Available online at www.sciencedirect.com
SCIENCE DIRECT
Metabolic Engineering 8 (2006) 315–323
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Metabolic engineering of malolactic wine yeast

John I. Husnik[a], Heinrich Volschenk[b], Jurgen Bauer[c], Didier Colavizza[d], Zongli Luo[a], Hennie J.J. van Vuuren[a,*]

[a]*Wine Research Centre, Faculty of Land and Food Systems, University of British Columbia, Suite 231, 2205 East Mall, Vancouver, BC, Canada V6T 1Z4*
[b]*Faculty of Applied Sciences, Cape Peninsula University of Technology, P.O. Box 652, Cape Town 8000, South Africa*
[c]*Gentana GmbH, Im Neuenheimer Feld, 515 D-69120 Heidelberg, Germany*
[d]*Lesaffre International, rue Gabriel Péri 137, F-59700 Marcq-en-Baroeul, France*

Received 6 January 2006; received in revised form 15 February 2006; accepted 27 February 2006
Dedicated to J.-P. Rossi
Available online 18 April 2006

Abstract

Malolactic fermentation is essential for the deacidification of high acid grape must. We have constructed a genetically stable industrial strain of *Saccharomyces cerevisiae* by integrating a linear cassette containing the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus oeni* malolactic gene (*mleA*) under control of the *S. cerevisiae PGK1* promoter and terminator sequences into the *URA3* locus of an industrial wine yeast. The malolactic yeast strain, ML01, fully decarboxylated 5.5 g/l of malate in Chardonnay grape must during the alcoholic fermentation. Analysis of the phenotype, genotype, transcriptome, and proteome revealed that the ML01 yeast is substantially equivalent to the parental industrial wine yeast. The ML01 yeast enjoys 'Generally Regarded As Safe' status from the FDA and is the first genetically enhanced yeast that has been commercialized. Its application will prevent the formation of noxious biogenic amines produced by lactic acid bacteria in wine.

Keywords: *Saccharomyces cerevisiae*; *Oenococcus oeni*; Malolactic fermentation; Malate permease; Malolactic enzyme

1. Introduction

The production of quality wine depends on the quality of the grapes, the microorganisms involved in fermentation, and the skills of the winemaker. Reduction of acidity in high acid grape musts is essential for the production of well-balanced wines. The main organic acids in grape must are L-malic and L-tartaric acids (Beelman and Gallander, 1979). Wineries currently employ the bacterial malolactic fermentation (MLF) to reduce wine acidity and to obtain microbially stable bottled wines. The MLF entails the decarboxylation of L-malic acid to L-lactic acid and carbon dioxide; this reaction is catalyzed by the malolactic enzyme (L-malate:NAD^+ carboxylyase) without the production of any free intermediates (Caspritz and Radler, 1983; Spettoli et al., 1984; Naouri et al., 1990). The MLF is ideally performed by *Oenococcus oeni* (Dicks et al., 1995) after the alcoholic fermentation is completed by wine yeast strains of *Saccharomyces* sp. (Lonvaud-Funel, 1995).

O. oeni, as well as other lactic acid bacteria, are inhibited by ethanol, low pH, sulfur dioxide, low temperature, fatty acids, decreased nutrient content, competitive interactions with yeast and other bacteria, and bacteriophage infections (see reviews Davis et al., 1985; Wibowo et al., 1985; van Vuuren and Dicks, 1993; Henick-Kling, 1995). Despite the use of bacterial malolactic starter cultures, lactic acid bacteria grow poorly and unpredictably in wine, especially Chardonnay wines, which leads to 'stuck' or 'sluggish' MLF and spoilage of wines (Davis et al., 1985). In addition to processing issues, several lactic acid bacterial contaminants produce toxic substances such as biogenic amines (see Lonvaud-Funel, 2001 for a review). More than 20 amines have been found in wine (Lehtonen, 1996), the most notable being histamine, cadaverine, phenylethylamine, putrescine and tyramine (Zee et al., 1983; Lonvaud-Funel, 2001). The presence of biogenic amines in wine can be of great concern for consumers since these molecules have

*Corresponding author. Fax: +1 604 822 5143.
E-mail address: hjjvv@interchange.ubc.ca (H.J.J. van Vuuren).

1096-7176/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2006.02.003

been shown to produce undesirable physiological effects in susceptible individuals. Histamine is known to cause headaches and other allergenic symptoms such as, hypotension, edema, palpitations, flushing, diarrhoea, and vomiting (Wantle et al., 1994; Santos, 1996; Soufleros et al., 1998). Tyramine and phenylethylamine have been associated with migraines and hypertension (Soufleros et al., 1998). Moreover, alcohol, acetaldehyde, antidepressive drugs and other biogenic amines such as cadaverine and putrescine can potentiate the toxic effect of histamine, tyramine and phenylethylamine (ten Brink et al., 1990; Straub et al., 1995). Biogenic amines are also linked to carcinogenesis. Nitrosable secondary amines (dimethylamine, piperidine, pyrrolidine, spermidine, spermine) detected in wine can react with nitrous acid and its salts to form carcinogenic nitrosoamines (Santos, 1996; Shalaby, 1996). Histamine and polyamines (putrescine, spermidine and spermine) can induce cell transformation and tumor pathogenesis (Medina et al., 1999; Pryme and Bardocz, 2001; Wallace and Caslake, 2001).

