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Research Article

Malo-ethanolic fermentation in grape must by recombinant strains of *Saccharomyces cerevisiae*

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Abstract

Recombinant strains of *Saccharomyces cerevisiae* with the ability to reduce wine acidity could have a significant influence on the future production of quality wines, especially in cool climate regions. L-Malic acid and L-tartaric acid contribute largely to the acid content of grapes and wine. The wine yeast *S. cerevisiae* is unable to effectively degrade L-malic acid, whereas the fission yeast *Schizosaccharomyces pombe* efficiently degrades high concentrations of L-malic acid by means of a malo-ethanolic fermentation. However, strains of *Sz. pombe* are not suitable for vinification due to the production of undesirable off-flavours. Heterologous expression of the *Sz. pombe* malate permease (*mae1*) and malic enzyme (*mae2*) genes on plasmids in *S. cerevisiae* resulted in a recombinant strain of *S. cerevisiae* that efficiently degraded up to 8 g/l L-malic acid in synthetic grape must and 6.75 g/l L-malic acid in Chardonnay grape must. Furthermore, a strain of *S. cerevisiae* containing the *mae1* and *mae2* genes integrated in the genome efficiently degraded 5 g/l of L-malic acid in synthetic and Chenin Blanc grape musts. Furthermore, the malo-alcoholic strains produced higher levels of ethanol during fermentation, which is important for the production of distilled beverages.

Keywords: malic acid; malo-ethanolic fermentation; wine; *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*

Introduction

Wine acidity and pH play an important role in the organoleptic quality and shelf life of wine. L-Tartaric acid and L-malic are the most prominent grape acids, contributing to more than 90% of the titratable acidity in wine (Beelman and Gallander, 1979; Radler, 1993; Henick-Kling, 1993; Gao and Fleet, 1995). The production of premium wines depends on the enologist's skill to accurately adjust wine acidity to obtain a balanced wine with optimum flavour and colour profile. In the warmer wine regions of Australia, South Africa, California and southern Europe, acidulating agents such as L-tartaric acid and D/L-malic acid are routinely added prior to fermentation to increase the titratable acidity of must (Beelman and Gallander, 1979; Boulton *et al.*, 1996). However, in the cooler wine regions of northern Europe, eastern USA and Canada, cold stabilization and malolactic fermentation are necessary to decrease the levels of L-tartaric and L-malic acid in the final product.

Strains of the lactic acid bacterium *Oenococcus oeni* are used to perform the malolactic fermentation in wine during which L-malic acid is converted to L-lactic acid and CO_2 (Wibowo *et al.*, 1985; van Vuuren and Dicks, 1993). Malolactic fermentation sufficiently decreases the acidic taste of wine, improves the microbial stability and modifies the organoleptic profile of the wine. However, stuck or sluggish malolactic fermentation often causes delays in cellar operations, such as sulphiting, which may result in the chemical oxidation and spoilage of wine as well as the production of biogenic amines by spoilage organisms (Lonvaud-Funel and Joyeux, 1994; Straub *et al.*, 1995). Even with the use of

starter cultures, malolactic fermentation may only be completed weeks or months after alcoholic fermentation (Henick-Kling, 1995). Factors such as pH, sulphur dioxide, ethanol, temperature, nutritional status of the wine and interactions with other wine flora synergistically influence the onset and completion of malolactic fermentation (Beelman and Gallander, 1979; Boulton *et al.*, 1996; Davis *et al.*, 1985; Henick-Kling, 1993). Furthermore, malolactic fermentation is usually undesirable in the production of the fruity-floral cultivars such as Sauvignon Blanc, Riesling and Gewurztraminer. The varietal flavours in these wines are essential to their aromatic character and are adversely modified during malolactic fermentation (Radler, 1972; Wagner, 1974).

