

Malolactic Fermentation in Grape Musts by a Genetically Engineered Strain of *Saccharomyces cerevisiae*

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Malate enters *Saccharomyces cerevisiae* by simple diffusion. Due to the lack of a malate transporter and the low affinity of the *S. cerevisiae* malic enzyme, this yeast is unable to degrade malate efficiently. We have constructed a malolactic yeast strain by co-expressing the malate permease gene (*mae1*) of the fission yeast *Schizosaccharomyces pombe* and the *Lactococcus lactis* malolactic gene (*mleS*) in *S. cerevisiae*. The recombinant strain of *S. cerevisiae* transported malate and actively metabolized malate to lactate within three days in Cabernet Sauvignon and Shiraz grape musts at 20°C. The malolactic fermentation in Chardonnay grape must was completed within seven days at 15°C. The efficient degradation of malate in grape musts is important to wineries and the availability of malolactic yeasts will allow the early application of cellar operations for storage and aging of wine.

KEY WORDS: malolactic fermentation, wine, malate transport, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Lactococcus lactis*

Most red wines and some white wines in colder wine regions are subjected to the secondary malolactic fermentation (MLF) during or soon after alcoholic fermentation. During MLF, lactic acid bacteria, primarily strains of *Leuconostoc oenos* (26), decarboxylate L-malate to L-lactate and carbon dioxide. Malolactic fermentation reduces the levels of titratable acidity and increases the pH of the wine. MLF leads to enhanced microbial stability of the wine and presumably improves the organoleptic complexity of wine (3,4,7,14). The deacidification of grape musts and wine is, therefore, essential for the production of well-balanced wines.

A major disadvantage of MLF is the unpredictability of its occurrence and control during vinification. Several factors influence the development, duration and completion of malolactic fermentation (3,4,14). Malolactic bacteria have fastidious nutritional requirements and growth depends on the availability of nutrients left after alcoholic fermentation. Other prevailing conditions such as low pH and temperatures, high alcohol and sulfur dioxide levels, bacteriophage infections and lysis of the malolactic bacteria, and even traces of fungicides may lead to stuck MLF. Spontaneous

MLF may occur during alcoholic fermentation or only months later (15), and even the application of bacterial starter cultures does not completely ensure rapid MLF. Sluggish MLF causes a delay in cellar operations, especially sulfiting, leading to chemical oxidation of wine and, more significantly, the proliferation of spoilage organisms that produce off-flavors and toxic bio-amines.

S. cerevisiae metabolizes only small and insignificant amounts of malate during alcoholic fermentation. The inability of *S. cerevisiae* to efficiently degrade malate is due to the absence of an active transport system for malate (27) and the low substrate affinity of its malic enzyme ($K_m = 50 \text{ mM}$) (10).

The fission yeast *S. pombe* efficiently degrades malate to ethanol and CO_2 under anaerobic conditions (19,20). The malo-ethanolic fermentation by *S. pombe* is accomplished by the constitutive synthesis of two proteins; malate permease encoded by the *mae1* gene (13) and the malic enzyme, encoded by the *mae2* gene (28). L-Malate and other C_4 -dicarboxylic acids is transported in *S. pombe* by a constitutive proton-dicarboxylate symport system which is not subject to glucose repression, not even in the presence of high glucose concentrations found in grape must (13,24). The NAD-dependent malic enzyme is responsible for the oxidative decarboxylation of L-malate to pyruvate, which is further metabolized to ethanol and CO_2 (17). The malic enzyme of *S. pombe* has a strong affinity for malate ($K_m = 3.2 \text{ mM}$) (25), compared to the malic enzyme of *S. cerevisiae* ($K_m = 50 \text{ mM}$) (10). Deacidification of grape must with *S. pombe* has been attempted with moderate success (3,5,11,21), as *S. pombe* produces foul tasting and malodorous metabolites.