Other methods used by the wine industry to reduce the acidity of wine or grape must include blending, carbonate additions, precipitation, dilution and carbonic maceration. Although these methods can reduce the acidity of wine they are laborious and often result in poor quality wine. Alternative technologies include the use of *S. pombe* (Gallander, 1977) and high density cell suspensions of yeasts (Gao and Fleet, 1995), and immobilization of *O. oeni*, *Lactobacillus* sp., or the malolactic enzyme on a variety of matrices (see Maicas, 2001 for a review). Unfortunately, these methods are not effective for the production of quality wines on a commercial scale. Wine yeast strains of *S. cerevisiae* cannot effectively metabolize extracellular malate due to the lack of an active malate transporter (Grobler et al., 1995; Volschenk et al., 1997b) and the low substrate affinity of its NAD-dependent malic enzyme ($K_m = 50$ mM) (Fuck et al., 1973) that is also subject to catabolite repression (Redzepovic et al., 2003). For more than three decades scientists have attempted to engineer a malolactic yeast by constitutive expression of the bacterial malolactic enzyme in *S. cerevisiae* (Lautensach and Subden, 1984; Williams et al., 1984; Ansanay et al., 1993; Denayrolles et al., 1995). These attempts failed due to the absence of a malate transporter in *S. cerevisiae*. We cloned and characterized a malate transport gene (*mae1*) from *S. pombe* (Grobler et al., 1995) and obtained an efficient MLF by co-expression of the *mae1* and the *L. lactis* malolactic enzyme (*mleS*) genes under the control of the *S. cerevisiae PGK1* promoter and terminator sequences on multicopy plasmids in a laboratory strain of *S. cerevisiae* (Volschenk et al., 1997a, b). The recombinant laboratory strain was able to efficiently decarboxylate 4.5 g/l of malate to lactate and carbon dioxide in 4 days.

We have now successfully integrated the malolactic cassette flanked by ura3 sequences into the genome of an industrial wine yeast strain. This genetically stable wine yeast decarboxylates 5.5 g/l of malate in Chardonnay grape must during the first 9 days of the alcoholic fermentation. Rigorous scientific examination of the phenotype, genotype, transcriptome and proteome revealed that the ML01 yeast is substantially equivalent to the parental strain S92. The ML01 yeast enjoys GRAS status from the FDA and has now been commercialized in Moldova and the USA.

2. Methods

2.1. Strains and culture conditions

Escherichia coli DH5α (F-, ϕ80d*lacZ*Δ*M*15, *endA*1, *recA*1, *hsdR*17 [r_k^-,m_k^+], *supE44, thi-1, gyrA96, relA1,* Δ[*lacZYA*-argF]U169, λ^-) was used for plasmid propagation and cultured according to standard methods (Ausubel et al., 1995). *S. cerevisiae* ML01 and S92 (Bio Springer, USA) were cultured according to standard methods. Active dry yeast (ADY) were used either directly or re-hydrated at 40 °C in 7% synthetic juice for 30 min. During re-hydration ADY was vortexed at the start, at 20 min, at 30 min and in between inoculations.

YEG (1% yeast extract, 2% dextrose, 1.5% Pastagar B [Bio-Rad, USA]) supplemented with 100 μg/ml of phleomycin (Invivogen, USA) was used for selecting co-transformed yeast colonies and testing for phleomycin sensitivity. Synthetic juice was prepared by adding 1.0 ml/l of Tween 80 to previously described synthetic broth (Denayrolles et al., 1995). The pH of the synthetic juice used in the L-lactic acid assay was adjusted to 3.5. The tartaric acid concentration of the synthetic juice for the DNA microarray, Real-Time PCR and iTRAQ analysis was adjusted to 4.5 g/l.

