression of the *A. niger GOX* gene showed a 5% decrease in ethanol. When AWRI1631 GOX was grown under aerobic conditions in the same medium, a decrease of 6% in ethanol production was achieved (data not shown), consistent with the requirement of glucose oxidase for oxygen, as noted previously by others (3). Interestingly, AWRI1631 GOX darkened the color of the medium relative to the parental strain, most likely due to hydrogen peroxide production during the formation of gluconic acid.

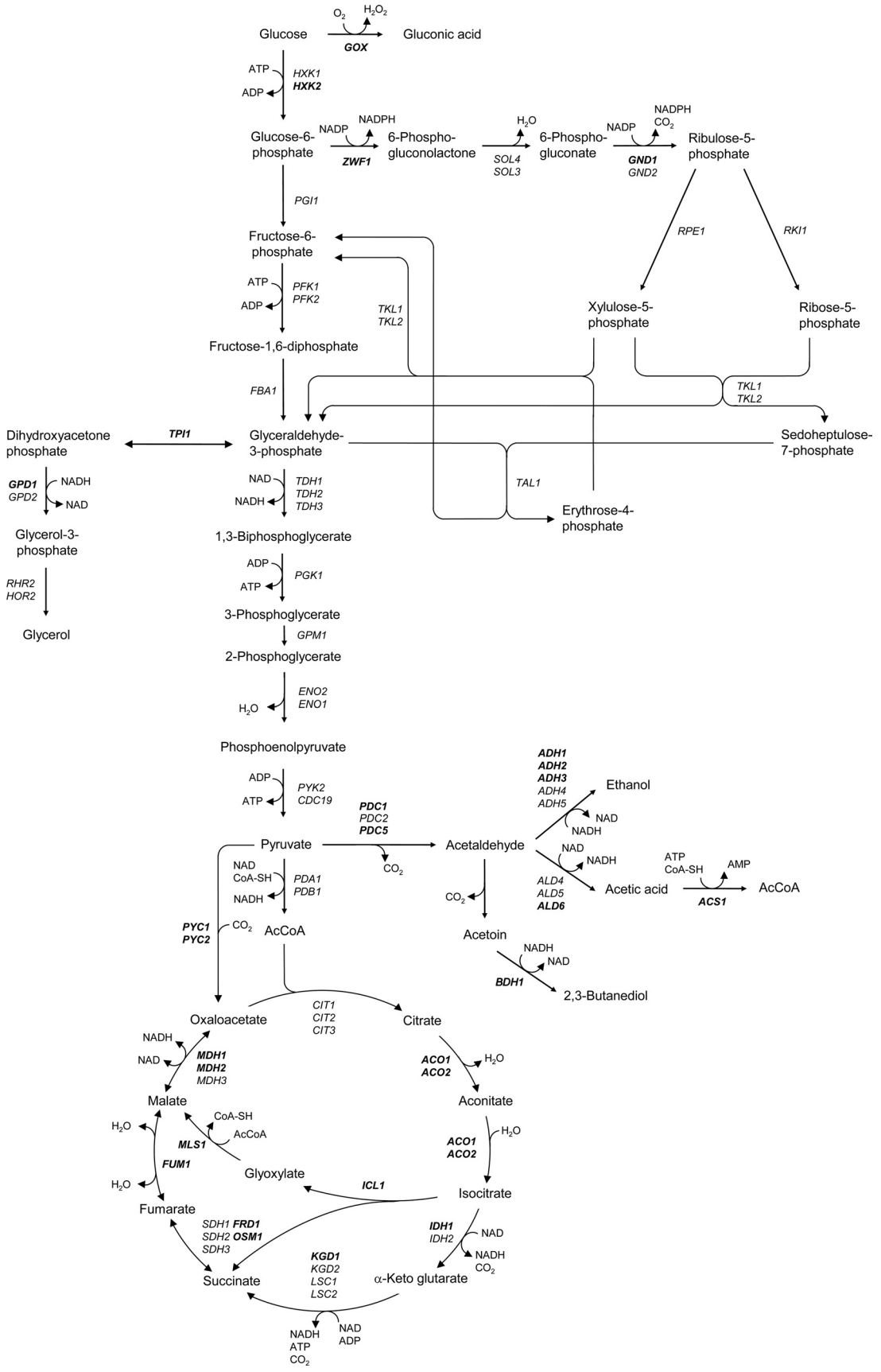
Glucose
O2
H2O2
GOX
Gluconic acid
ATP
ADP
HXK1
HXK2
Glucose-6-phosphate
NADP
NADPH
ZWF1
6-Phospho-gluconolactone
H2O
SOL4
SOL3
6-Phospho-gluconate
NADP
NADPH
CO2
GND1
GND2
Ribulose-5-phosphate
PGI1
RPE1
RKI1
Fructose-6-phosphate
ATP
ADP
PFK1
PFK2
TKL1
TKL2
Xylulose-5-phosphate
Ribose-5-phosphate
Fructose-1,6-diphosphate
