Genetic Engineering of an Industrial Strain of *Saccharomyces cerevisiae* for L-Malic Acid Degradation via an Efficient Malo-Ethanolic Pathway

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The optimal ratio of L-malic and L-tartaric acid in relation to other wine components is one of the most important aspects that ultimately determine wine quality during winemaking. Winemakers routinely rely on the judicious use of malolactic fermentation (MLF) after alcoholic fermentation to deacidify and stabilise their wines. However, due to the unreliability of the process and unsuitable sensory modifications in some grape cultivars, especially for fruity-floral wines, MLF is often regarded as problematic and undesirable. Alternative methods for reducing the amounts of L-malic acid in wine will contribute to improving the production of quality wines in the future, especially in cool-climate regions. Most wine yeast strains of *Saccharomyces* are unable to effectively degrade L-malic acid, whereas the fission yeast *Schizosaccharomyces pombe* efficiently degrades high concentrations of L-malic acid by means of malo-ethanolic fermentation. However, strains of *S. pombe* are not suitable for vinification due to the production of undesirable off-flavours. Previously, the *S. pombe* malate permease *(mae1)* and malic enzyme *(mae2)* genes were successfully expressed under the 3-phosphoglycerate kinase *(PGK1)* regulatory elements in *S. cerevisiae*, resulting in a recombinant laboratory strain of *S. cerevisiae* with an efficient malo-ethanolic pathway. Stable integration of the *S. pombe* malo-ethanolic pathway genes has now been obtained through the construction of a unique integration strategy in a commercial wine yeast strain. Co-transformation of the linear integration cassette containing the *mae1* and *mae2* genes and *PGK1* regulatory elements and a multi-copy plasmid containing the phleomycin-resistance marker into a commercial *Saccharomyces cerevisiae* strain resulted in the successful transformation and integration of the malo-ethanolic genes. The recombinant *S. cerevisiae* strain was successfully cured of phleomycin-resistance plasmid DNA in order to obtain malo-ethanolic yeast containing only yeast-derived DNA. The integrated malo-ethanolic genes were stable in *S. cerevisiae* and during synthetic and grape must fermentation, L-malic acid was completely fermented to ethanol without any negative effect on fermentation kinetics and wine quality.

The conversion of L-malic acid to lactic acid and CO_2 during malolactic fermentation (MLF) is one of the four possible metabolic conversions found for L-malic acid in nature. L-Malic acid can also serve as a carbon source for yeast, which can transform L-malic acid into one of three other compounds, i.e. oxaloacetic acid (via a malate dehydrogenase), fumaric acid (via reverse reaction of fumarase) and pyruvic acid (via malic enzyme). Based on yeasts' ability to utilise L-malic acid and other TCA cycle intermediates as sole carbon or energy source, yeasts are divided into a K (-) or K (+) group (Barnett & Kornberg, 1960). Strains of *Saccharomyces sensu stricto,* including commercial wine yeast strains, as well as *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii,* are all classified as K (-) yeasts that can utilise TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources (Barnett & Kornberg, 1960).

Strains of *Saccharomyces* cannot degrade L-malic acid completely in grape must during alcoholic fermentation, resulting in relatively minor modifications in total acidity during vinification (Rankine, 1966; Radler, 1993; Subden *et al*., 1998). A number of reasons for the weak degradation of L-malic acid in *S. cerevisiae* have been postulated. Firstly, *S. cerevisiae* lacks the machinery for active transport of L-malic acid found in *S. pombe* and relies on rate-limiting simple diffusion for the uptake of extracellular L-malic acid. Secondly, the malic enzyme of *S. cerevisiae* has a significantly lower substrate affinity for L-malic acid (K_m = 50 mM) than that of *S. pombe* (K_m = 3.2 mM), which contributes to weaker metabolism of this acid in *S. cerevisiae* (Temperli *et al.*, 1965; Fuck *et al*., 1973). Lastly, compartmentalisation of the malic enzyme in mitochondria may contribute to the weak L-malic acid degradation in *S. cerevisiae*. The mitochondrial location of the malic enzyme of *S. cerevisiae*, in contrast to the cytosolic location of the *S. pombe* malic enzyme, suggests that this enzyme is inherently subject to the regulatory effect of glucose (Redzepovic *et al*., 2002). Furthermore, mitochondrial deterioration, a well-documented phenomenon in Crabtree-positive yeast, might exacerbate the already weak L-malic acid metabolism of *S. cerevisiae*.

The ability of yeast to efficiently degrade extracellular L-malic acid depends on an efficient uptake system for L-malic acid, i.e.

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active transport via a malate permease, and an effective L-malic acid-converting enzyme, such as the malic enzyme. The malate permease gene (*mae1*) and the malic enzyme gene (*mae2*) of *S. pombe* were therefore cloned (Viljoen *et al*., 1994; Grobler *et al*., 1995) and co-expressed in multi-copy and single copy under the *S. cerevisiae* constitutive 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences in a laboratory strain of *S. cerevisiae* (Volschenk *et al*., 2001). A strong malo-ethanolic phenotype was introduced in *S. cerevisiae,* where L-malic acid was rapidly and efficiently degraded in synthetic and Chardonnay grape must with the concurrent production of higher ethanol levels (Volschenk *et al*., 2001). Functional expression of the malo-ethanolic pathway genes of *S. pombe* in a laboratory strain of *S. cerevisiae* paved the way for the genetic modification of industrial wine yeast strains of *Saccharomyces* for commercial winemaking applications.

Stable integration of the malo-ethanolic pathway genes into the genome of industrial wine yeast strains is a prerequisite for becoming an inherited component of the yeast genome. Genetic engineering of wine yeast strains of *Saccharomyces* is, however, complicated by the homothallic, multiple ploidy and prototrophic nature of industrial strains of *Saccharomyces* (Pretorius, 2000). Transformation and stable integration of heterologous genes into industrial strains of *Saccharomyces* require the use of dominant selectable markers, antibiotic or toxic compound resistance markers. However, integration of these markers into the yeast genome is not acceptable for commercial application, mainly due to the absence of long-term risk assessment and to consumer disapproval.

The integration of the malo-ethanolic expression cassettes in industrial wine yeast strains previously reported by Volschenk *et al*. (2001) was based on resistance to the herbicide sulphometuron methyl (SMM) via the *SMR1-410* gene. In this study we report a novel integration strategy for the *S. pombe mae1* and *mae2* expression cassettes without the incorporation of any non-yeast derived DNA sequences. Integration and expression of the malo-ethanolic genes in *S. cerevisiae* S92 resulted in rapid and complete degradation of L-malic acid and increased ethanol production during the early stages of alcoholic fermentation. Furthermore, this had no adverse effect on the yeast's fermentative ability, and sensory evaluation and chemical analysis of Chardonnay wine indicated an improvement in wine flavour perception compared to the control wines.

MATERIALS AND METHODS

Strains and maintenance

The microbial strains and plasmids used in this study are listed in Table 1. Cells of *Escherichia coli* JM109 were transformed by electroporation and selected on LB agar medium supplemented with 200 mg/L ampicillin (Ausubel *et al*., 1995). *S. cerevisiae* S92 (a galactose positive strain of *S. cerevisiae* previously referred to as *Saccharomyces bayanus*) was maintained on YPD agar, while transformants were plated onto YEG media containing 0.5% yeast extract, 2% glucose, 3% Pastagar B (Difco Laboratories, Detroit, MI) and 250 µg/mL phleomycin.