2.2. Construction of integration cassette

The *mae1* expression cassette was isolated from plasmid pHV3 (Grobler et al., 1995; Volschenk et al., 1997b) by PCR amplification (Takara, Japan) using primers J13 (5′-AACCAAAAAT**GGTACC**AAGCTTTCTAACTGATCTATCCAAAACTGA-3′) and J14 (5′-AAGGAAAAA**AGGTACC**AAGCTTTAACGCAGAATTTCGAGTT-3′) and cloned between sequences homologous to the *URA3* locus on pHVJH1 yielding pJH1 (Husnik, 2001). The *mleA* gene was isolated from *O. oeni* Lo 8413, isolated in the Bordeaux region of France (Labarre et al., 1996), by PCR amplification using primers J18 (5′-GAGGA**GAATTC**ATGACAGATCCAG-3′) and J19 (5′-ACG**AGATCT**CATTAGTATTTCGGATCC-3′). The *mleA* gene was cloned into the *Eco*RI and *Bgl*II sites of pHVX2 (Grobler et al., 1995; Volschenk et al., 1997b) yielding the *mleA* expression cassette. The *mleA* expression cassette was isolated by PCR amplification using primers J15 (5′-ATGCTTTTTTGCGGCCGCAAGCTTTCTAACTGATCTATCCAAAACTGAAA-3′) and J16 (5′-ATGCATATAAGCGGCCGCAAGTTTAACGAACGAACGCAGAATTTTCGAGTTAT-3′) and cloned into pBluescript KS^+, the *mleA* cassette was then isolated as a *Not*I fragment and subcloned into pJH1

yielding pJH2 (Husnik, 2001). The final construct (malolactic cassette) was isolated from plasmid pJH2 by digestion with *Srf*I and subsequent gel extraction. This resulted in the purification of a 8683 bp fragment containing the *mae1* and *mleA* expression cassettes flanked by homologous ura3 sequences.

2.3. Co-transformation and screening

S. cerevisiae S92 was co-transformed with the malolactic cassette and pUT332 (Gatignol et al., 1987) combined at a 10:1 (malolactic cassette:pUT332) molar ratio. After electroporation (Ausubel et al., 1995) 1 ml of ice-cold YPD media was used to recover the yeast. Yeast cells were gently transferred to a 1.5 ml microfuge tube and gently mixed at 30 °C for 4 h. Aliquots of the yeast suspension were then directly plated onto YEG + phleomycin plates and incubated at 30 °C for 3 days.

Co-transformed yeast colonies were inoculated into sterile 96 micro-well plates (round bottom) containing 200 μl of synthetic juice. Inoculated micro-well plates (wrapped in parafilm) were incubated at 30 °C for 3–5 days. After incubation, 75 μl of the supernatant from each well was removed and placed into a new micro-well plate. 25 μl of the L-lactic acid reaction mixture (Subden et al., 1982) was added to each well containing the 75 μl of the test sample. The micro-well plate was then incubated at 37 °C in the dark for 30 min. Wells containing L-lactic acid were indicated by a purple/blue color and yeast cells from the corresponding plate were recovered by inoculation into 5 ml of synthetic juice or YPD and incubated at 30 °C for 1–2 days. After recovery, clones were streaked onto YEG plates and incubated at 30 °C until colonies appeared. Confirmation of integration was performed by PCR on genomic DNA (Promega, Wizard® Genomic DNA Purification kit) from each clone identified in the screening. The PCR reaction was done according to manufacturer's recommendations (Life Technologies Inc., GibcoBRL®) using primers 5′-GTTGTAATGTGACCAATGAG-3′ (inside the cassette) and 5′-CTCTTTATATTTACATGCTAAAAATGG-3′ (outside the cassette). The 1095 bp PCR product was visualized by 0.8% agarose gel electrophoresis and ethidium bromide staining (Ausubel et al., 1995).

2.4. Genetic characterization of ML01

Southern blotting, labeling of probes, hybridization, stringency washes and detection were completed as recommended by the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, USA). Genomic DNA was digested with *Eco*RV, *Hinc*II, *Nco*I, *Nsi*I, or *Pvu*II. After fractionation by electrophoresis in a 1% agarose gel the DNA was blotted onto positively charged membranes (Roche, Germany) and fixed by heating at 80 °C for 1 h. Probes corresponding to the MLF cassette were excised from pJH2 with the following restriction enzymes: *Xba*I/*Kpn*I to produce the 5′ end 938 bp *URA3* probe, *Sph*I/*Bam*HI to produce the 842 bp *mae1* probe, *Xho*I/*Pme*I to produce the 1556 bp *mleA* probe, and *Cla*I/*Not*I to produce the 674 bp *PGK1* promoter probe. Probes corresponding to pUT332 were either excised from the plasmid or PCR amplified. The 471 bp probe for the Tn5*Ble* gene was prepared by PCR amplification using primers 5′-AATGACCGACCAAGCGACG-3′ and 5′-ATCCTGGGTGGTGAGCAG-3′ and Pwo polymerase (Roche, Germany). A probe for the remaining non-*Saccharomyces* sequences of pUT332 was prepared by digesting pUT332 with *Ssp*I/*Cla*I resulting in a 1758 bp fragment.

Sporulation was done according to standard protocols (Ausubel et al., 1995). The only modification was that cells were plated on SAA medium (6.5 g/sodium acetate and Pastagar B 15 g/l) and incubated for 72 h at ambient temperature.