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Patent. The *mae1* gene and its application in oenology in combination with the malic enzyme gene and the malolactic gene has been patented internationally.

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Genetic manipulation of *S. cerevisiae* to perform alcoholic and malolactic fermentation simultaneously, has been explored for several years. The malolactic gene from *Lactobacillus delbrueckii* (29) and *Lactococcus lactis* (1,8) as well as genes involved in malate assimilation in *L. oenos* have been cloned (16). The malolactic gene (*mleS*) of *L. lactis* encodes a NAD-dependent malolactic enzyme which converts L-malate to L-lactate and CO₂. Expression of bacterial malolactic genes in *S. cerevisiae* did not significantly increase the rate of malate degradation (1,9,29), due to the absence of a transport system for the uptake of malate in *S. cerevisiae*.

In this study a recombinant strain of *S. cerevisiae* containing both the *S. pombe mae1* and *L. lactis mleS* genes, was constructed. The ability of the recombinant strain to conduct malolactic fermentation in Cabernet Sauvignon, Shiraz and Chardonnay grape musts was investigated. Malolactic fermentation in Cabernet Sauvignon and Shiraz grape musts was completed within three days, and after seven days in Chardonnay grape must.

Materials and Methods

Strains and plasmids: The different strains and plasmids employed in this study are listed in Table 1.

Subcloning of the *mae1* and *mleS* genes: DNA manipulations were performed in the yeast-*E. coli* shuttle vector YEplac181 (12). The expression vector pHVX2 (Table 1) was obtained by subcloning a *Hind*III fragment from plasmid pJC1 (6), containing the *PGK1* promoter and terminator sequences into the *Hind*III site of YEplac181 (Fig. 3). The *mae1* ORF was isolated as a *Bal*I-*Nde*I fragment from plasmid pJG1 (13) and subcloned into YEplac181 containing a multiple cloning site with *Eco*RI, *Bal*I, *Nde*I and *Bgl*II restriction sites. The *mae1* ORF was re-isolated as an *Eco*RI-*Bgl*II fragment and subcloned into the *Eco*RI/*Bgl*II site of pHVX2 to yield plasmid pHV3 (Fig. 3). The cloning and expression of the *L. lactis mleS* gene in *S. cerevisiae* have previously been described (9).

Culture conditions: *E. coli* JM109 (Table 1) was cultured as described previously (22). *E. coli* was transformed by electroporation and transformants were selected on LB medium supplemented with ampicillin.

Yeast cells were cultured in liquid YPD media (1% yeast extract, 2% bactopectone, 2% glucose) at 30°C. *S. cerevisiae* was co-transformed (lithium acetate procedure) with plasmids pHV3 and pMDMALO together, or with pHVX2, pHV3 or pMDMALO, individually (Table 1). Transformants were isolated on selective YNB agar plates (0.17% yeast

nitrogen base (YNB) without amino acids (aa) and ammonium sulphate [Difco Laboratories, Detroit, MI], 0.5% (NH₄)₂SO₄, 2% glucose and 1.7% agar) supplemented with amino acids as required. The transformants were cultured to high cell density in 10 mL YNB liquid medium at 30°C, harvested by centrifugation and resuspended in sterile grape juice before inoculation into grape must.

Malolactic fermentation in grape musts: Recombinant strains of *S. cerevisiae* containing the different plasmids were inoculated to a final concentration of 2 × 10⁶ cells/mL in 200 mL must (preheated to 15 or 20°C) in 250-mL glass containers. Cabernet Sauvignon (2.8 g/L L-malate) and Shiraz (3.2 g/L L-malate) were fermented at 20°C and Chardonnay must (3.4 g/L L-malate) at 15°C without shaking. Both red and white grape musts were supplemented with 0.075% diammonium phosphate before inoculation.

The L-malate and L-lactate concentrations during fermentation were measured enzymatically using the L-Malic Acid and L-Lactic Acid Test Kits (Boehringer Mannheim, Germany). Malate to lactate conversion was visualised by paper chromatography according to standard methods.