Yeast species capable of utilizing tricarboxylic acid (TCA) cycle intermediates, such as L-malic acid, are classified into two groups: K(+) yeasts utilize TCA intermediates as sole energy and carbon source, while K(−) yeasts can only utilize TCA cycle intermediates when glucose or other fermentable carbohydrates are present. The K(−) group includes yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Zygosaccharomyces balii* (Baranowski and Radler, 1984; Kuczynski and Radler, 1982; Rodriquez and Thornton, 1989). However, wine yeast strains of *S. cerevisiae* cannot efficiently utilize L-malic acid during alcoholic fermentation (Subden *et al.*, 1998; Volschenk *et al.*, 1997b). The inefficient degradation of L-malic acid by *S. cerevisiae* is ascribed to the slow uptake of L-malic acid by diffusion (Ansanay *et al.*, 1996; Baranowski and Radler, 1984; Volschenk *et al.*, 1997a,b) and the low substrate affinity of its malic enzyme ($K_m = 50$ mM) (Fuck *et al.*, 1973). Furthermore, transcriptional analysis of the *S. cerevisiae* malic enzyme gene (*MAE1*), indicated relatively low but constitutive levels of expression for this gene (Boles *et al.*, 1998).

In contrast to *S. cerevisiae*, the fission yeast *Sz. pombe* can efficiently degrade up to 29 g/l of L-malic acid (Taillandier *et al.*, 1988; Taillandier and Strehaiano, 1991). Cells of *Sz. pombe* actively transport L-malic acid via a H^+-symport system (Sousa *et al.*, 1992) provided by the malate permease encoded by the *mae1* gene (Grobler *et al.*, 1995). Intracellularly, *Sz. pombe* decarboxylates L-malic acid to pyruvate and CO_2 by means of a cytosolic malic enzyme encoded by the *mae2* gene (Viljoen *et al.*, 1994). Under fermentative conditions, pyruvate is further metabolized to ethanol and CO_2 (Mayer and Temperli, 1963; Osothsilp and Subden, 1986), resulting in the so-called malo-ethanolic fermentation. Although strains of *Sz. pombe* have been used for the degradation of L-malic acid in grape must, it is unsuitable for the fermentation of wine due to the production of off-flavours and the high fermentation temperature required (Beelman and Gallander, 1979; Carré *et al.*, 1983; Gallander, 1977; Radler, 1993).

We have cloned and co-expressed the *mae1* and *mae2* genes responsible for the malo-ethanolic fermentation in *Sz. pombe* in a laboratory strain of *S. cerevisiae*. Since the native promoters of these genes are not recognized by *S. cerevisiae*, the genes were expressed under control of the constitutive 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences of *S. cerevisiae*. The recombinant yeast strain rapidly degraded L-malic acid in synthetic and Chardonnay grape musts and consistently produced higher levels of ethanol in the presence of malic acid, relative to the control yeast strain. The linear *mae1* and *mae2* expression cassettes were also integrated in the *ILV2* locus of *S. cerevisiae* to overcome possible artifacts due to the instability of 2 μm plasmids. Recombinant strains of *S. cerevisiae* containing a single genomic copy of the *mae1* and *mae2* genes effectively degraded L-malic acid in synthetic and Chenin Blanc grape musts and compared well with the malolactic yeast strains previously reported (Volschenk *et al.*, 1997a).

Materials and methods

Strains and maintenance

The bacterial and yeast strains and plasmids used in this study are listed in Table 1. Cells of *E. coli* JM109 were transformed by electroporation and selected on LB medium supplemented with ampicillin (Ausubel *et al.*, 1995). Cells of *S. cerevisiae* were cultured in liquid YPD media at 30°C and competent cells (LiOAc method) were transformed or co-transformed with plasmids pHVX2, pHV3 and pHV7 (Table 1). Transformants were isolated on selective YNB agar plates (Difco Laboratories, Detroit, MI) supplemented with amino acids as required. The transformants were cultured to high cell density in 50 ml selective YNB media [0.17% Yeast Nitrogen Base (Difco Laboratories, Detroit,

Table 1. Strains and plasmids used in the genetic construction of malo-ethanolic strains of *S. cerevisiae*