Sequencing was completed at the Nucleic Acid Protein Service Unit (NAPS Unit, University of British Columbia, Canada) using an Applied Biosytems PRISM 377 sequencer and AmpliTaq FS Dyedeoxy Terminator Cycle sequencing chemistry. Using *PfuTurbo* (Stratagene, USA) two unique sets of templates spanning the entire malolactic cassette were obtained by PCR. One set of templates was used for the sequencing of one strand while the other set was used for the sequencing of the reverse strand. The two complete sequences were aligned and analyzed for differences. If anomalies occurred, another round of PCR and sequencing of the region in doubt was run to determine the correct sequence.

2.5. Microarray analyses

One milliliter of re-hydrated ML01 (25 mg/ml) and S92 (25 mg/ml) were each inoculated in triplicate into 500 ml sterile flasks (with 130 ml head space), containing 500 ml of synthetic juice and a magnetic stir bar. Each fermentation flask was fitted with a disinfected vapor lock. Additional flasks were similarly prepared in order to monitor weight loss, measure optical density and take samples for chemical analyses. All flasks were stirred once daily and incubated at 20 °C. After stirring, samples for monitoring optical density were obtained by puncturing the #6.5 rubber stopper (Fermenthaus, USA) with a sterile syringe and 18G × 3″ needle (Air-Tite, USA). After absorbance readings, samples were centrifuged and the supernatant stored at −30 °C until chemical analyses were completed.

At 48 and 144 h, five 30 ml volumes from each culture were centrifuged in 40 ml tubes for 3 min at 3500*g*. The supernatant was decanted and the pellet re-suspended (briefly vortexed) in 10 ml of dH_2O. A second centrifugation was completed for 3 min at 3500*g*. The supernatant was decanted and the cell pellet was placed in liquid nitrogen for 30 s and then stored at −80 °C. Optical density measurements and chemical analyses were conducted on the remaining fermentation broth and the initially decanted

supernatant. Total RNA, poly $(A)^+$ RNA purification, cDNA synthesis and biotin-labeled cRNA synthesis and fragmentation procedures have been previously described (Erasmus et al., 2003). The only modification was that the isolated total RNA was also passed through an RNA clean-up kit (RNeasy Midi kit, Qiagen). Twelve YGS98 oligonucleotide arrays (Affymetrix, USA) were used as targets for hybridization. Hybridization, fluidics and scanning procedures have been described previously (Erasmus et al., 2003). Data were analyzed using Affymetrix Microarray Suite v 5.0. All detection and comparison tunable parameters were set to default values (Affymetrix, USA). Absolute analysis was completed on triplicate data for ML01 and S92 at 48 and 144 h. Nine comparative analyses were generated for each time point. Only probe sets that had a change call of 'I' or 'MI' and change *P*-value of $\leqslant 0.003$ across all nine comparison were called 'increasers', probe sets with 'D' or 'MD' and a *P*-value $\geqslant 0.997$, 'decreasers'. Average Signal Log (base 2) Ratio values were used to calculate the fold change. Probe sets were linked to their target descriptions and to their gene ontology (GO) annotations using NetAffx (www.affymetirx.com/analysis/index.affx) and SGD (*Saccharomyces* Genome Database; http://genome-www.stanford.edu/Saccharomyces).

2.6. Analysis of the proteome

Cell pellets obtained for microarray analysis at 48 h were also used for protein extraction. Each cell pellet was re-suspended in ice-cold 5 ml lysis buffer (50 mM Tris, pH 8.5, 0.1% SDS) and aliquoted into four 1.5 ml tubes also on ice containing sterile glass beads. Cells were lysed in a cold room using a mini-beadbeater with intermittent vortexing. After lysis the samples were centrifuged at 18 000*g* for 2 min at 2 °C. The supernatant was transferred to clean tubes and centrifuged at 18 000*g* for 10 min at 2 °C. TCA precipitation of the proteins required adding an equal volume of ice cold 40% trichloroacetic acid and incubation (on ice) for 60 min. Samples were centrifuged at 18 000*g* for 30 min at 2 °C. Precipitated proteins were washed twice with 100% acetone (−20 °C) and centrifuged at 2 °C for 30 and 20 min. Pellets were dried at room temperature until all visible liquid was evaporated and then stored at −80 °C. Protein pellets were shipped on dry ice to University of Victoria–Genome BC Proteomics Centre for iTRAQ analysis.

Total protein determination was completed using a commercial Bradford assay reagent (BioRad, Canada). Denaturation, blocking of cysteines, digestion with trypsin and labeling with iTRAQ tags were done according to the iTRAQ protocol (Applied Biosystems,USA). Protein samples were labeled with the iTRAQ tags as follows: ML01 replicate 1, iTRAQ114; S92 replicate 1, iTRAQ115; ML01 replicate 2, iTRAQ116; and S92 replicate 2, iTRAQ117.