FBA1
TKL1
TKL2
Dihydroxyacetone phosphate
TPI1
Glyceraldehyde-3-phosphate
Sedoheptulose-7-phosphate
GPD1
GPD2
NADH
NAD
NAD
NADH
TDH1
TDH2
TDH3
TAL1
Glycerol-3-phosphate
Erythrose-4-phosphate
1,3-Biphosphoglycerate
RHR2
HOR2
ADP
ATP
PGK1
Glycerol
3-Phosphoglycerate
GPM1
2-Phosphoglycerate
H2O
ENO2
ENO1
Phosphoenolpyruvate
ADP
ATP
PYK2
CDC19
PDC1
PDC2
PDC5
ADH1
ADH2
ADH3
ADH4
ADH5
Ethanol
NAD
NADH
Pyruvate
CO2
Acetaldehyde
NAD
NADH
ALD4
ALD5
ALD6
Acetic acid
ATP
CoA-SH
AMP
ACS1
AcCoA
NAD
CoA-SH
NADH
PDA1
PDB1
CO2
PYC1
PYC2
AcCoA
Acetoin
NADH
NAD
BDH1
2,3-Butanediol
CIT1
CIT2
CIT3
Oxaloacetate
Citrate
NADH
NAD
MDH1
MDH2
MDH3
ACO1
ACO2
H2O
Malate
Aconitate
H2O
CoA-SH
AcCoA
MLS1
ACO1
ACO2
H2O
FUM1
H2O
Glyoxylate
ICL1
Isocitrate
Fumarate
SDH1 FRD1
SDH2 OSM1
SDH3
IDH1
IDH2
NAD
NADH
CO2
KGD1
KGD2
LSC1
LSC2
Succinate
α-Keto glutarate
NADH
ATP
CO2
NAD
ADP