Plasmid construction

Standard recombinant DNA techniques were employed essentially as described by Ausubel *et al*. (1995). Restriction enzymes, modification enzymes and DNA purification kits were used as prescribed by the manufacturer (Roche Diagnostics, Germany). All polymerase chain reactions (PCR) were executed with Takara Ex *Taq* (Takara Bio Inc, Japan). All subcloning and DNA manipulations were performed in the 2µm-based plasmid YEp352 (Hill *et al*., 1986). Prior to any subcloning, the *Kpn*I restriction site located in the multiple cloning region of YEp352 was eliminated

TABLE 1

Strains and plasmids used for the integration of the *S. pombe* malo-ethanolic genes into a commercial strain of *Saccharomyces cerevisiae.*

Strains	**Description**	**Reference**
***E. coli* JM109**	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 [r_k-, m_k+], *relA*1, *supE*44, λ^-, Δ(*lac-proAB*), [F', *traD*36, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15]	Yanisch-Perron, 1985
***S. cerevisiae* S92**	Commercial wine yeast	Lesaffre
Plasmids	**Description**	**Reference**
YEp352	Yeast/*E. coli* shuttle vector with a *URA3* marker	Hill *et al*., 1986
pUT332	Yeast episomal plasmid containing the *Tn5ble* gene for selection of phleomycin resistance	Gatignol *et al*., 1990; Wenzel *et al*., 1992
pHV3	pHVX2 containing the *mae1* ORF subcloned between the *PGK1* promoter and terminator sequences.	Volschenk *et al*., 1997[a, b]
pHV7	YEplac195 (*URA3* marker gene) containing the *mae2* ORF subcloned between the *PGK1* promoter and terminator sequences.	Volschenk *et al*., 2001
pHV9	YEp352 without the *Kpn*I restriction site	This study
pHVJH1	pHV9 containing the mutated *URA3* gene	This study
pHV11	pHVJH1 containing the *PGK1*$_p$-*mae1*-*PGK1*$_t$ expression cassette subcloned into the *Kpn*I site in the mutated *URA3* gene	This study
pHV13	pHV11 containing the *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassette subcloned into the *Not*I site in the mutated *URA3* gene	This study

by *Kpn*I digestion, filled to blunt-ends with Klenow enzyme and religated to yield pHV9. A 944 bp upstream *URA3* fragment was PCR amplified from *S. cerevisiae* S92 genomic DNA using primer set 5'-XBASFRURA3 and 3'-URA3KPN (Table 2), while a 959 bp downstream *URA3* fragment was PCR amplified using primer set 5'-KPNNOTURA3 and 3'-URA3SFRXBA.

Both the upstream and downstream *URA3* fragments were digested with *Kpn*I and fused by T_4 DNA ligation. The resulting linear product, which was isolated after 1% agarose gel electrophoresis and purified by the High Pure Gel Extraction Kit, served as template for PCR amplification with primer set 5'-XBASFRURA3 and 3'-URA3SFRXBA. The modified *URA3* fragment containing unique cloning sites (*Kpn*I, *Not*I) and excision sites (*SrfI* and *Xba*I) was subcloned in the *Xba*I restriction site of pHV9, resulting in pHVJH1 (Fig. 1).

Construction of the expression vectors and pHV3 and pHV7 (Table 1) was previously described (Volschenk *et al*., 1977a,b; Volschenk *et al*., 2001). The *PGK1*$_p$-*mae1*-*PGK1*$_t$ expression cassette was PCR amplified using primer set 5'-KPNPGK and 3'-PGKKPN with plasmid pHV3 as template, while primers 5'-NOTPGK and 3'-PGKNOT were used for PCR amplification of the *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassette from plasmid pHV7 (Volschenk *et al*., 2001). The *PGK1*$_p$-*mae1*-*PGK1*$_t$ PCR product was subcloned as a *Kpn*I fragment into pHVJH1 to yield pHV11 (Fig. 2). Similarly, the *PGK1*$_p$-*mae2*-*PGK1*$_t$ PCR product was subcloned as a *Not*I fragment into pHV11 to yield pHV13. *SrfI* digestion of pHV13 resulted in the excision of a linear *PGK1*$_p$-*mae1*-*PGK1*$_t$ -*PGK1*$_p$-*mae2*-*PGK1*$_t$ fragment flanked by ca. 500 to 600 bp *URA3* sequences, which excludes any vector-derived DNA sequences.

Phleomycin and geneticin resistance of industrial wine yeast strains

The minimum inhibition concentration (MIC) of phleomycin and geneticin for *S. cerevisiae* S92 was determined. Yeast cells were cultured overnight in 10 mL YEG broth and plated with or without electroporation (in the absence of any DNA) onto YEG plates with Pastagar B (Difco Laboratories, Detroit, MI), containing a range of 5 µg/mL to 500 µg/mL phleomycin or geneticin. A minimum concentration of 100 µg/mL geneticin was required for complete inhibition *S. cerevisiae* S92 prior to electroporation. However, electroporated cells of *S. cerevisiae* S92 cells gave rise to background colonies (false positives) even at a concentration of 500 µg/mL geneticin. The minimum inhibitory concentration of phleomycin was determined at 250 µg/mL for electroporated cells of *S. cerevisiae* S92.

Adaptation of GMIA media for optimised malo-ethanolic phenotype selection

A plate assay method was developed to simplify the selection of positive transformants with a malo-ethanolic phenotype after electroporation and integration. The malo-ethanolic plate assay was also used to determine the dominance of the malo-ethanolic recombinant yeast strain during the subsequent wine fermentations. The Glucose-Malate-Indicator Agar (GMIA) selection medium was previously developed for the malo-ethanolic yeast *S. pombe* (Osothsilp *et al*., 1986). The plates produce blue colonies with a surrounding blue halo when L-malic acid in the media is degraded by *S. pombe* due to a shift in pH (pH 3.3 to 5.2) when L-malic acid is converted to pyruvic acid. Initial attempts with a recombinant strain of *S. cerevisiae* containing the malo-ethanolic genes on a multi-copy plasmid (Volschenk *et al*., 2001) did not produce a clear

TABLE 2

List of PCR primers used in this study to construct the linear integration cassette containing the *PGK1*$_p$-*mae1*-*PGK1*$_t$ and *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassettes flanked by *URA3* sequences.