Strains	**Description**	**Reference**
E. coli JM109	*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* [r_k-, m_k+], *relA1*, *supE44*, λ^-, Δ(*lac-proAB*), [F′, *traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15]	(Yanisch-Perron, 1985)
S. cerevisiae YPH259	*MATα*, *ura3-52*, *lys2-801*^{amber}, *ade2-101*^{ochre}, *his3Δ200*, *leu2-Δ1*	(Sikorski and Hieter, 1989)
Plasmids	**Description**	**Reference**
pHVX2	YEplac181 (*LEU2* marker gene) containing the *PGK1* promoter and terminator sequences	(Volschenk *et al.*, 1997a,b)
pHV3	pHVX2 containing the *mae1* ORF subcloned between the *PGK1* promoter and terminator sequences	(Volschenk *et al.*, 1997a,b)
pHV7	YEplac195 (*URA3* marker gene) containing the *mae2* ORF subcloned between the *PGK1* promoter and terminator sequences	This study
pHVS2	pBluescript KS+ containing the *SMR1-410* gene. The *PGK1*p–*mae1*–*PGK1*t and *PGK1*p–*mae2*–*PGK1*t cassettes were subcloned in the terminator region of the *SMR1* gene	This study

MI)], 0.5% $(NH_4)_2SO_4$, 2% D-glucose, 0.13% of drop-out amino acid pool (Ausubel *et al.*, 1995) at 30°C, harvested by centrifugation and resuspended in 5 ml sterile grape juice before inoculation into grape must.

Plasmid construction

Standard recombinant DNA techniques were performed essentially as described by Ausubel *et al.* (1995). All subcloning and DNA manipulations, except the construction of the integration plasmid pHVS2, were performed in the 2μ-based plasmids YEplac181 and YEplac195 (Gietz and Sugino, 1988). The construction of the expression vectors pHVX2 and pHV3 (Table 1) were previously described (Volschenk *et al.*, 1997a,b). The *PGK1*p–*mae2*–*PGK1*t expression cassette from pHV4 (Volschenk *et al.*, 1997b) was subcloned as a *Hin*dIII fragment into YEplac195, resulting in pHV7.

Integration of *mae1* and *mae2* genes in *S. cerevisiae*

Integration of the *mae1* and *mae2* genes into *S. cerevisiae* genome was obtained by selection of resistance to the herbicide sulphometuron methyl (SMM) via the *SMR1-410* gene (Casey *et al.*, 1988). The *SMR1-410* gene was subcloned from pWX509 (Casey *et al.*, 1988) by *Kpn*I–*Bam*HI digestion into pBluescript KS+ to yield pDLG42 (provided by Dr D. C. la Grange, Dept of Microbiology, University of Stellenbosch). The *PGK1*p–*mae2*–*PGK1*t cassette from pHV7 was subcloned as a *Hin*dIII fragment into the *SMR1* terminator region in pDLG42 (Figure 1), while *PGK1*p–*mae1*–*PGK1*t was subcloned as a *Pvu*II fragment from pHV3 into the Klenow-treated *Nde*I site in the *SMR1* terminator region in pDLG42 to yield pHVS2 (Figure 1). pHVS2 was linearized with *Apa*I and transformed into LiOAc-competent cells of *S. cerevisiae* YPH259. Transformants were selected on YNB agar plates (Difco Laboratories, Detroit, MI) containing 200 μg/ml SMM and supplemented with all amino acids except isoleucine and valine. Transformants were maintained on YPD plates for more than 200 generations.

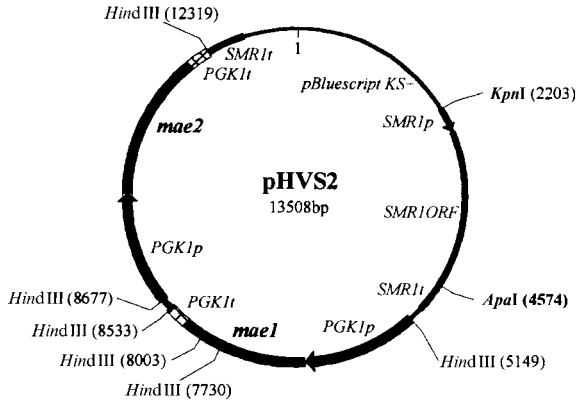

Figure 1. Plasmid map of integration plasmid pHVS2. The *PGK1*p–*mae2*–*PGK1*t fragment was cloned as a *Hin*dIII fragment into the *SMR1* terminator region in pDLG42. The *PGK1*p–*mae1*–*PGK1*t fragment was cloned as *Pvu*II fragment in the *Nde*I site (blunt-ended) in the terminator region of *SMR1* in pDLG42. Digestion with *Apa*I linearized the plasmid in the *SMR1* ORF region