TABLE 3 Chemical composition of wines obtained by fermentation of CDGJM

Strain	Concn (g/liter) of[a]:					
	Residual sugar	Malic acid	Succinic acid	Glycerol	Acetic acid	Ethanol
AWRI1631	0.0 ± 0.0	2.6 ± 0.0	0.7 ± 0.0	5.7 ± 0.1	0.2 ± 0.0	102 ± 0.3
AWRI1631 aspTPI1	0.0 ± 0.0	4.2 ± 0.1	0.0 ± 0.0	7.3 ± 0.2	0.3 ± 0.0	100 ± 0.4
AWRI1631 3GPD1	0.0 ± 0.0	2.4 ± 0.1	0.5 ± 0.0	34.3 ± 0.1	4.1 ± 0.1	82 ± 0.4
AWRI 2531	0.0 ± 0.0	2.1 ± 0.0	0.8 ± 0.0	35.0 ± 0.1	0.6 ± 0.0	81 ± 0.2
AWRI 2532	118 ± 5.2	4.7 ± 0.3	0.0 ± 0.0	14.7 ± 0.3	0.3 ± 0.0	24 ± 0.5

[a] Values are averages and standard deviations from three replicates.

Both *TPI1* deletion strains (AWRI1631 ΔTPI1 and AWRI1631 GPD1 ΔTPI1) were unable to complete fermentation; however, they showed the greatest glycerol-to-glucose yields (data not shown). For this reason, four additional modifications aimed at decreasing *TPI1* expression or Tpi1p activity were tested, but only one showed a significant effect on ethanol production. Substituting glutamic acid-165 with aspartic acid in the active site of Tpi1p was previously shown to lower the activity of this enzyme (54); this mutation resulted in 6% decreased ethanol production compared to AWRI1631.

Fps1p, the glycerol transporter, has been shown to regulate the intracellular concentration of glycerol by closing the pore channel (57). Removal of the channel gate allows glycerol to leak out of the cell and increases glycerol production (56, 57). AWRI1631 FPS1Δ11, carrying the "open" transporter, diverted carbon toward glycerol generation and showed an 8% decrease in ethanol production.

Seven of the yeast constructs delivering reduced amounts of ethanol involved increased expression of *GPD1*. AWRI1631 GPD1 carried the single gene modification most effective at reducing ethanol production (10% decrease, compared to AWRI1631). Introduction of two (AWRI1631 2GPD1) and three copies (AWRI1631 3GPD1) of the *FBA1*p-*GPD1* cassette enabled further reductions in ethanol production, of 19% and 29%, respectively. AWRI1631 GPD1 FPS1Δ11, carrying a high-*GPD1*-expression construct and the open glycerol transporter, exhibited a 13% decrease in ethanol concentration.

As described previously, increased glycerol production leads to a higher concentration of acetic acid (10, 46), which is detrimental to wine quality. Two strategies were attempted to reduce acetic acid production: increased expression of *ACS1* and deletion of *ALD6* (6, 15). AWRI1631 2GPD1 ACS1 produced amounts of ethanol similar to those produced by AWRI1631 2GPD1 (Table 1) with no effect on elevated levels of acetic acid (data not shown), while AWRI2531 (with two copies of the *FBA1*p-*GPD1* cassette and an *ALD6* deletion) showed a further 10% decrease in ethanol concentration compared to AWRI1631 2GPD1. The same behavior was observed when *ALD6* was deleted in the strain carrying three copies of the *FBA1*p-*GPD1* cassette. Thus, AWRI2532 produced the fermentation product with the lowest ethanol concentration, 35% lower than that of AWRI1631.

Characterization of low-ethanol strains under winemaking conditions in CDGJM. Four strains selected from the above screening were assessed for their ability to ferment CDGJM in winemaking conditions (Table 3). Only AWRI2532 was not able to complete fermentation, as was evident from the high concentration of residual sugar in the medium. All four strains produced fermentation products with higher glycerol and lower ethanol concentrations than AWRI1631. AWRI1631 aspTPI1 produced more malic acid, acetic acid, and glycerol with less succinic acid and a 2% lower ethanol concentration than the parental strain. Compared to AWRI1631, AWRI1631 3GPD1 produced slightly less malic acid and succinic acid, 6 times more glycerol, 20 times more acetic acid, and 20% less ethanol. AWRI2531 produced less malic acid, slightly more succinic acid, 6.1 times more glycerol, 3 times more acetic acid, and 22% less ethanol.

Characterization of low-ethanol strains in grape juice. Only two strains, AWRI2531 and AWRI2532, were tested for their ability to ferment Chardonnay and Cabernet Sauvignon grape juice. Both strains fermented these juices to dryness, although AWRI 2532 took longer to consume all sugar (Fig. 2). Cell numbers were lower for the modified strains than for the parental strain in both Chardonnay and Cabernet Sauvignon (Fig. 2). AWRI 2531 and AWRI 2532 produced higher concentrations of glycerol, acetic acid, acetaldehyde, acetoin, and 2,3-butanediol and less ethanol than the parental strain (Tables 4 and 5).