Strong cation exchange (SCX) chromatography, fractionation and LC MS/MS analyses have been previously described (DeSouza et al., 2005). The modifications to the SCX method were that the flow rate was set to 0.5 ml/min and the gradient applied was 0–35% buffer B in 30 min. Fractions were collected every minute. Prior to LC MS/MS analysis fractions were brought up to 20 µl with 5% acetonitrile and 3% formic acid and transferred to autosampler vials (LC Packings, Amsterdam). The modifications to the LC MS/MS procedure were that the mobile phase (solvent A) consisted of water/acetonitrile (98:2 [v/v]) with 0.5% formic acid for sample injection and equilibration on the guard column at a flow rate of 100 µl/min. A linear gradient was created upon switching the trapping column inline by mixing with solvent B that consisted of acetonitrile/water (98:2 [v/v]) with 0.5% formic acid and the flow rate reduced to 200 nl/min. A 10 µl of sample was injected in 95% solvent A and allowed to equilibrate on the trapping column for 10 min. Upon switching inline with the MS, a linear gradient from 95% to 40% solvent A developed for 40 min and in the following 5 min the composition of mobile phase was decreased to 20% A before increasing to 95% A for a 15 min equilibration before the next sample injection. The MS data acquisition method consisted of a 1 s TOFMS survey scan of mass range 400–1200 amu and two 2.5 s product ion scans of mass range 100–1500 amu. The two most intense peaks over 20 counts, with charge state 2–5 were selected for fragmentation and a 6 amu window was used to prevent the peaks from the same isotopic cluster from being fragmented again. Once an ion was selected for MS/MS fragmentation it was put on an exclude list for 180 s. Curtain gas was set at 23, nitrogen was used as the collision gas and the ionization tip voltage used was 2700 V.

Data files were processed using the ProQuant software (Ver. 1.0) (AB, Foster City, USA) in Analyst using the following parameters: the MS and MS/MS tolerances were set to 0.15. A *S. cerevisiae* subset database of the Celera Discovery Systems database (Ver. 3.0, 01/12/2004) was used for the protein searches. Methyl methanethiosulfonate (MMTS) modification of cysteines was used as a fixed modification. All results were written to a Microsoft Access database. In order to reduce protein redundancy, experimental software, ProGroup viewer (RC 20, Foster city, USA) was used to assemble and report the data. Four comparative analyses were generated using S92 replicate 1 and S92 replicate 2 as denominators. A weighted average ratio was calculated for protein ratios with a *P*-value <0.05 across all comparisons.

2.7. Phenotypic characterization

ADY of ML01 (50 mg/l) and S92 (50 mg/l) were inoculated into 500 ml sterile flasks containing 500 ml of Chardonnay must (Okanagan, BC, Canada, 2004). The juice contained 108.3 g/l of glucose, 113.9 g/l of fructose, and 5.5 g/l of malate. Yeast assimilable nitrogen content was 401.2 mg/l. Total free SO_2 was adjusted to 25 mg/l prior to inoculation with potassium metabisulfite. The pH

of the juice was 3.41. Flasks for chemical analyses were stirred once before daily sampling in order to obtain a homogenous sample. Sampling was done anaerobically as described for the transcriptome/proteome study. Flasks to monitor weight loss were weighed daily. The must was incubated initially at 19 °C for 35 h, then 13 °C for 1 week and finally 19 °C until completion of the fermentation. After completion of the alcoholic fermentation one-half of the wines produced by S92 were inoculated with *O. oeni* as per manufacturer's recommendations (Viniflora, CHR Hansen). Malate, lactate, glucose, fructose, acetate and ethanol determinations were done by enzymatic analysis (Megazyme, Ireland). A single-factor analysis of variance (ANOVA) with Fisher's least-significant difference (LSD) post hoc test was used to evaluate differences in acetic acid production ($P<0.05$). Differences in residual sugar and ethanol concentrations in wines produced by ML01 and S92 were evaluated using a two-tailed independent samples t tests ($P<0.05$).

3. Results

3.1. Functionality and phenotype of the ML01 yeast

After screening approximately 2000 yeast transformants for integration of the malolactic cassette (Fig. 1), five clones were found that produced lactic acid from malic acid. Only one clone, ML01, produced an equimolar amount of lactic acid from malic acid in laboratory media. In small-scale vinification trials with ML01 in filter sterilized Chardonnay grape must, the ML01 efficiently decarboxylated 5.5 g/l of malate and produced equimolar amounts of lactate without affecting the production of ethanol (Fig. 2). In contrast, *O. oeni* was unable to complete the MLF after 4 months and 2.59 and 2.62 g/l of malic acid remained in the wines despite the fact that these wines were re-inoculated four times with *O. oeni* and the temperature increased to 25 °C. Similar growth and fermentation kinetics were observed for the ML01 and the parental strain S92 (data not shown). The residual sugar in wine fermented with the parental strain S92 was 0.47 ± 0.02 g/l and the residual sugar in wine fermented with ML01 was 1.23 ± 0.05 g/l. As expected for wines after a MLF, the pH of the wines produced by ML01 was 0.21 pH units higher than wines produced by S92. Acetic acid (main component of volatile acidity) was significantly lower in wines produced with the ML01 strain (249.5 ± 12.4 mg/l of acetic acid) than those produced by S92 (328.4 ± 8.5 mg/l) and S92/*O. oeni* (424.4 ± 10.0 mg/l) ($P<.001$). GC/MS analysis of wines produced by ML01 and S92/*O. oeni* revealed that wines were similar as far as other volatile metabolites are concerned (data not shown).