Results and Discussion

The production of well-balanced wines requires the reduction of excess acidity, especially in colder regions of the world. Strains of *S. cerevisiae* metabolize only small and insignificant amounts of L-malate and many wines are, therefore, subjected to the crucial process of MLF during or soon after alcoholic fermentation.

The metabolic engineering of *S. cerevisiae* to carry out the alcoholic and malolactic fermentations simultaneously, has been explored for several years. Recombinant strains of *S. cerevisiae* containing the *L. lactis mleS* gene have been shown to stoichiometrically convert small amounts of L-malate to L-lactate (1,9). However, due to the absence of a transport system for L-

Table 1. Different strains and plasmids employed in the genetic construction of malolactic strains of *S. cerevisiae*.

Strains	Description	Reference
<i>E. coli</i> JM109	<i>endA1, recA1, gyrA96, thi, hsdR17</i> [<i>r_k</i> ⁻ , <i>m_k</i> ⁺], <i>relA1, supE44, λ</i> ⁻ , Δ (<i>lac-proAB</i>), [<i>F</i> ⁺ , <i>traD36, proA⁺B⁺, lac⁺ZΔM15</i>]	(30)
<i>S. cerevisiae</i> YPH259	α <i>ura3-52, lys2-801^{amber}, ade2-101^{ochre}, his3Δ200, leu2-Δ1</i>	(23)
Plasmids		
1. pHVX2	YEplac181 containing the <i>PGK1</i> promoter and terminator sequences.	(This study)
2. pHV3	pHVX2 containing the <i>mae1</i> ORF subcloned between the <i>PGK1</i> expression cassette.	(This study)
3. pMDMALO	Multicopy episomal plasmid containing the <i>URA3</i> gene and the <i>mleS</i> ORF inserted between the <i>PGK1</i> promoter and terminator sequences.	(9)