Production of malic and succinic acids was grape juice dependent. In Chardonnay, AWRI2531 produced less malic and succinic acids than the parental strain, while in Cabernet Sauvignon, the concentration of these acids was higher than for the parent. AWRI2531 produced 3.6-fold more glycerol in both wines. This increase in glycerol production generated 15% less ethanol in both wines. Acetic acid production was similar to that of the parent in Chardonnay, while in Cabernet Sauvignon the concentration was

FIG 1 Central carbon metabolism in *S. cerevisiae*, including glycolysis, the pentose phosphate pathway, and the TCA cycle. Genes in bold were modified in this work. *ACO1* and *-2*, aconitase; *ACS1*, acetyl coenzyme A (CoA) synthetase; *ADH1* to *-5*, alcohol dehydrogenase; *ALD4* to *-6*, aldehyde dehydrogenase; *BDH1*, butanediol dehydrogenase; *CDC19*, pyruvate kinase; *CIT1* to *-3*, citrate synthase; *ENO1* and *-2*, enolase; *FBA1*, fructose-bisphosphate aldolase; *FRD1*, fumarate reductase; *FUM1*, fumarase; *GND1* and *-2*, 6-phophogluconate dehydrogenase; *GOX*, glucose oxidase; *GPD1* and *-2*, glycerol-3-phosphate dehydrogenase; *GPM1*, phosphoglycerate mutase; *HOR2*, glycerol-3-phosphatase; *HXK1* and *-2*, hexokinase; *ICL1*, isocitrate lyase; *IDH1* and *-2*, isocitrate dehydrogenase; *KGD1* and *-2*, α-ketoglutarate dehydrogenase; *LSC1* and *-2*, succinyl-CoA ligase; *MDH1* to *-3*, malate dehydrogenase; *MLS1*, malate synthase; *OSM1*, fumarate reductase; *PDA1*, pyruvate dehydrogenase alpha unit; *PDB1*, pyruvate dehydrogenase beta unit; *PDC1*, *-2*, and *-5*, pyruvate decarboxylase; *PFK1* and *-2*, phosphofructokinase; *PGK1*, phosphoglycerate kinase; *PGI1*, phosphoglucose isomerase; *PYC1* and *-2*, pyruvate carboxylase; *PYK2*, pyruvate kinase; *RKI1*, ribose-5-phosphate ketol-isomerase; *RHR2*, glycerol-3-phosphatase; *RPE1*, ribulose-5-phosphate epimerase; *SDH1* to *-3*, succinate dehydrogenase; *SOL3* and *-4*, 6-phosphogluconolactonase; *TAL1*, transaldolase; *TDH1* to *-3*, glyceraldehyde-3-phosphate dehydrogenase; *TKL1* and *-2*, transketolase; *TPI1*, triosephosphate isomerase; *ZWF1*, glucose-6-phosphate dehydrogenase.