Pulsed-field gel electrophoresis and Southern blotting

S. cerevisiae YPH259 and the integrated malo-ethanolic strain of *S. cerevisiae* was grown in 200 ml YPD overnight. Chromosomal DNA plugs were prepared by lyticase enzyme treatment (Boehringer-Mannheim, Germany) and the chromosomes separated by counter-clamped homogeneous electric field (CHEF) electrophoresis, as described by van der Westhuizen and Pretorius (1992).

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 (Boehringer-Mannheim, Germany). An internal 622 bp *Xho*I DNA fragment of *mae1* and a internal 900 bp *Bgl*II DNA fragment of *ILV2* was labelled with [α-^{32}P]dCTP, using the random-primed DNA-labelling kit (Boehringer-Mannheim, Germany) and used as probes to detect the *mae1*/*mae2* cassette and *ILV2* gene.

Malo-ethanolic fermentation in grape must

The synthetic grape must consisted of 0.17% YNB (without amino acids and ammonium sulphate), 0.5% $(NH_4)_2SO_4$, 10% glucose, 8 or 5 g/l L-malic acid and amino acids supplemented as required. The pH was adjusted to 3.3 with 1 N KOH. The recombinant strains of *S. cerevisiae* were inoculated to a final concentration of 2×10^6 cells/ml in 100 ml synthetic grape must in 250 ml Erlenmeyer flasks and incubated at 28°C while shaking.

Chardonnay must (6.75 g/l L-malic acid, pH 3.29) and Chenin Blanc must (5 g/l L-malic acid, pH 3.42) were also inoculated with 2×10^6 cells/ml into 800 ml must in 1 l flasks and incubated at 20°C without aeration. The Chardonnay and Chenin Blanc grape musts were supplemented with 0.075% diammonium phosphate before inoculation to ensure a sufficient nitrogen source during fermentation. The concentration of L-malic acid, D-glucose and ethanol were measured at regular intervals during the fermentation using enzymatic assays (Roche Diagnostics, Germany).

Results and discussion

Although both *S. cerevisiae* and *Sz. pombe* are classified as K(−) yeasts, their ability to degrade L-malic acid differs significantly. The method of L-malic acid uptake contributes largely to this phenomenon: cells of *S. cerevisiae* rely on simple diffusion for the uptake of L-malic acid, whereas *Sz. pombe* actively transports L-malic acid via a H^+-malate symporter. The malic enzyme of *S. cerevisiae* has strong homology with the malic enzyme of *Sz. pombe*, but they differ significantly in their co-factor specificity, substrate affinity and subcellular localization (Boles *et al.*, 1998). The malic enzyme of *S. cerevisiae* requires either $NADP^+$ or NAD^+ as electron acceptor and is located in the mitochondria, whereas the cytosolic malic enzyme from *Sz. pombe* is NAD^+-specific (Figure 2). Furthermore, the affinity ($K_m = 50$ mM; Fuck *et al.*, 1973) of the *S. cerevisiae* malic enzyme is much lower for the substrate L-malic acid than the malic enzyme from *Sz. pombe* ($K_m = 3.2$ mM; Temperli *et al.*, 1965).

These data strongly suggest that the malic enzyme of *S. cerevisiae* plays an entirely different physiological role than that of *Sz. pombe*. In *S. cerevisiae*, malic acid is mainly metabolized through malate dehydrogenase and the oxidative reactions of the TCA cycle. It has been suggested that the *S. cerevisiae* malic enzyme plays a role in providing intramitochondrial NADPH or pyruvate for biosynthetic pathways under anaerobic conditions (Figure 2) (Boles *et al.*, 1998). The role of the highly efficient degradation of L-malic acid by the yeast *Sz. pombe* is somewhat enigmatic, since L-malic acid is not incorporated into biomass but stoichiometrically converted to ethanol and CO_2 under anaerobic conditions (Taillandier *et al.*, 1988; Taillandier and Strehaiano, 1991; Subden *et al.*,