Primer name	Primer sequence
5'-XBASFRURA3	5'-GATC***TCTAGA***<u>*GCCCGGGC*</u>AACGGTTCATCATCTCATGGATCTGC-3'
3'-URA3KPN	5'-GATC**GGTACC**TACTTCTTCCGCCGCCTGCTTCAAACCGCT-3'
5'-KPNNOTURA3	5'-GATC**GGTACC***GCGGCCGC*ACAAAGGAACCTAGAGGCCTTTTGATGTTAG-3'
3'-URA3SFRXBA	5'-GATC***TCTAGA***<u>*GCCCGGGC*</u>TACACCAGAGATACATAATTAGATAT-3'
5'-KPNPGK	5'-GATC**GGTACC**AACCTTTCTAACTGATC-3'
3'-PGKKPN	5'-GATC**GGTACC**AAGCTTTAACGAACGCA-3'
5'-NOTPGK	5'-GATC*GCGGCCGC*AACCTTTCTAACTGATCTATCCAAAACTG-3'
3'-PGKNOT	5'-GATC*GCGGCCGC*AAGCTTTAACGAACGCAGAATTTTCG-3'
5'-mae1	5'-GATCGAATTCATGGGTGAACTCAAGGAAAT-3'
3'-mae1	5'-GATCAGATCTTTAAACGCTTTCATGTTCACT-3'
5'-mae2	5'-GATCGAATTCATGCCTGCAGGAACCAAAGAA-3'
3'-mae2	5'-GATCCTCGAGTTATACAAAAGGCTTGTATTC-3'
5'-mae1DIG	5'-CTTTCAATATCCACGTTCATCGACA-3'
3'-mae1DIG	5'-GAGACAGTAACACCAAGCAGCAAGA-3'
5'-mae2DIG	5'-GAACCAAAGAACAAATCGAGTGTCC-3'
3'-mae2DIG	5'-GAGAACAATGGGCAAGAATCGATTA-3'

TCTAGA = *Xba*I, <u>*GCCCGGGC*</u> = *SrfI*, **GGTACC** = *Kpn*I, *GCGGCCGC* = *Not*I.

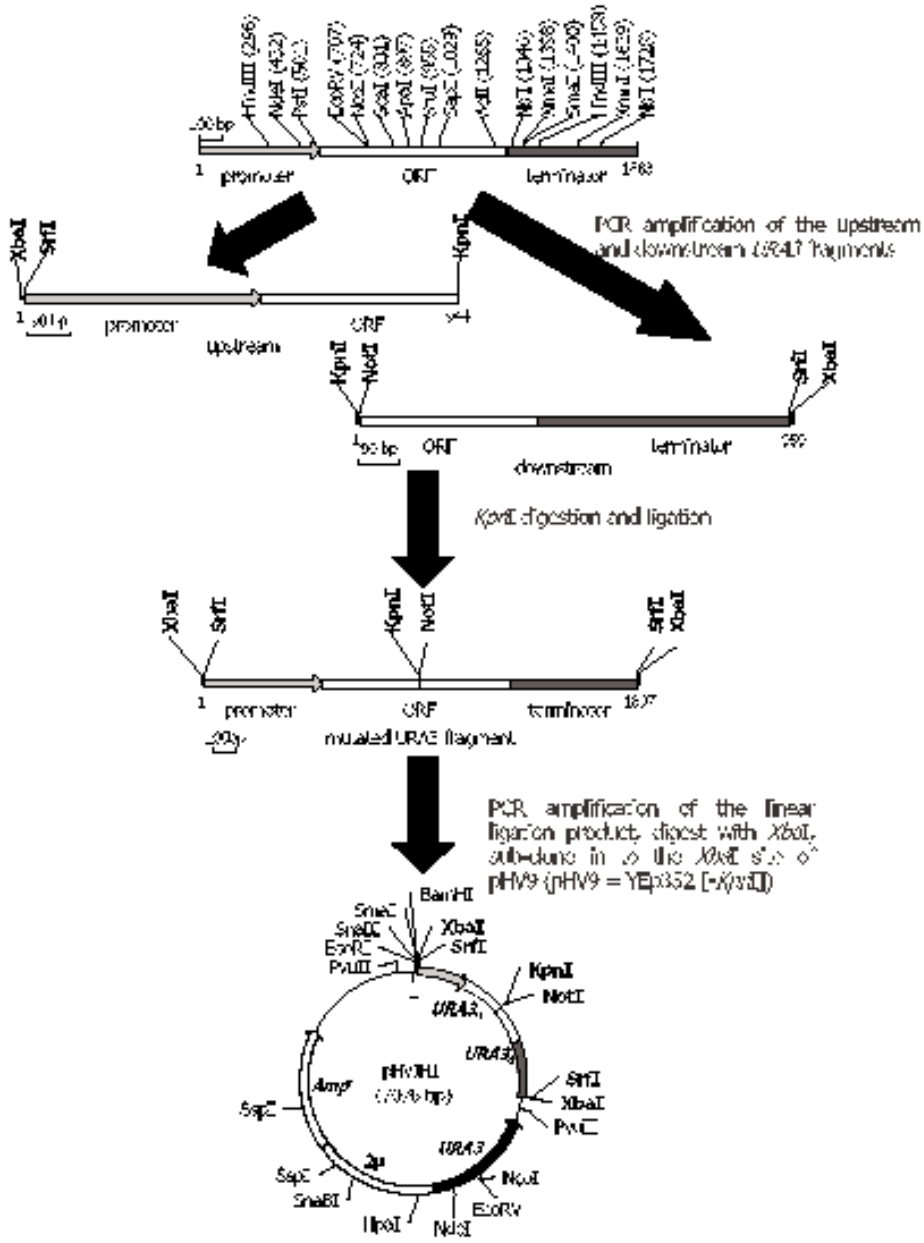

FIGURE 1

Construction of plasmid pHVJH1 by subcloning an upstream and downstream region of the *URA3* gene synthesised by PCR amplification to create unique restriction sites (*Kpn*I and *Not*I) for subcloning and excision sites (*Srf*I and *Xba*I).

phenotype for L-malic acid degradation on the GMIA plates. The original GMIA medium was therefore modified to contain 0.17% Yeast Nitrogen Base (Difco Laboratories, Detroit, MI), 0.5% $(NH_4)_2SO_4$, 10% glucose (to simulate glucose levels in grape must), 10% L-malic acid, 0.01% bromocresol green and 2% Noble agar (Difco Laboratories, Detroit, MI), instead of the Bacto-agar. The pH of the optimised GMIA media was adjusted to 3.3 with KOH.

Co-transformation and integration of *mae1* and *mae2* genes in *S. cerevisiae* S92

Integration of the *mae1* and *mae2* genes into the genome of commercial wine yeast strains was obtained by co-transformation of the linear *URA3*-flanked $PGK1_p$-*mae1*-$PGK1_t$-$PGK1_p$-*mae2*-$PGK1_t$ integration cassette and plasmid pUT332, which contains the *Tn5ble* gene for selection of phleomycin resistance (Gatignol *et al*., 1990; Wenzel *et al*., 1992). Initial screening on phleomycin-containing media was required to select for successful transformation and to minimise the number of colonies to be screened for the malo-ethanolic phenotype.