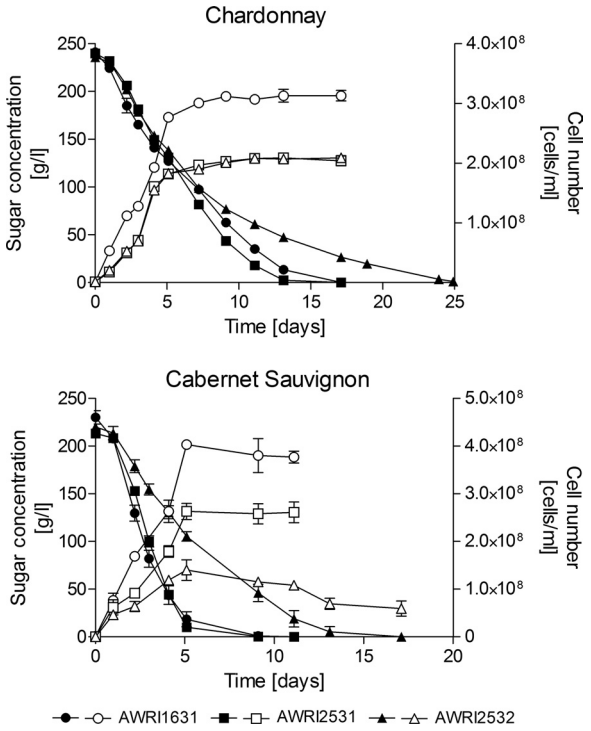

FIG 2 Fermentation kinetics and cell numbers in Chardonnay and Cabernet Sauvignon. Closed symbols, sugar consumption; open symbols, cell number.

slightly higher than for AWRI1631. In Chardonnay, acetaldehyde, acetoin, and 2,3-butanediol concentrations increased 9-fold, 19-fold, and 6.5-fold, respectively, whereas in Cabernet Sauvignon, these concentrations were increased 5.2-fold, 75.6-fold, and 22.5-fold, respectively.

AWRI2532 produced less malic and succinic acids than AWRI1631 in Chardonnay. In Cabernet Sauvignon, however, malic acid production was higher than for the parental strain, while the concentration of succinic acid was less than that produced by AWRI1631. AWRI2532 produced, on average, 4.9-fold more glycerol than the parent in both Chardonnay and Cabernet Sauvignon. Consequently, ethanol production by this strain was 22% less in both wines. Acetic acid production was higher in both Chardonnay and Cabernet Sauvignon but still lower than levels considered detrimental to wine quality (55). Acetaldehyde, acetoin, and 2,3-butanediol production in Chardonnay increased 8.5-fold, 37.5-fold, and 5.8-fold, respectively, whereas in Cabernet Sauvignon these concentrations increased 7.8-fold, 179-fold, and 22.6-fold, respectively.

TABLE 5 Chemical composition of wines obtained by fermentation of Cabernet Sauvignon

Component	AWRI1631	AWRI2531	AWRI2532
Residual sugar (g/liter)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Malic acid (g/liter)	3.9 ± 0.2	6.9 ± 0.4	7.9 ± 0.0
Succinic acid (g/liter)	7.7 ± 0.4	7.9 ± 0.1	7.0 ± 0.2
Glycerol (g/liter)	9.0 ± 0.1	33.3 ± 0.6	42.2 ± 0.4
Acetic acid (g/liter)	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.0
Ethanol (g/liter)	122.3 ± 0.6	103.7 ± 0.3	93.8 ± 0.6
Acetaldehyde (mg/liter)	29 ± 1.6	150 ± 16	227 ± 21
Acetoin (mg/liter)	33 ± 1	2495 ± 83	5923 ± 99
2,3-Butanediol (mg/liter)	9.4 ± 0.6	226 ± 36	212 ± 42
Glycerol yield (g/g sugar)	0.04 ± 0.0	0.15 ± 0.0	0.19 ± 0.0
Ethanol yield (g/g sugar)	0.55 ± 0.0	0.48 ± 0.0	0.44 ± 0.0

[a] Values are averages and standard deviations from three replicates.

As high concentrations of acetoin negatively impact the organoleptic properties of the resulting wine (16), AWRI2531 and AWRI2532 were genetically modified to convert acetoin into the sensorially neutral compound 2,3-butanediol. Expression of *BDH1*, which encodes 2,3-butanediol dehydrogenase, was therefore increased in both strains. Compared to AWRI2531, AWRI2531 BDH1 showed a significant decrease in both acetaldehyde and acetoin concentrations, with an accompanying increase in the concentration of 2,3-butanediol; levels of all other metabolites remained similar (Table 4). In contrast, while AWRI2532 BDH1 also showed significantly lower acetaldehyde and acetoin concentrations combined with a higher 2,3-butanediol concentration than AWRI2532, AWRI2532 BDH1 also produced considerably less glycerol and more ethanol than its parent.