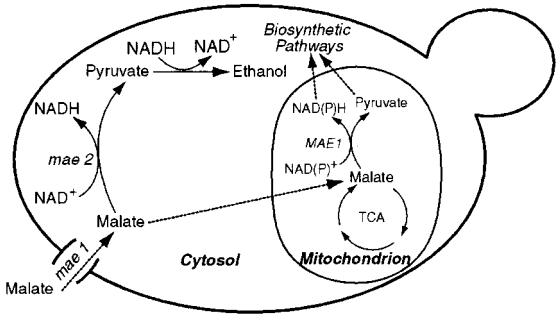

Figure 2. A schematic representation of the pathway for L-malic acid degradation in *S. cerevisiae* with the newly introduced malo-ethanolic pathway from *Sz. pombe*. *MAE1*, malic enzyme gene of *S. cerevisiae*; *mae1*, *Sz. pombe* malate permease gene and *mae2*, *Sz. pombe* malic enzyme gene

1998). Analysis of the transcriptional regulation of the malic enzyme gene of *Sz. pombe* suggests that this enzyme may help to maintain the redox potential under fermentative conditions (Viljoen *et al.*, 1999).

Winemakers rely on malolactic fermentation to balance the acidity levels of wine after alcoholic fermentation. However, the malolactic fermentation is often erratic and difficult to manage. The use of genetically improved strains of *S. cerevisiae* with the ability to reduce L-malic acid levels during alcoholic fermentation could be of great benefit to winemakers. We previously reported the construction of a malolactic strain of *S. cerevisiae* able to efficiently carry out malolactic fermentation by co-expressing the malate permease gene (*mae1*) of *Sz. pombe* and the malolactic enzyme gene (*mle*S) of *Lactococcus lactis* (Volschenk *et al.*, 1997a). Since malolactic fermentation is, however, not always desired in certain cultivars, we investigated an alternative pathway to reduce the levels of L-malic acid in these wines. This pathway uses the *Sz. pombe* malate permease and malic enzyme to perform a so-called malo-ethanolic fermentation.

Recombinant strains of *S. cerevisiae* containing both the *Sz. pombe mae1* and *mae2* genes were constructed by co-transformation of plasmid pHV3 and pHV7 into *S. cerevisiae* YPH259. The malo-ethanolic fermentation by the recombinant *S. cerevisiae* strain was completed within 3 days in synthetic grape must containing 8 g/l L-malic acid, while 6.75 g/l L-malic acid was fully degraded within 11 days in Chardonnay grape must (Figure 3). In contrast, the control yeast strain containing only the *PGK1*-expression cassette (plasmid pHVX2) was not able to degrade the malic acid present in the media. Furthermore, the recombinant strain containing only the *mae2* expression cassette (plasmid pHV7) had no effect on the levels of L-malic acid (results not shown). This confirmed the essential contribution of the *Sz. pombe* malate permease for the effective degradation of malic acid in *S. cerevisiae*.

In *Sz. pombe*, the malic enzyme catalyses the oxidative decarboxylation of L-malic acid to L-pyruvate. Under fermentative conditions, pyruvate is further metabolized to ethanol and CO_2 by alcohol dehydrogenase (Maconi *et al.*, 1984). The malo-ethanolic strain of *S. cerevisiae* containing the malate transport (*mae1*) and malic enzyme (*mae2*) genes from *Sz. pombe*, consistently produced higher