An adapted electroporation method was used in this study for the transformation of industrial wine yeast strains. Yeast cells were pre-cultured overnight in 10 mL YPD at 30°C followed by 500 mL YPD in a 2 L flask to an optical density at 600 nm (OD_{600}) of 0.1. The culture was shaken vigorously at 30°C until an OD_{600} of 1.3 to 1.5 was reached. Yeast cells were harvested by

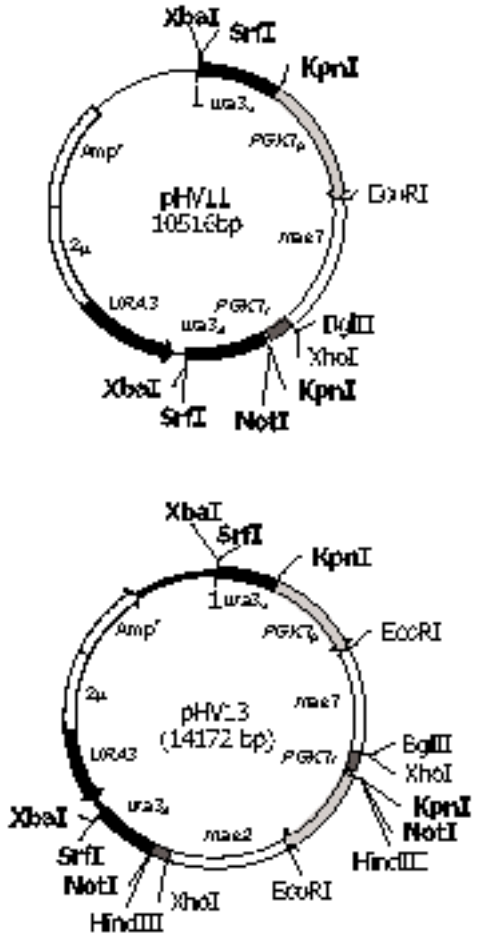

FIGURE 2

Plasmid maps of pHV11 containing the *PGK1*$_p$-*mae1*-*PGK1*$_t$ expression cassette and plasmid pHV13 containing both the *PGK1*$_p$-*mae1*-*PGK1*$_t$ and *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassettes. *Srf*I digestion of pHV13 yielded a linear integration cassette without any vector, bacterial or other foreign DNA sequences that were used for co-transformation with plasmid pUT332.

centrifugation at 4000 x g at 4°C and re-suspended in 80 mL double-distilled water (sterilised). While swirling, 10 mL 10 X TE buffer (pH 7.5) was added, followed by 10 mL 1 M LiOAc. After incubation for 45 min at 30°C with gentle agitation, 2.5 mL fresh 1 M DTT was added to the yeast suspension, while swirling, with a continued incubation for 15 min at 30°C with gentle agitation. The yeast suspension was subsequently diluted to a volume of 500 mL with double-distilled water, washed and concentrated three times at 4000 x g, 4°C. Cell pellets were re-suspended first in 250 mL ice-cold double-distilled water, then in 30 mL ice-cold 1 M sorbitol and finally in 0.5 mL ice-cold 1 M sorbitol. This yielded a final volume of 1 – 1.5 mL cells with an approximate OD_{600} of 200. After the cell pellet was re-suspended, 40 µL of the concentrated yeast cells was mixed with 5 µL DNA in a sterile, ice-cold 1.5 mL tube. A 10:1 molar ratio of linear:plasmid DNA was used, with ideally 50 ng of pUT332 and an appropriate 10-fold molar increase of linear DNA. The cell-DNA mixtures were transferred to an ice-cold 0.2 cm gap electroporation cuvette (Biorad, South Africa) and subjected to a pulse of 1.5 kV, 25 mF and 200 ohms (Gene Pulser II Electroporator, Biorad, South Africa). Immediately after the pulse was administered, 1 mL ice-cold YPD (1% yeast extract, 2% peptone and 2% glucose) was added to the cuvette, followed by a gentle mix for 2 to 4 h at 30°C. Aliquots of 250 µL yeast suspension were spread directly onto YEG plates containing 250 µg/mL phleomycin. Transformants were incubated for 3 to 4 days at 30°C. Putative transformants were inoculated in 10 mL YPD (non-selective conditions) and cultured successively for > 200 generations at 30°C to cure the yeast of plasmid pUT332. After the loss of plasmid pUT332 was confirmed on phleomycin media (data not shown), transformants were streaked onto modified GMIA plates.

PCR confirmation of integration and Southern blotting

Initial proof of the integration of the linear *PGK1*$_p$-*mae1*-*PGK1*$_t$ -*PGK1*$_p$-*mae2*-*PGK1*$_t$ fragment was obtained through PCR amplification of the entire *mae1* and *mae2* open reading frames using primer sets 5'-mae1/3'-mae1 and 5'-mae2/3'-mae2, respectively (Table 2). Integration of the linear *PGK1*$_p$-*mae1*-*PGK1*$_t$-*PGK1*$_p$-*mae2*-*PGK1*$_t$ fragment in the genomic *URA3* locus was confirmed through Southern blot analysis. Standard procedures (Ausubel *et al*., 1995) were used to prepare the gel for Southern blotting and to transfer the DNA to a positively charged nylon membrane (Roche Diagnostics, Germany). Genomic DNA was isolated from *S. cerevisiae* (Hoffman & Winston, 1987), digested with *Hpa*I and separated on a 1% agarose gel. An internal 944 bp *URA3* fragment corresponding to the upstream *URA3* region used for construction of the linear integration cassette was DIG-labelled (PCR Probe Synthesis Kit, Roche Diagnostics, Germany) using primer set 5'-XBASFRURA3 and 3'-URA3KPN. The presence of the *URA3* gene was visualised with the Chemiluminescent Detection Kit (Roche Biochemicals, Germany).

Malo-ethanolic fermentation in grape must

Synthetic grape must

The host yeast strain, *S. cerevisiae* S92, and three transformants (MEF2) containing the integrated *mae1* and *mae2* genes were inoculated at 2 x 10^6 cells /mL into duplicate sets of 200 mL synthetic grape must in 250 mL Erlenmeyer flasks (Denayrolles *et al*., 1995). The synthetic grape must contained 0.94% L-malic acid (Sigma, St. Louis, MO) and the pH was adjusted with 1 M KOH to 3.3. Fermentations were carried out at 20°C without shaking and sealed with fermentation caps filled with 2.5% SO_2 solution for approximately 15 days. Growth of yeast cells was monitored spectrophotometrically at OD_{600}.

Small-scale grape must fermentation

Small-scale fermentations were also performed in Chardonnay (3 g/L L-malic acid, pH 3.40), Cabernet Sauvignon (2.5 g/L L-malic acid, pH 3.77), Colombard (4.5 g/L L-malic acid, pH 3.42) and Ruby Cabernet (3.5 g/L L-malic acid, pH 3.54) grape musts. To ensure dominance in the final yeast population, the host and the malo-ethanolic recombinant strain, MEF2, containing the integrated *mae1* and *mae2* genes were inoculated at 2 x 10^6 cells/mL into 400 mL must in 500 mL flasks and incubated at 20°C without shaking. The flasks were sealed with fermentation caps filled with 2.5% SO_2 solution. White and red grape musts were supplemented with 50 ppm and 30 ppm SO_2 respectively, while 0.075% diammonium phosphate was added to all flasks before inoculation to ensure a sufficient nitrogen source during fermentation. The weight of the fermentation flasks was measured at regular intervals as an indication of fermentation speed by indi-

rectly measuring CO_2 production and evaporation, while the presence and dominance of the recombinant malo-ethanolic strains were verified by regular serial dilutions of the fermenting juice and screening on the optimised GMIA media.