3.2. Genetic characterization of ML01

A Southern blot was performed using a 5′*ura3* probe on *Nsi*I-digested genomic DNA of ML01 and S92. Two signals were detected for ML01 corresponding to 1.7 and 2.8 kbp DNA fragments, and one signal was detected for S92 corresponding to a 1.7 kbp DNA fragment (Fig. 3). The 1.7 kbp fragment matches the expected fragment size for a non-disrupted *URA3* locus and the 2.8 kbp fragment is in accordance with the presence of a malolactic cassette integrated into the *URA3* locus. To clearly characterize the integration event in the ML01 strain, Southern blot analyses were performed using probes corresponding to the *mae1* and *mleA* genes, and the *PGK1* promoter. Blots with the *mae1, mleA* and *PGK1* promoter probes confirmed

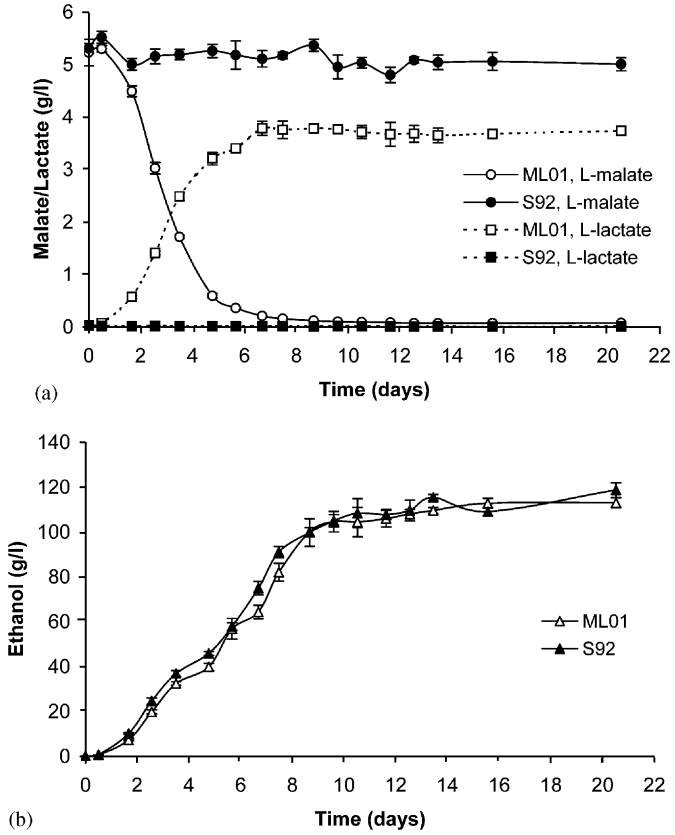

Fig. 2. Vinification trails with ML01 and S92 in Chardonnay must. (**a**) Efficient conversion of L-malate (○) to L-lactate (□) during alcoholic fermentation by ML01; no significant degradation of L-malate (●) or production of L-lactate (■) was observed for S92. (**b**) Production of ethanol by ML01 (△) and S92 (▲); introduction of the malolactic cassette did not affect ethanol formation or growth (data not shown).

Fig. 1. Schematic representation of the malolactic cassette integrated into the *URA3* locus of *S. cerevisiae* S92. The linear cassette was co-transformed with pUT332 that contains the Tn*5ble* gene encoding for resistance to phleomycin. A L-lactic acid screen was performed on phleomycin-resistant colonies to identify malolactic clones.

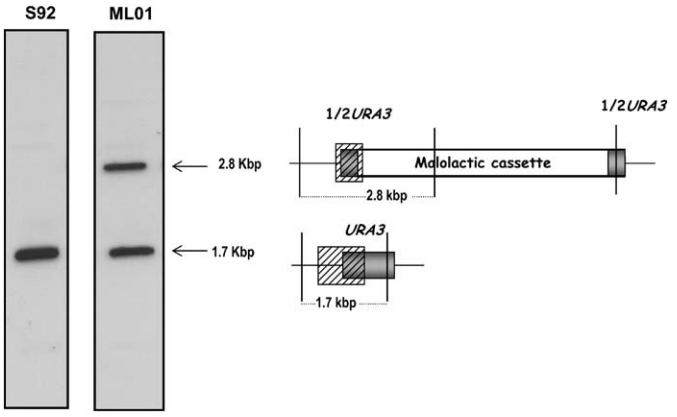

Fig. 3. Southern blots confirming integration of the malolactic cassette into the *URA3* locus of S92. The schematic representation illustrates the *Nsi*I restriction sites with vertical lines and the *URA3* probe (area with hatched boxes in panel on the right).