DISCUSSION

There is growing interest in alcoholic beverage industries to reduce the level of alcohol in beers and wines. There are many strategies available to achieve this end, but all have serious limitations (49). It is widely believed that microbiological approaches have the capacity to deliver the best outcome. This paper describes a survey of a large number of target *S. cerevisiae* genes that have been manipulated to test their potential for the development of a low-ethanol-wine yeast strain.

Genes involved in glucose repression, glycolysis, the pentose

TABLE 4 Chemical composition of wines obtained by fermentation of Chardonnay[a]

Component	AWRI1631	AWRI2531	AWRI2532	AWRI2531 BDH1	AWRI2532 BDH1
Residual sugar (g/liter)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Malic acid (g/liter)	4.8 ± 0.4	3.3 ± 0.1	4.2 ± 0.1	3.4 ± 0.2	4.1 ± 0.1
Succinic acid (g/liter)	3.2 ± 0.0	2.7 ± 0.0	2.6 ± 0.0	2.9 ± 0.1	3.2 ± 0.1
Glycerol (g/liter)	8.7 ± 0.1	31.1 ± 0.5	43.4 ± 0.1	30.3 ± 0.2	34.4 ± 0.4
Acetic acid (g/liter)	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Ethanol (g/liter)	124.3 ± 0.8	105.7 ± 0.7	96.9 ± 0.2	107.0 ± 1.1	105.6 ± 0.6
Acetaldehyde (mg/liter)	32 ± 1.8	290 ± 20	272 ± 22	175 ± 5	143 ± 29
Acetoin (mg/liter)	25 ± 8	478 ± 7	939 ± 94	105 ± 25	100 ± 26
2,3-Butanediol (mg/liter)	14 ± 1.3	91 ± 21	81 ± 20	191 ± 23	148 ± 24
Glycerol yield (g/g consumed sugar)	0.03 ± 0.0	0.11 ± 0.0	0.13 ± 0.0	0.11 ± 0.0	0.10 ± 0.0
Ethanol yield (g/g consumed sugar)	0.48 ± 0.0	0.44 ± 0.0	0.42 ± 0.0	0.45 ± 0.0	0.46 ± 0.0

[a] Values are averages and standard deviations from three replicates.

phosphate pathway, and the TCA cycle and a heterologous (*A. niger*) gene involved in glucose oxidation were separately engineered in a wine yeast with the aim of reducing ethanol production. Specifically, genes were manipulated to divert carbon away from ethanol production to other endpoints.

One of the obvious strategies to lower ethanol production is to decrease the activity of the main enzyme responsible for ethanol formation. Deletion of *ADH1*, which encodes alcohol dehydrogenase, has been shown to decrease ethanol production in laboratory yeast strains (9, 12, 13). In the present work, however, deletion of *ADH1* did not affect ethanol formation, suggesting that other alcohol dehydrogenase isozymes compensate for loss of this enzyme in AWRI1631 wine yeast background under the conditions used in this study. Indeed, Adh3p has been shown to mimic the function of Adh1p in a genetic background lacking *ADH1*, *ADH2*, *ADH4*, and *ADH5* (12). However, deletion of both *ADH1* and *ADH3*, together and individually, had no effect on ethanol production. This might indicate that one or more of the other Adh isozymes compensates for loss of *ADH1* and *ADH3* in the genetic background of the yeast strain used for this work. *ADH2*, which encodes an alcohol dehydrogenase that metabolizes ethanol (12), had no impact on ethanol yield when overexpressed.

Several studies have shown that deletion or overexpression of specific genes involved in the TCA cycle affect not only organic acid production but also ethanol yields (1, 37, 51). Deletion of *KGD1*, *KGD2*, or *FUM1* decreased production of ethanol in a lab strain (51), whereas *ACO1* deletion resulted in a modest decrease in ethanol formation in a sake yeast (1).