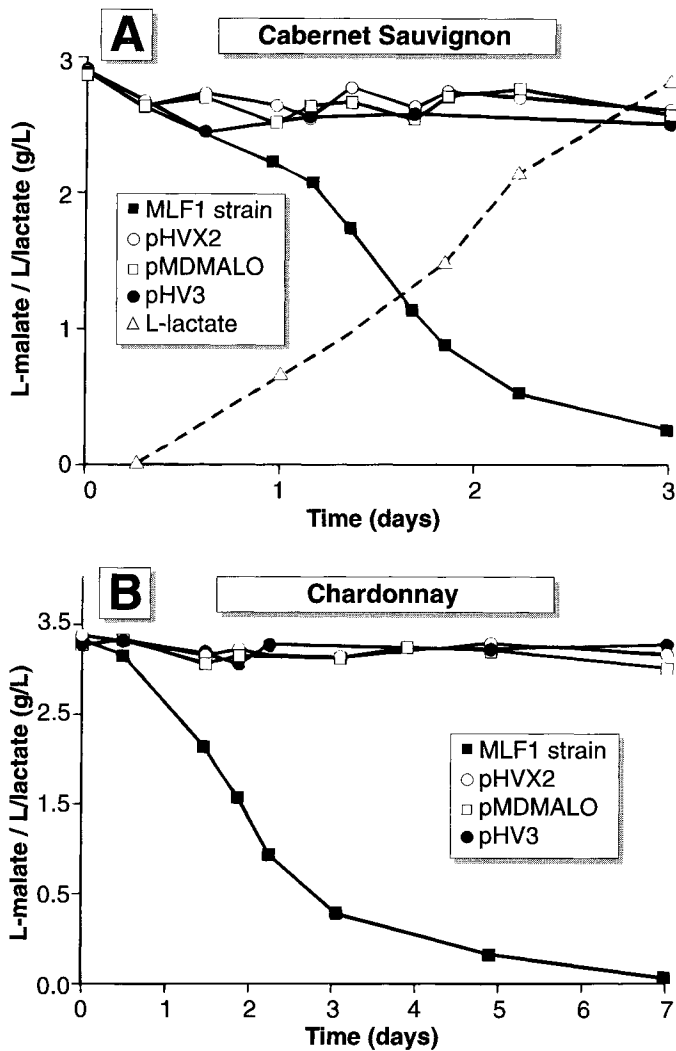


Fig. 1. Degradation of L-malate in Cabernet Sauvignon (A) and Chardonnay (B) grape must by recombinant strains of *S. cerevisiae*. Malolactic fermentation was regarded as complete when the concentration of L-malate reached 0.3 g/L (18). The MLF1 strain [■] of *S. cerevisiae* containing the malate permease gene (*mae1*) of *S. pombe* and the malolactic gene (*mleS*) of *L. lactis* rapidly degraded L-malate in both Cabernet Sauvignon and Chardonnay grape must. Malate was not significantly degraded by the control yeasts containing the *PGK1* expression cassette (pHVX2 [○]), the *mleS* gene (pMDMALO [□]), or the *mae1* gene (pHV3, [●]). Equimolar amounts of L-lactate[Δ] was produced from L-malate by the MLF1 strain [■].

malate in *S. cerevisiae*, L-malate degradation was slow and incomplete (2).

In contrast to previously engineered strains of *S. cerevisiae*, our recombinant strain (MLF1) containing both the *S. pombe mae1* and *L. lactis mleS* genes, efficiently and rapidly degraded L-malate to L-lactate in grape must in a significantly short period of time (Figs. 1 and 2). The control yeast strains containing only the *PGK1*-expression cassette (pHVX2), the *mleS* gene (pMDMALO) or the *mae1* gene

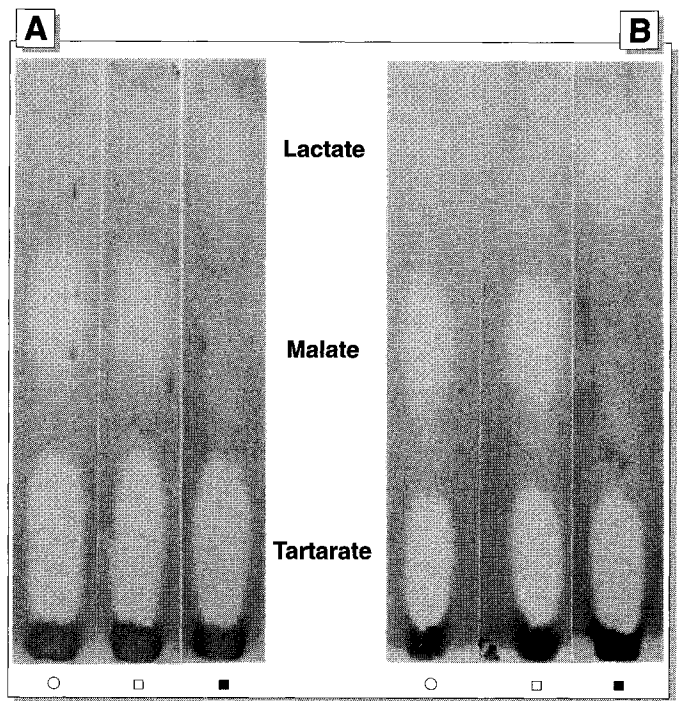


Fig. 2. Paperchromatographic assays of Cabernet Sauvignon (A) and Chardonnay (B) wine fermented with recombinant strains of *S. cerevisiae* (from left to right: yeast transformed with pHVX2, pMDMALO, and pHV3 plus pMDMALO, respectively). Only the MLF1 strain (pHV3 and pMDMALO) containing the *mae1* and *mleS* genes degraded L-malate to L-lactate.