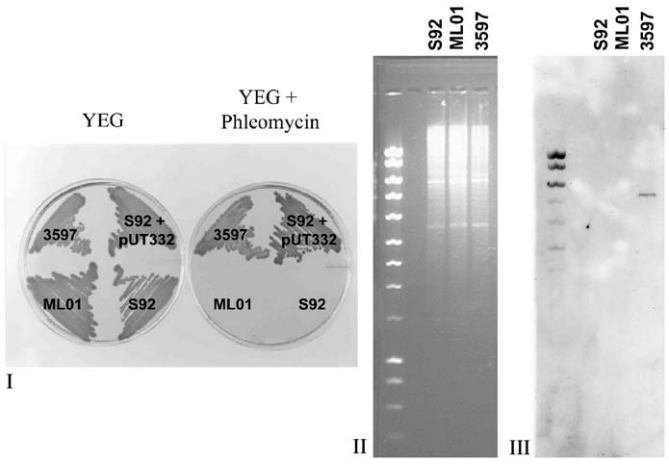

Fig. 4. The phleomycin resistance gene and pUT332 non-*Saccharomyces* vector sequences including the ampicillin resistance gene are absent in the genome of ML01. Growth of ML01, S92, S92 transformed with pUT332 and *S. cerevisiae* 3597*Δ*78-1 (integrated Tn5*ble* gene) yeast strains on YEG media with or without 100 μg/ml phleomycin (I). Agarose gel showing fractionated genomic DNA of S92, ML01 and *S. cerevisiae* 3597*Δ*78-1 digested with *Nco*I restriction enzyme (II). Southern blot analysis probing for the presence of the Tn5*ble* gene in the genomes of S92, ML01 and *S. cerevisiae* 3597*Δ*78-1 (III). Absence of pUT332 vector sequences including the ampicillin resistance gene were also confirmed by Southern analysis (data not shown).

the presence of these genes in the ML01 strain and correct integration into only the *URA3* locus. Chromoblots using *mae1*, *URA3* and *PGK1* promoter probes further verified that only one chromosome (V) contained the malolactic cassette (data not shown). Both S92 and ML01 form ascospores. Analysis of the ML01 spores revealed 2:2 segregation; cells from two spores were auxotrophic for uracil but positive for MLF while cells from the remaining two ascopores had the opposite phenotype.

After transformation ML01 was cultured successively for 7 days on a non-selective medium to eliminate pUT332, whose only purpose was to serve in the early steps of screening for transformants. A single colony was plated on non-selective media and on phleomycin containing media (Fig. 4). A Southern blot using a probe specific to the Tn5*Ble* genes of pUT332 revealed that this gene was absent in ML01 (Fig. 4). We also verified that ML01 contains neither the *bla* gene nor bacterial derived pUT322 sequences by Southern analysis (data not shown).

The sequence of the malolactic cassette integrated at the *URA3* locus as well as 35 bp upstream and 229 bp downstream of the genomic flanking sequences were verified by sequencing. Comparison to previously published sequences obtained from the *Saccharomyces* Genome Database for *URA3*, *PGK1* promoter and terminator, and the National Center for Biotechnology Information for *mae1* and *mleA* showed four nucleotide differences in the *URA3* flanking sequences and two nucleotide differences in one of the *PGK1* promoter sequences. These anomalies could be due to genetic polymorphisms or amplification errors during the construction of the cassette. Two changes were found in the coding sequence of the *mleA* gene. The first difference resulted in a change from aspartic acid to glutamic acid (amino acid position 538); both amino acids are acidic and did not affect the functionality of the malolactic enzyme. The second change (C–T, *mleA* nt position 996) resulted in a silent mutation (aspartic acid, aa position 332). The *mae1* sequence was identical to the published sequence.

In silico analysis of the integrated cassette revealed that four putative ORFs were created during construction of the malolactic cassette; these ORFs were entirely composed of *Saccharomyces* sequences (Fig. 5).

3.3. Effect of the integrated malolactic cassette on the transcriptome of ML01

Global gene expression patterns in ML01 and S92 were investigated using the Affymetrix GeneChip® Yeast Genome S98 Array. The transcriptome of ML01 and S92 was analyzed at 48 h, corresponding to log phase; at this stage the ML01 strain had consumed 1.73 ± 0.04 g/l of malate and produced 0.92 ± 0.04 g/l of lactate. The transcriptomes of ML01 and S92 were also compared at 144 h, corresponding to the stationary growth phase and completion of MLF. Transcription of 19 genes was affected $\geqslant$two-fold in ML01 after 48 h; 11 genes were expressed at higher levels and eight genes were expressed at lower levels (Table 1). In contrast, the parental strain S92 had consumed 0.33 ± 0.24 g/l of malate and only 0.03 ± 0.01 g/l of lactate was detected in the media. Equal amounts of glucose/fructose remained; 169.9 ± 4.0 and 166.7 ± 7.3 g/l for ML01 and S92, respectively.