Large-scale vinification for sensory evaluation

Chardonnay grapes (23.7°B) were harvested during the 2001 season, de-stemmed, crushed and pressed. The must was treated with 50 mg/L SO_2 and allowed to settle overnight. Chemical analysis indicated that the Chardonnay juice contained 2.97 g/L L-malic acid, a pH of 3.53 and a total acidity of 6.27 g/L. Similarly, the Cabernet Sauvignon grapes (21.8°B) were de-stemmed and crushed, treated with 30 mg/L SO_2 and divided into 12 lots of 15 L each, followed by direct inoculation with yeasts as described above. The Cabernet Sauvignon must contained 3.5 g/L L-malic acid, a pH of 3.77 and a total acidity of 7.16 g/L. After three days of skin contact, the must was pressed and returned to fermentation flasks for further alcoholic fermentation.

The Chardonnay and Cabernet Sauvignon juice was divided into 12 lots of 15 L each for three repetitions of two different treatments, i.e. (i) inoculation with the host yeast strain, *S. cerevisiae* S92, as a control fermentation, or (ii) three positive transformants containing the integrated malo-ethanolic genes. The yeast was inoculated at a final concentration of 2 X 10^6 cells/mL to ensure dominance in the final yeast population. The presence and dominance of the recombinant malo-ethanolic strains were confirmed at the beginning, halfway mark and at the end of alcoholic fermentation by serial dilutions of the fermenting juice and screening on the optimised GMIA media. Fermentations in Chardonnay must were carried out at 15°C, while Cabernet Sauvignon must was fermented at 23°C. After alcoholic fermentation was completed, one set of control wines (*S. cerevisiae* S92) from both Chardonnay and Cabernet Sauvignon was inoculated with Viniflora Oenos (Chris Hansen, Denmark) for MLF according to the manufacturer's recommendations. All other wines were decanted and treated with 30 mg/L SO_2 and stored at 0°C for seven days for cold stabilisation. An experienced panel of 15 judges performed organoleptic evaluation of the Chardonnay wine six months after bottling. A ranking method was used to determine differences between the treatments and statistical significance was determined according to Amerine & Roessler (1976).

Chemical analysis

The concentrations of L-malic acid, D-glucose, glycerol and ethanol were determined using enzymatic assays (Roche Diagnostics, Germany). In-depth analysis of large-scale fermented wines were done by Capillary Electrophoresis (HP3D CE system, Hewlett-Packard) and GrapeScan 2000 (FOSS Electric A/S, Denmark) to determine glucose, fructose, glycerol, ethanol, tartaric, malic, citric, succinic, acetic and lactic acid concentrations. CE analysis was carried out with a diode array detector. The CE detector wavelength was fixed at 200 nm with 350 nm as the reference wavelength. A bare silica capillary with an internal diameter of 50 µm (total length = 80.5 cm and effective length = 72 cm) was used for wine analysis and samples were injected hydrodynamically (50 mbar for 2 sec). A constant voltage of -25 kV was applied during the separation run and the temperature of the column was set at 25°C. The "HP organic acid buffer" was used as the separation buffer. Wine samples were centrifuged (8 min x 12 000 rpm) before diluting them 20-fold in MilliQ water. A standard solution of L-tartaric acid (60 mg/L), L-malic acid (40 mg/L), citric acid (20 mg/L), succinic acid (20 mg/L), acetic acid (20 mg/L) and lactic acid (20 mg/L) was prepared freshly and run between samples to create valid calibration curves for each component. HP Chemstation Software was used to calculate the concentrations of L-tartaric, L-malic, citric, succinic, acetic and lactic acids in the wine using data obtained from the standard and sample runs. The commercial calibration for the Grapescan 2000 was verified and adjusted to South African wines and conditions to ensure the correct intercepts. Standard methods were used to confirm the results obtained for residual sugar, final ethanol content, pH, total and volatile acidity of the finished wines (Ough & Amerine, 1987).

RESULTS AND DISCUSSION

Transformation of *S. cerevisiae* S92 with integration cassette

Electroporation of competent cells of *S. cerevisiae* with the linear malo-ethanolic integration cassette and pUT332 resulted in 100 to 200 phleomycin-resistant transformants per µg of linear DNA. The optimised GMIA medium allowed for the effective screening of the transformants with the integrated malo-ethanolic cassette after the initial screening of transformants on phleomycin-containing medium. Phleomycin-resistant transformants were transferred to GMIA plates and screened accordingly for the malo-ethanolic phenotype. Transformants with the ability to degrade L-malic acid appeared as blue-coloured colonies that could be easily distinguished from transformants lacking the malo-ethanolic phenotype (yellow/brown colonies).

To cure the transformants of the pUT332 plasmid, the transformants were individually picked and cultured in non-selective conditions (YPD broth) for more than 200 generations to obtain a phleomycin-sensitive phenotype that corresponded to the loss of pUT332 carrying the resistance marker gene, *Tn5ble*. Transformants cured of pUT332 were subsequently spotted onto GMIA media to confirm the presence of a malo-ethanolic phenotype. Colonies with a positive malo-ethanolic phenotype were re-inoculated into non-selective media and repeated in triplicate on GMIA media to determine the stability of the malo-ethanolic phenotype. The malo-ethanolic phenotype was considered to be stable in transformants if less than 1/10,000 revertant colonies appeared after each round of non-selective growth. Genetically engineered yeasts produced in this manner should be more acceptable for industrial application, since no antibiotic resistance markers are present in the recombinant yeast strain.

PCR amplification and Southern blot analysis of integration

The presence of the *mae1* and *mae2* open reading frames in the genome of *S. cerevisiae* S92 transformants (MEF) was confirmed by PCR amplification of a 1317 bp and 1698 bp fragment, corresponding to the complete open reading frame of the *mae1* and *mae2* genes, respectively (Fig. 3A). The host strain (wt) did not yield any PCR products under the same conditions.

The PCR product yield was significantly higher in transformants where multiple integrations of the malo-ethanolic cassette occurred (MEF1) compared to the single integration events (MEF2). Integration of the malo-ethanolic cassette in the *URA3* gene was also confirmed with Southern blot analysis that clearly

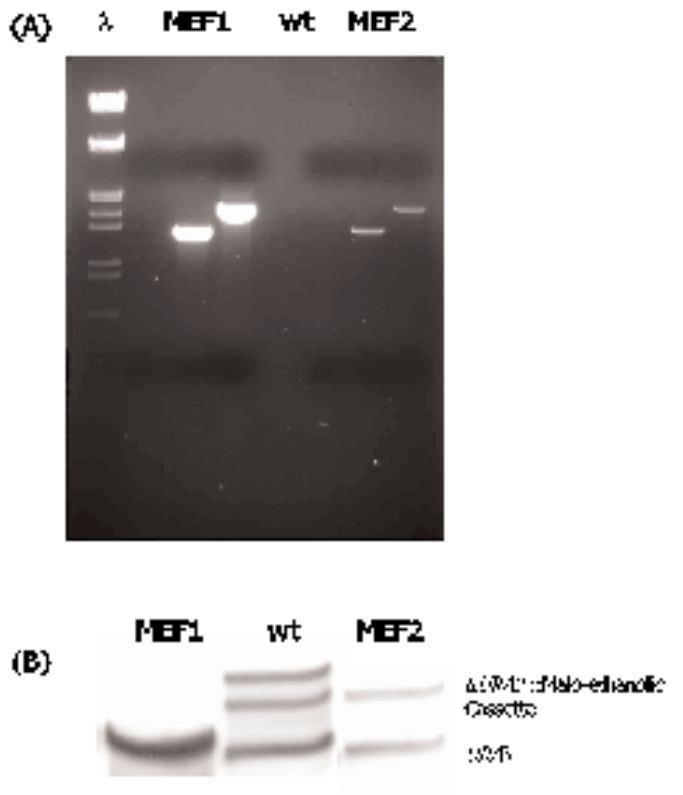

FIGURE 3

(A) PCR amplification of the *mae1* (1317 bp) and *mae2* (1698 bp) open reading frames using genomic DNA from *S. cerevisiae* S92 and selected transformants as template. (B) Southern blot results showing single (MEF2) or multiple (MEF1) integration of the malo-ethanolic cassette in the *URA3* locus. wt = host strain *S. cerevisiae* S92.