In the work described here, manipulation of most of the genes involved in the oxidative or reductive branches of the TCA cycle affected the formation of organic acids but did not impact ethanol production. At least in some cases, this differs from what has been previously reported, perhaps reflecting differences in genetic backgrounds and/or different environmental conditions. Only increased expression of *MDH2* and *FRD1*, involved in the reductive branch, led to a decrease in ethanol concentration, but this was rather modest. Increased expression of the cytosolic malate dehydrogenase (*MDH2*) can increase malate, fumarate, and citrate production (38), but the authors of that study noted that the activity of pyruvate carboxylase (encoded by *PYC1* and *PYC2*) appeared to be rate limiting for malate synthesis. Therefore, increasing expression of more than one of the genes involved in the reductive branch of the TCA cycle simultaneously might divert more carbon away from ethanol production.

Another strategy with the potential to decrease ethanol production is to lift glucose repression from genes encoding enzymes involved in respiration and therefore burn carbon that would otherwise feed into ethanol production. Glucose repression involves a great number of genes and several signal transduction pathways (28, 47). Nevertheless, deletions of *HXK2*, *GRR1*, and *MIG1* have each been shown to decrease ethanol production by redirecting carbon to biomass formation in continuous cultures (44). In the work described here, the only genetic modifications targeting glucose repression found to decrease ethanol formation were the deletions of *HXK2* and *MIG1*. Regardless, the minor effect of these gene deletions on ethanol production, even in the presence of oxygen, suggests that in the AWRI1631 genetic background, other pathways related to glucose repression prevent respiration during wine fermentation.

The pentose phosphate pathway represents another potential sink for carbon. However, Cadiere et al. (5) found that yeast strains exhibiting higher carbon fluxes through this pathway showed no effect on ethanol production. Mutants lacking *PGI1* and therefore channeling carbon through the pentose phosphate pathway were not able to sustain growth even when overexpressing *ZWF1*, indicating that another reaction or factor limits flux through this pathway (24). Therefore, it is perhaps not surprising that increased expression of *ZWF1* and *GND1* showed unchanged ethanol production in this study.

Expression in *S. cerevisiae* of the *A. niger* glucose oxidase gene has been previously shown to decrease ethanol production by diverting sugar metabolism toward gluconic acid formation (33). In the present work, this approach only showed a modest decrease in ethanol formation; additionally, hydrogen peroxide, a product of the glucose oxidase reaction, was most likely responsible for darkening the medium color. Therefore, to avoid perturbations to wine color when expressing glucose oxidase, other strategies, such as coexpressing a catalase, should be explored.

Glycerol proved to be the best carbon sink in the current study. All modifications intended to increase the formation of glycerol (*TPI1* deletion, *FPS1* modification, and *GPD1* overexpression) made a substantial impact on ethanol production.

Although yeast strains lacking *TPI1* have been shown to grow in rich medium (7), neither AWRI1631 ΔTPI1 nor AWRI1631 GPD1 ΔTPI1 was able to ferment high concentrations of sugar. Interestingly, however, these strains showed the greatest yields of glycerol on glucose relative to all other mutants tested, which is consistent with previous reports (8). In order to decrease Tpi1p activity without affecting fermentation performance, we explored two strategies, decreasing expression of *TPI1* and lowering Tpi1p activity. Deleting REB1-, RAP1-, and GCR1-binding sites in the promoter region of *TPI1* has been shown to decrease *TPI1* expression in yeast (50). However, deletion of RAP1- and GCR1-binding sites had no impact on glycerol or ethanol concentrations. Replacement of glutamic acid-165 with aspartic acid in the active site of the chicken Tpi1p has been shown to considerably decrease the activity of the enzyme (54). Yeast Tpi1p also contains a glutamic acid in position 165; when this residue was replaced with aspartic acid, we observed an increase in glycerol formation and a decrease in ethanol production but no effect on fermentation performance.

