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. Copyright © 2001 John Wiley & Sons, Ltd.

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 CO₂ (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 t-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 baili (Baranowski and Radler, 1984; Kuczynski and Radler, 1982; Rodriguez and Thornton, 1989). However, wine yeast strains of S. cerevisiae cannot efficiently utilize t-malic acid during alcoholic fermentation (Subden et al., 1998; Volschenk et al., 1997b). The inefficient degradation of t-malic acid by S. cerevisiae is ascribed to the slow uptake of t-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 S. pombe can efficiently degrage up to 29 g/l of t-malic acid (Taillandier et al., 1988; Taillandier and Strehaianno, 1991). Cells of S. pombe actively transport t-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, S. pombe dehydroxylates t-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 S. pombe have been used for the degradation of t-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 S. 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 t-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 t-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,
MI), 0.5% (NH₄)₂SO₄, 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 PGK1p–mae2–PGK1t expression cassette from pHV7 (Volschenk et al., 1997b) was subcloned as a HindIII 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 sulphoturon methyl (SMM) via the SMRI-410 gene (Casey et al., 1988). The SMRI-410 gene was subcloned from pWX509 (Casey et al., 1988) by KpnI–BamHI digestion into pBluescript KS+ to yield pDLG42 (provided by Dr D. C. la Grange, Dept of Microbiology, University of Stellenbosch). The PGK1p–mae2–PGK1t cassette from pHV7 was subcloned as a HindIII fragment into the SMRI terminator region in pDLG42 (Figure 1), while PGK1p–mae1–PGK1t was subcloned as a PvuII fragment from pHV3 into the Klenow-treated NdeI site in the SMRI terminator region in pDLG42 to yield pHVS2 (Figure 1). pHVS2 was linearized with Apal 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.

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JM109</td>
<td>endA1, recA1, gyrA96, thi, hisdR17 [r6–, mll+], relA1, supE44, λ–, Δ(lac-proAB), [F, traD36, proA+ B–, lacETFΔM15]</td>
<td>(Yanisch-Perron, 1985)</td>
</tr>
<tr>
<td>S. cerevisiae YPH259</td>
<td>MATa, ura3-52, lys2-801 Δ(met14), ade2-101 Δ(ade2-101), his3Δ200, leu2-3,112</td>
<td>(Sikorski and Hieter, 1989)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td><strong>Description</strong></td>
<td><strong>Reference</strong></td>
</tr>
<tr>
<td>pHVX2</td>
<td>YEplac181 (LEU2 marker gene) containing the PGK1 promoter and terminator sequences</td>
<td>(Volschenk et al., 1997a,b)</td>
</tr>
<tr>
<td>pHV3</td>
<td>pHVX2 containing the mae1 ORF subcloned between the PGK1 promoter and terminator sequences</td>
<td>(Volschenk et al., 1997a,b)</td>
</tr>
<tr>
<td>pHV7</td>
<td>YEplac195 (URA3 marker gene) containing the mae2 ORF subcloned between the PGK1 promoter and terminator sequences</td>
<td>This study</td>
</tr>
<tr>
<td>pHVS2</td>
<td>pBluescript KS+ containing the SMRI-410 gene. The PGK1p–mae1–PGK1t and PGK1p–mae2–PGK1t cassettes were subcloned in the terminator region of the SMRI gene</td>
<td>This study</td>
</tr>
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Figure 1. Plasmid map of integration plasmid pHVS2. The PGK1p–mae2–PGK1t fragment was cloned as a HindIII fragment into the SMRI terminator region in pDLG42. The PGK1p–mae1–PGK1t fragment was cloned as PvuII fragment in the NdeI site (blunt-ended) in the terminator region of SMRI in pDLG42. Digestion with Apal linearized the plasmid in the SMRI 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 an internal 900 bp *Bgl*II DNA fragment of *ILV2* was labelled with \(^{\alpha}\)-32PdCTP, 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% (NH₄)₂SO₄, 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 \(\times 10^6\) 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 \(\times 10^6\) 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 H¹-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; Fock 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; Templeri 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 CO₂ under anaerobic conditions (Taillandier et al., 1988; Taillandier and Strehaiano, 1991; Subden et al., 1991).
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 wine-makers. 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 (mleS) 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 CO2 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 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.
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 *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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References