demonstrated single (MEF2) or multiple integration (MEF1) events into the *URA3* locus (Fig. 3B). MEF2 transformants containing a single integration of the malo-ethanolic cassette were used for subsequent fermentation and sensory evaluation experiments.

Malo-ethanolic fermentation in synthetic and actual grape must

Rapid and efficient degradation of ca. 9.5 g/L L-malic acid within 5 days was obtained in synthetic grape must during small-scale fermentations by a recombinant strain of *S. cerevisiae* S92 (MEF2), which contains a single integrated copy of the *PGK1*$_p$-*mae1*-*PGK1*$_t$ and *PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassettes (Fig. 4A). The host strain (*S. cerevisiae* S92) showed no significant degradation of L-malic acid during the first 5 days of fermentation, but after 15 days almost 32% of the total L-malic acid was degraded by this strain. This reduction in L-malic acid concentration by the control yeast strain is not ascribed to the active metabolism of L-malic acid by the yeast cells, but rather to the release of intracellular enzymes, i.e. malate dehydrogenases and the native malic enzyme during yeast autolysis at the late stationary phase of fermentation. Comparison of the growth rate and the rate of glucose consumption between the MEF2 and host

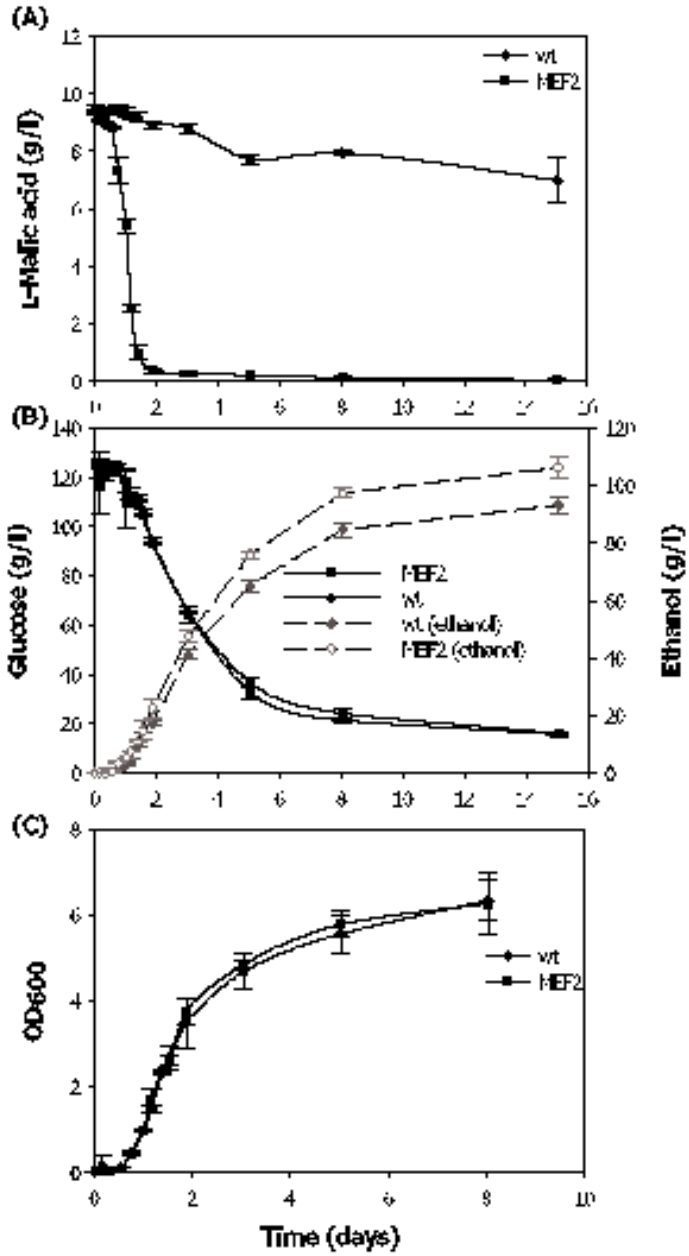

FIGURE 4

(A) L-malic acid degradation by MEF2 compared to the control yeast (*S. cerevisiae* S92, wt); (B) Glucose utilisation and ethanol production by the MEF2 strain compared to the control yeast during alcoholic fermentation, and (C) growth curve of the malo-ethanolic yeast (MEF2) and control strain in synthetic grape must as measured by cell density at OD_{600}.

strain showed no significant aberrations (Fig. 5B and C). This suggested that the introduction of the heterologous genes had no adverse effect on the recombinant yeast's growth and fermentation capacity.

During fermentative sugar metabolism, pyruvic acid is further decarboxylated to acetaldehyde by pyruvate decarboxylase and subsequently reduced to ethanol by alcohol dehydrogenase. Theoretically, the introduction of an efficient malo-ethanolic pathway in yeast should contribute additional pyruvic acid to the existing intracellular pool, promoting the production of elevated levels of ethanol. As previously reported for laboratory strains

(Volschenk *et al*., 2001), the MEF2 strain consistently produced higher levels of ethanol relative to the host strain (Fig. 4B), confirming that the two *S. pombe* genes enabled cells of *S. cerevisiae* to metabolise the extra-cellular L-malic acid to ethanol under fermentative conditions.