Although *FPS1* deletion has been shown to decrease glycerol yield and increase ethanol formation (60), increased expression of *FPS1* failed to deliver the opposite phenotype (9). Indeed, glycerol transport, and hence glycerol production, is controlled by a short regulatory domain in the N-terminal extension of Fps1p (56, 57). When this domain is deleted, the channel is hyperactive, remaining open for glycerol efflux and resulting in continuous glycerol leakage from the cell, which is compensated for by increased glycerol production (45, 56). We observed that this increased glycerol production, as a consequence of deleting the regulatory domain of Fps1p lowered ethanol formation considerably.

Increased expression of *GPD1* delivered the greatest impact on ethanol production. Moreover, increasing the number of copies of highly expressed *GPD1* further decreased ethanol yield. However, increased glycerol production leads to increased acetic acid formation (10, 46), which is detrimental to wine quality. Two strategies were tried in the current work to decrease acetic acid production, *ALD6* deletion and increased expression of *ACS1*. Only the deletion of *ALD6* was effective at decreasing acetic acid concentration; this approach was shown previously to lower acetic

acid production when glycerol 3-phosphate dehydrogenase is overexpressed (6, 15).

After all modified strains had been evaluated in rich medium, it was crucial to assess the most promising modified yeasts in synthetic must and then in grape juice. To the best of our knowledge, this is the first time low-alcohol-producing GM strains have been used to ferment must from different grape varieties. AWRI 2531 and AWRI 2532 showed similar metabolic profiles in Chardonnay and Cabernet Sauvignon musts. However, AWRI 2532 was slower to complete fermentation in both grape juices than AWRI 2531. In addition, AWRI 2532 was unable to complete fermentation in chemically defined juice, suggesting that this strain might struggle in highly clarified musts. Compared to the parental strain, AWRI 2531 generated wines with an ethanol concentration that was 2.4% (vol/vol) lower on average (from 15.6% [vol/vol] to 13.2% [vol/vol]), while AWRI 2532 produced wines that were 3.6% [vol/vol] lower on average (from 15.6% [vol/vol] to 12% [vol/vol]). Acetic acid concentrations in wines fermented with both strains were within the range considered acceptable for wine quality (55). However, consistent with previous reports (6, 15), other metabolites were increased in wines produced with these modified strains. Acetaldehyde concentration was over its sensory threshold described in wine (100 mg/liter) (52), eliciting a "bruised-apple" smell in these wines, which is detrimental to their sensory properties. Levels of acetoin, because of its low sensory threshold, are also likely to affect negatively the organoleptic properties of wine; nevertheless, acetoin can be converted into the sensorially neutral compound 2,3-butanediol by increasing expression of *BDH1*, which encodes 2,3-butanediol dehydrogenase (16). In the current work, increasing *BDH1* expression not only enabled the conversion of acetoin to 2,3-butanediol but also resulted in lower acetaldehyde concentration. Unexpectedly, increasing *BDH1* expression altered the production of glycerol and ethanol in AWRI2532, most likely driven by changes in redox balance. Although acetaldehyde and acetoin production was significantly decreased in wines fermented with strains AWRI2531 BDH1 and AWRI2532 BDH1, acetaldehyde concentrations still exceeded its sensory threshold, while acetoin concentrations were below the sensory threshold described in the literature (150 mg/liter) (17) and hence, at least in the case of acetaldehyde, may still affect negatively the sensory properties of the resulting wine.

In summary, of all strategies aimed at decreasing ethanol production evaluated in this study, those intended to increase glycerol formation were the most effective. The efficiencies of several strategies that have been shown to alter ethanol formation, including diverting sugar to lactate production (11), manipulating hexose transporter genes (23), and expressing a H_2O-forming NADH oxidase (25), as well as novel strategies, such as diverting carbon to the formation of reserve carbohydrates, remain to be tested.

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