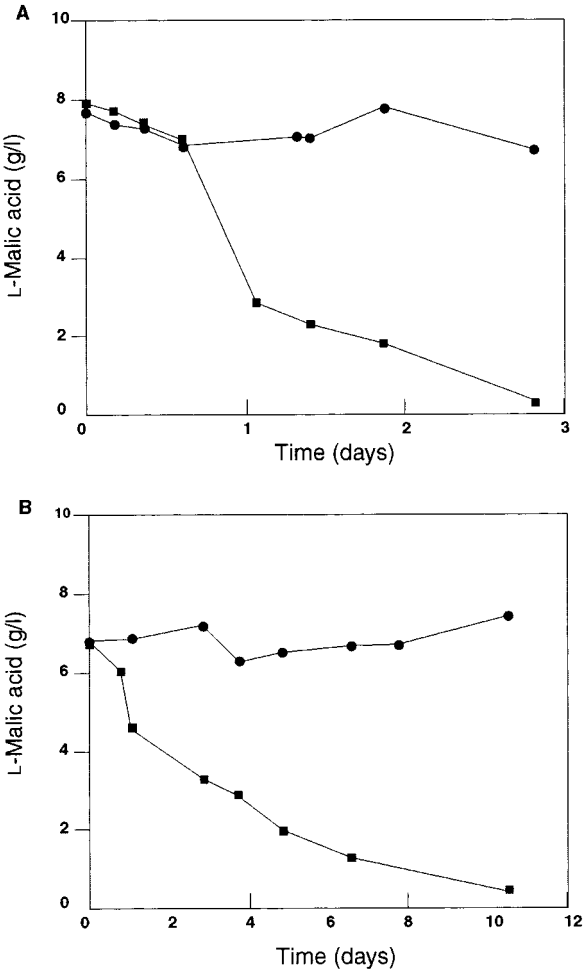

Figure 3. Malo-ethanolic ferentation in (A) synthetic grape must containing 8 g/l L-malic acid, or (B) Chardonnay grape must containing 6.75 g/l L-malic acid. The recombinant strain of *S. cerevisiae* contained the *Sz. pombe mae1* and *mae2* genes (■), whereas the control yeast contained only the pHVX2 expression vector (●)

levels of ethanol, relative to the control strain (Figure 4). This confirmed that the two *Sz. pombe* genes enabled cells of *S. cerevisiae* to metabolize the extracellular L-malic acid to ethanol under fermentative conditions.

The linear *Sz. pombe mae1* and *mae2* expression cassettes were successfully integrated into the genome of *S. cerevisiae* strain YPH259. Southern analysis of a CHEF gel confirmed that the *mae1* and *mae2* genes are located on the same chromosome (XIII) as the *ILV2* gene (Figure 5). Cells of *S. cerevisiae* containing the integrated *mae1* and *mae2* genes efficiently degraded 5 g/l L-malic acid

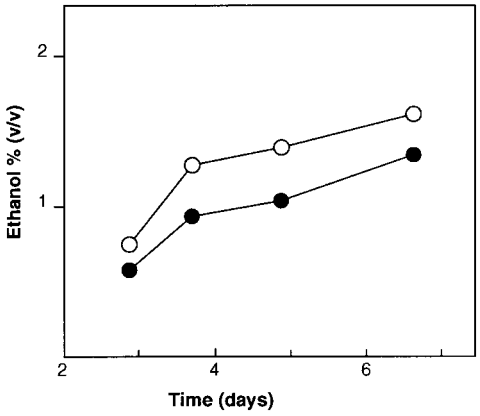

Figure 4. Ethanol production by the malo-ethanolic strain of *S. cerevisiae* containing the *Sz. pombe mae1* and *mae2* genes (○) in a Chardonnay grape must, compared to the control yeast strain containing only plasmid pHVX2 (●)

within 34 h and 10 days in synthetic and Chenin Blanc grape musts, respectively (Figure 6). Results obtained in this study suggest that *S. cerevisiae* containing integrated single copies of the *mae1* and *mae2* genes of *Sz. pombe* under the control of the

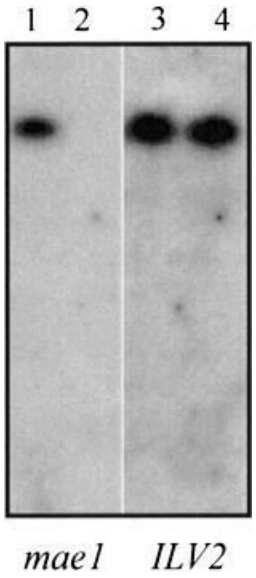

Figure 5. Chromosomal blot indicating that the *mae1*/*mae2* gene cassette was integrated into the *ILV2*/*SMR1* locus of *S. cerevisiae* strain YPH259. Lanes 1 and 2 were probed with the internal 622 bp *Xho*I DNA fragment of *mae1* and lanes 3 and 4 were probed with an internal 900 bp *Bgl*II DNA fragment of *ILV2*. Lanes 1 and 3 contained chromosomes from the malo-ethanolic strain and lanes 2 and 4 represents chromosomal banding patterns from the parental strain