The ability of the malo-ethanolic MEF2 wine yeast strain to degrade L-malic acid during alcoholic fermentation was also investigated during small-scale fermentations in Cabernet Sauvignon, Chardonnay, Colombard and Ruby Cabernet grape musts (Fig. 5A). Rapid and complete degradation of extracellular L-malic acid degradation was observed for MEF2 within 1, 1.5, 2 and 5 days in Cabernet Sauvignon, Ruby Cabernet, Colombard and Chardonnay grape musts, respectively. The host yeast strain did not contribute significantly to the degradation of L-malic acid

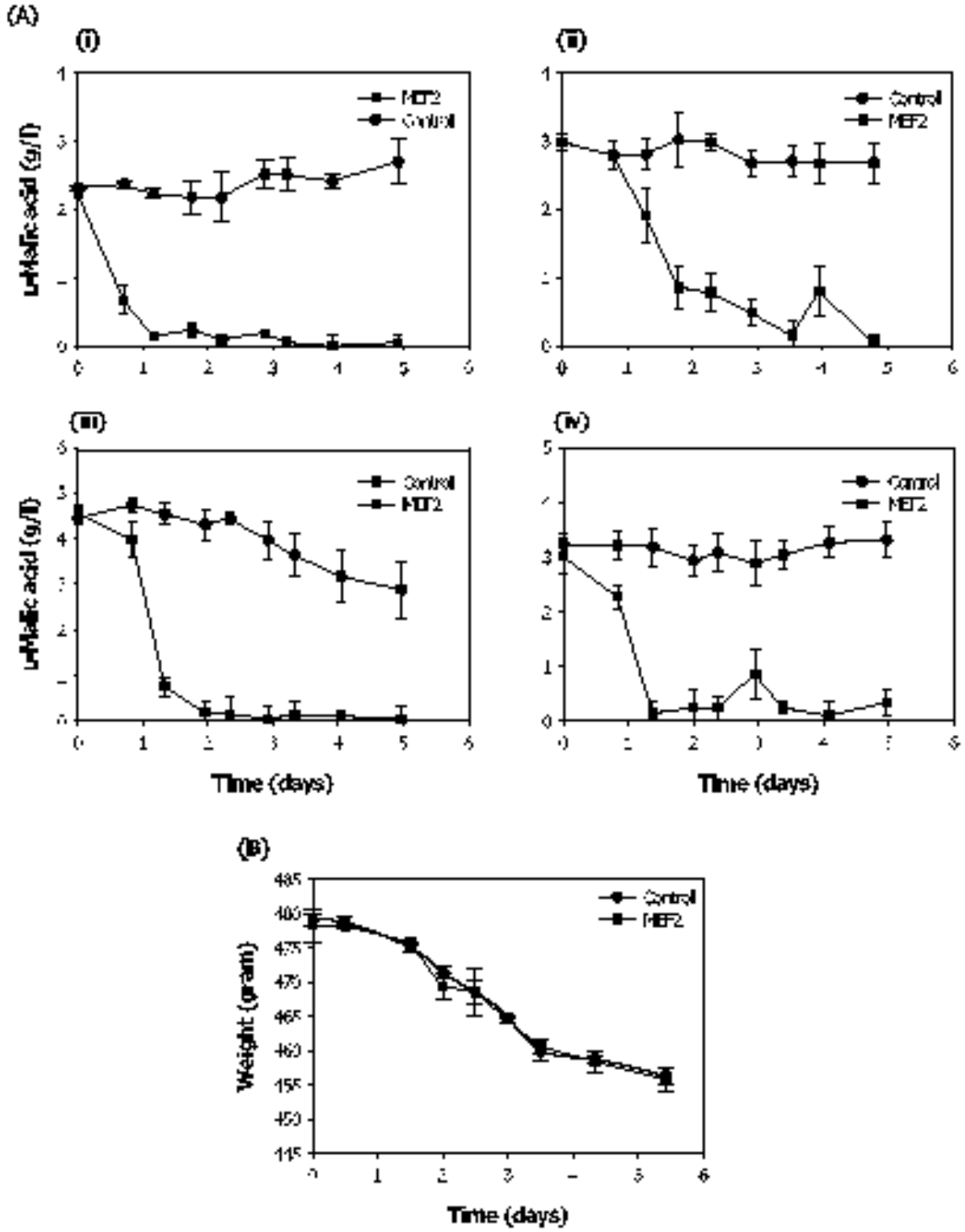

FIGURE 5

(A) Malo-ethanolic fermentation in (i) Cabernet Sauvignon, (ii) Chardonnay, (iii) Colombard and (iv) Ruby Cabernet grape musts by MEF2 and the control yeast strain *S. cerevisiae* S92. (B) Fermentation rate of MEF2 and the control yeast strain in Chardonnay grape must as measured by the loss of CO_2 during alcoholic fermentation.

in the corresponding control fermentations. Furthermore, the fermentation rate measured as the loss of weight (CO_2 evaporation) was almost identical for MEF2 and the host yeast strain (Fig. 5B), confirming that the expression of integrated *mae1* and *mae2* genes did not adversely affect the alcoholic fermentation capacity in the recombinant yeast. Serial dilutions of the fermenting juice followed by plating on optimised GMIA agar (results not shown) consistently indicated that the population of recombinant MEF2 yeast showed rapid growth to reach 10^8 and 10^9 cells per mL and dominance in the fermentation (≥ 85% of total yeast population) after 2 days and throughout the remainder of alcoholic fermentation.

Effect of malo-ethanolic fermentation on organoleptic quality of wine

The ability of the MEF2 recombinant strain to produce a wine of quality was also evaluated during larger-scale vinification of Chardonnay and Cabernet Sauvignon grape musts. Standard winemaking practices were employed during the vinification, including the inoculation of the malolactic bacterium *O. oeni*, after alcoholic fermentation with *S. cerevisiae* S92 was completed. The malo-ethanolic yeast (MEF2) efficiently degraded all the L-malic acid in both Chardonnay and Cabernet Sauvignon grape musts, whereas the host strain, *S. cerevisiae* S92, had little effect on the L-malic acid concentration (Fig. 6). Serial dilutions of the fermenting juice followed by plating on optimised GMIA agar (results not shown) indicated that the recombinant strain of *S. cerevisiae* S92 (MEF2) became the dominant yeast species (≥ 88% of total yeast population) after 1 and 2 days in Chardonnay and Cabernet Sauvignon grape musts, respectively, and maintained dominance throughout the remainder of alcoholic fermentation, confirming that the reduction in the L-malic acid is due the presence of the recombinant yeast strain and not by non-*Saccharomyces* strains also present in the juice.

The decrease in L-malic acid concentration also correlated with the decrease in total acidity (Table 3). In the wine fermented with MEF2, total acidity decreased by 2.3 g/L in agreement with complete L-malic acid decomposition. Total acidity was decreased by 1.34 g/L in the wine that underwent MLF after alcoholic fermentation by *S. cerevisiae* S92, and by 0.54 g/L for wine produced by *S. cerevisiae* S92 without MLF. The decrease in acidity was also reflected in the pH of the different wines; the pH of the wine fermented with MEF2 increased by 0.46 units, whereas fermentation with *S. cerevisiae* S92 with and without MLF resulted in a pH increase of only 0.28 and 0.16 units, respectively. Chemical analysis of the final wines indicated no significant changes to the other organic acids in the wine fermented with MEF2 and the control yeast. The tartaric, citric and succinic acid concentrations remained relatively unchanged for the different treatments (Table 3). The concentration of lactic acid remained relatively constant for the control and MEF2 wine, while a significant increase in lactic acid could be seen in the wine that underwent MLF. Furthermore, volatile acidity as measured by the acetic acid concentration, was slightly increased in the wine fermented with MEF2, but still within the threshold value for acetic acid in wine compared to the control yeast fermentation.