(pHV3) under the control of the *PGK1* promoter, were unable to degrade significant amounts of L-malate to L-lactate and CO₂ (Fig.1, 2).

Rapid and complete metabolism of 2.8 g/L L-malate in Cabernet Sauvignon must was obtained within three days at 20°C. In Chardonnay must 3.4 g/L L-malate was degraded to L-lactate after seven days at 15°C (Figs. 1 and 2). The slower rate of malate degradation in Chardonnay must can probably be ascribed to the lower fermentation temperature. Rapid malolactic fermentation (2 days) with the recombinant strain was also achieved in Shiraz grape must (results not shown). The recombinant strain of *S. cerevisiae* completed malolactic fermentation within days after the onset of the

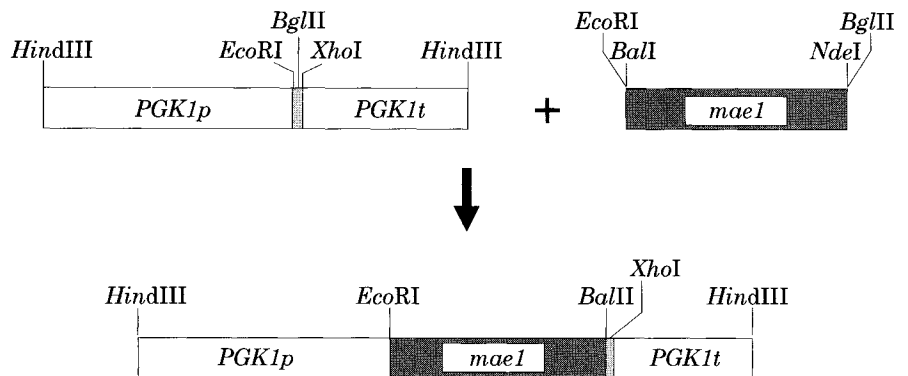


Fig. 3. Subcloning of the *S. pombe mae1* ORF under control of the *PGK1* promoter and terminator sequences in pHVX2, a derivative of YEplac181 (12).

alcoholic fermentation, a major improvement on the traditional MLF by lactic acid bacteria which can take weeks or even months before it is completed (15).

The early completion of MLF in wine is of great importance to winemakers as sluggish or stuck fermentations lead to scheduling problems in cellars. Furthermore, wines cannot be stabilized by sulfite and this may lead to the proliferation of spoilage organisms which often produce off-odors or noxious bio-amines. The application of malolactic strains of *S. cerevisiae* can circumvent these problems.

It is well known that multicopy 2 μ -based plasmids in *S. cerevisiae* are unstable. We are currently integrating the *mae1* and *mleS* genes into the genomes of wine yeast strains to overcome this problem. Recombinant strains containing single copies of the *mae1* and *mleS* genes should also be able to degrade L-malate to L-lactate and CO₂ during the alcoholic fermentation. Once we have obtained these ethanol tolerant malolactic wine strains of *S. cerevisiae*, we will compare and evaluate their fermentation kinetics and the organoleptic quality of wines produced by application of these yeasts and, wines fermented by the recipient yeast strain in combination with malolactic bacteria. An alternative approach would be to construct ethanol sensitive malolactic strains of *S. cerevisiae* which can be used as co-cultures together with industrial wine yeast strains. The use of ethanol-sensitive malolactic strains of *S. cerevisiae* during vinification should result in a rapid and complete degradation of L-malate to L-lactate. However, the spread of malolactic yeasts in a cellar will be minimized as most of these yeast cells should be killed during the latter stages of fermentation due to ethanol toxicity.

Many problems are associated with the bacterial MLF. We have successfully engineered a strain of *S. cerevisiae* that efficiently decarboxylates L-malate to L-lactate during the initial stages of vinification. The efficient degradation of L-malate in grape musts by malolactic yeasts will allow the early application of cellar operations for the production of safer wines of a higher quality.

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