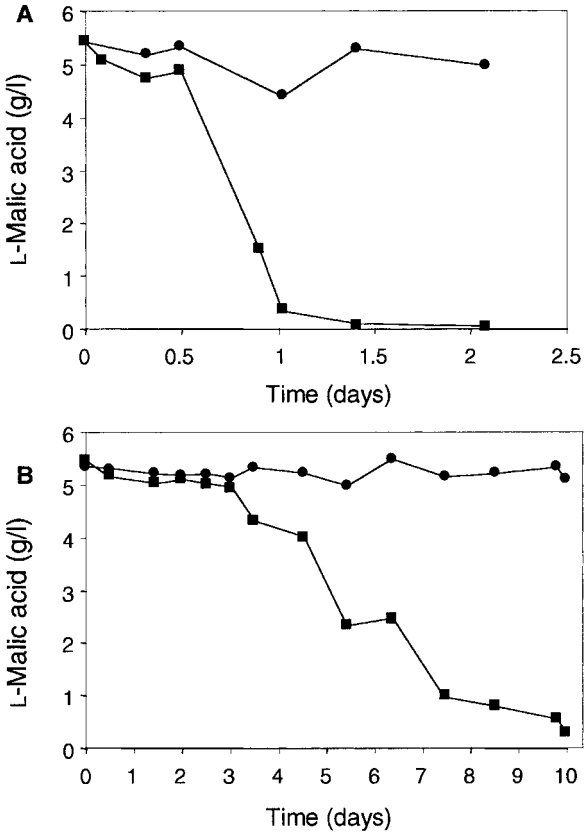

Figure 6. Malo-ethanolic fermentation in (A) synthetic grape must and (B) Chenin Blanc grape must containing 5 g/l L-malic acid. The recombinant strain of *S. cerevisiae* contained the integrated *Sz. pombe mae1* and *mae2* genes (■), whereas the control yeast is the parent strain (*S. cerevisiae* YPH259) (●)

S. cerevisiae PGK1 promoter and terminator is sufficient for rapid degradation of L-malic acid. Malo-ethanolic strains of *S. cerevisiae* could play an important role in the deacidification of white wines in cool climate regions in the future.

We have successfully introduced a malo-ethanolic pathway into a strain of *S. cerevisiae* on multicopy plasmids, enabling this yeast to degrade ~7 g/l L-malic acid during fermentation of grape must. The degradation of L-malic acid by the malo-ethanolic yeast was as efficient as the conversion of L-malic acid to L-lactic acid by the malolactic yeast strain previously reported (Volschenk *et al.*, 1997a). Stable expression of the malo-ethanolic genes in *S. cerevisiae* was also obtained by integration in the *ILV2*/*SMR1* locus. Strains of *S. cerevisiae* containing a single copy of the malo-ethanolic

genes efficiently degraded ~5 g/l L-malic acid within 10 days during grape must fermentation.

Strains of *S. cerevisiae* able to conduct the malo-ethanolic fermentation in grape musts will be well-suited for the production of aromatic wines such as Sauvignon Blanc, Riesling and Gewurztraminer, where the reduction of malic acid is required without the negative effects that the malolactic fermentation has on the organoleptic profile of these wines.

Strains of *S. cerevisiae* with the capacity to produce higher levels of ethanol during vinification are of particular importance to the distilled beverage industry for the production of a higher-alcohol *rabate* wines for distillation purposes. To ensure stable expression of the genes under the non-selective conditions associated with wine, current research is focused on the integration of the *mae1* and *mae2* genes into the genomes of selected commercial wine yeast strains. Once industrial malo-ethanolic strains of *S. cerevisiae* are obtained, we will determine their fermentation kinetics and evaluate the organoleptic quality of wines produced by the recombinant yeasts.

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