Comparison of the final ethanol values of the wines indicated an insignificant increase in ethanol concentration of the wine fermented with MEF2 in relation to the control wine fermented with

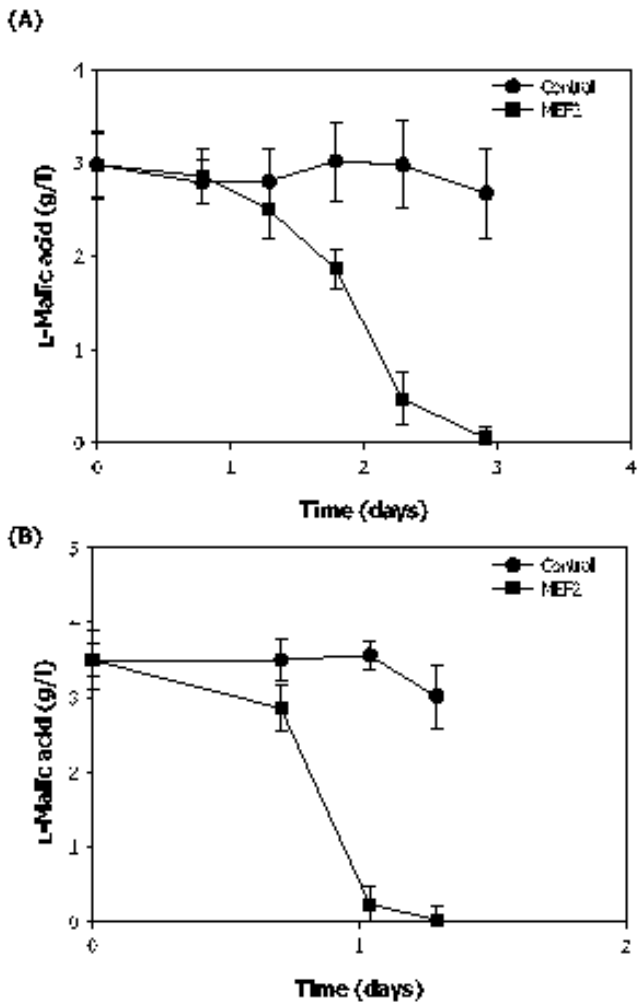

FIGURE 6

L-malic acid degradation during larger-scale fermentation of (A) Chardonnay and (B) Cabernet Sauvignon grape must with the recombinant MEF2 yeast containing the integrated malo-ethanolic expression cassette. The control fermentation was performed using the host yeast strain, *S. cerevisiae* S92.

S. cerevisiae S92. Glycerol production in yeast acts as a mechanism to rectify a possible NAD^+/NADH imbalance during yeast metabolism. Therefore, changes in the redox balance in yeast metabolism, such as during oxidative or osmotic stress conditions, are associated with changes in the amounts of glycerol produced by yeast (Nordström, 1968; Oura, 1977; Van Dijken & Scheffers, 1986; Larson *et al*., 1998). Since the conversion of L-malic acid to pyruvic acid by the malic enzyme involves the reduction of NAD^+ to NADH, the level of glycerol in wine was also determined after alcoholic fermentation. Wine fermented by the MEF2 strain showed an increase of ca. 1 g/L glycerol relative to the wine fermented by the control yeast.

Organoleptic evaluations of the fermented Chardonnay wine indicated a significant difference between wines produced with *S. cerevisiae* S92, the MEF2 recombinant strain and wines that underwent MLF (Table 4). Based on the perceived aroma of the wines, the lowest score was obtained for the wine fermented by *S. cerevisiae* S92, while wine made by MEF2 scored the highest, even higher than the wine that underwent MLF. No off-flavours

TABLE 3
Chemical composition of fermented Chardonnay wine.

	Malic Acid (g/L)	Tartaric Acid (g/L)	Citric Acid (g/L)	Succinic Acid (g/L)	Acetic Acid (g/L)	Lactic Acid (g/L)	Glycerol (g/L)	Ethanol (g/L)	Residual Glucose (g/L)	Residual Fructose (g/L)	Total Acidity (g/L)	pH
Prior to fermentation	2.97								115	n.d.	6.27	3.53
	±0.03								±0.6		±0.02	±0.01
S. cerevisiae **S92**	2.82	1.26	0.36	0.26	0.38	0.09	6.5	14.3	0.44	3.13	5.73	3.69
	±0.2	±0.01	±0.01	±0.02	±0.01	±0.1	±0.1	±0.1	±0.6	±0.3	±0.6	±0.01
S. cerevisiae **S92+MLF**	0.29	1.16	0.24	0.27	0.38	1.32	6.4	14.48	1.31	1.75	4.93	3.81
	±0.2	±0.2	±0.1	±0.01	±0.02	±0.2	±0.1	±0.1	±0.5	±0.5	±0.4	±0.06
MEF2	0.22	1.47	0.44	0.27	0.56	0.13	7.2	14.6	1.24	2.64	3.97	3.99
	±0.1	±0.02	±0.18	±0.01	±0.01	±0.2	±0.2	±0.2	±0.4	±0.2	±0.2	±0.2

n.d. = not determined.
± = standard deviation.

TABLE 4
Results of the organoleptic evaluation of Chardonnay wine.

Treatment	Aroma order	Palate order
S. cerevisiae S92	3*	1**
S. cerevisiae S92+ MLF	2	2
MEF2	1**	3*

* – significant at $p<0.05$.

** significant at $p<0.01$.

were detected, while an increase in fruitiness was noted, possibly due to the absence of the masking effect of L-malic acid. These results indicate that the malo-ethanolic strain was more successful in producing fruity-floral aromas in wine, a definite advantage in the production of cultivars such as Muscat, Riesling, Sauvignon Blanc and Gewürztraminer. However, based on the perceived palate of the wine, the best results were obtained with wine fermented by the control yeast *S. cerevisiae* S92, while wine produced with MEF2 scored the lowest. The taste panel detected an imbalance in the acid:sugar ratio in the wine made by the malo-ethanolic yeast (MEF2). This could be expected since all the L-malic acid was completely removed from the wine and resulted in a sub-optimal final total acidity (Table 3). The Chardonnay and Cabernet Sauvignon musts used in this study were harvested from a warm-climate viticultural region and contained low levels of L-malic acid in the grape must, i.e. 2.97 and 3.5 g/L L-malic acid, respectively. Future evaluation of the malo-ethanolic yeast in high-acid wines from a cool-climate viticultural region will be required to determine the actual organoleptic influence of this recombinant yeast on wine.

CONCLUSIONS

In this study the commercial wine yeast *S. cerevisiae* S92 was successfully transformed through integration of a malo-ethanolic cassette containing the *PGK1*$_p$-*mae1*-*PGK1*$_t$ and *PGK1*$_p$-*mae2*- *PGK1*$_t$ linear integration cassette flanked by large *URA3* homologous sequences. A single genomic copy of the malo-ethanolic cassette in *S. cerevisiae* S92 was sufficient to yield a strong malo-ethanolic phenotype, i.e. the conversion of L-malic acid to ethanol, in the recombinant yeast in synthetic and grape must fermentations. Sensory evaluation and chemical analysis of a Chardonnay wine produced by the malo-ethanolic yeast indicated an improvement in wine aroma compared to the traditional MLF. Commercial availability of malo-ethanolic wine yeast will be especially beneficial in the production of fruity-floral wines and the de-acidification of high-acid wines in the cool-climate viticultural regions of the world.

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