At 144 h the transcription of six genes was affected $\geqslant$two-fold; three genes were expressed at higher levels and three genes were expressed at lower levels (Table 2). At this time point the ML01 had consumed all of the malate (4.47 ± 0.002 g/l) and produced 3.18 ± 0.12 g/l of lactate,

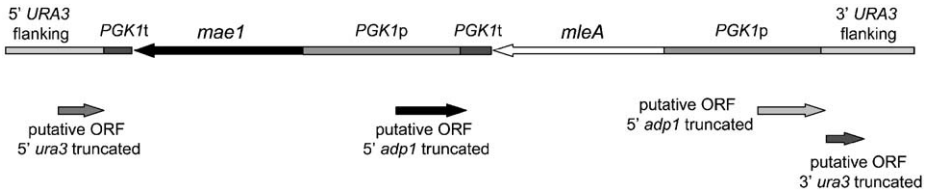

Fig. 5. A schematic representation of putative open reading frames (ORFs) of more than 100 codons generated during construction of the malolactic cassette; four putative ORFs composed of *S. cerevisiae* sequences, were created.

Table 1
Effect of the integrated malolactic cassette in the genome of S92 on global gene expression patterns in *S. cerevisiae* ML01 at 48 h (⩾2-fold change)

Gene symbol	Fold change	Biological process
Genes expressed at higher levels in ML01		
DIP5	2.81	Amino acid transport
YLR073C	2.79	Unknown
PCL1	2.69	Cell cycle
SUL1	2.47	Sulfate transport
OPT2	2.32	Oligopeptide transport
RPL7B	2.19	Protein biosynthesis
PHO84	2.18	Manganese ion transport and phosphate transport
RLP24	2.08	Ribosomal large subunit biogenesis
YOR315W	2.08	Unknown
MAK16	2.02	Ribosomal large subunit biogenesis and host–pathogen interaction
HAS1	2.01	rRNA processing
Genes expressed at lower levels in ML01		
SUE1	−5.13	Protein catabolism
PRR2	−3.44	MAPKKK cascade
CTT1	−3.29	Response to stress
PUT4	−3.13	Neutral amino acid transport
YGR243W	−2.41	Unknown
YRO2	−2.31	Unknown
JID1	−2.23	Unknown
YPC1	−2.18	Ceramide metabolism

Table 2
Effect of the integrated malolactic cassette in the genome of S92 on global gene expression patterns in *S. cerevisiae* ML01 at 144 h (⩾2-fold change)

Gene symbol	Fold change	Biological process
Genes expressed at higher levels in ML01		
ENA2	5.27	Sodium ion transport
RDH54	2.18	Double-strand break repair via break-induced replication, meiotic recombination, and heteroduplex formation
YOL048C	2.10	Unknown
Genes expressed at lower levels in ML01		
AQR1	−3.229	Monocarboxylic acid transport and drug transport
YML089C	−3.143	Unknown
YIL152W	−2.458	Unknown

whereas the control strain consumed 0.53 ± 0.08 g/l of malate; lactate was not detected in the medium. The fermentation rate was similar to that of the parental yeast S92 (data not shown) and the residual sugar concentrations (glucose/fructose) at this time point were 37.09 ± 2.79 and 36.5 ± 1.74 g/l for ML01 and S92, respectively. Ethanol concentrations at 144 h were $9.04 \pm 0.14\%$ (v/v) and $9.18 \pm 0.14\%$ (v/v) for ML01 and S92, respectively.

The introduction of the MLF cassette into *S. cerevisiae* S92 thus had a minimal effect on the transcription of the 5773 ORFs (*Saccharomyces* Genome Database; 1 June 2005) in the ML01 strain. Moreover, no metabolic pathway was affected by the presence of the malolactic expression cassette integrated into the genome of ML01.

3.4. Characterization of the proteome of ML01

The proteomes of ML01 and S92 were analyzed after 48 h using iTRAQ (DeSouza et al., 2005) and multi-dimensional liquid chromatography and tandem mass spectrometry. Proteins were extracted from the same samples used for microarray analysis. iTRAQ analysis identified 559 proteins (confidence level >94%) using a subset of the *S. cerevisiae* Celera Discovery System database (Ver 3.0, 01/12/2004). Only one protein, lanosterol 14-demethylase cytochrome P450 was shown to be different at a P-value <0.05 (default parameters) and across duplicate experiments. Lanosterol 14-demythylase cytochrome P450 had a weighted average ratio of 0.799 (using the S92 data as the denominator); Erg11p is involved in ergosterol biosynthesis. Furthermore, 199 of the 559 proteins detected are involved in many of the major metabolic pathways including carbohydrate and amino acid metabolism, and pyrimidine, purine, fatty acid, ergosterol and formate biosynthesis. Other than Erg11p, no difference in protein ratios for ML01 and S92 were detected for any other identified protein.

In order to search for unique ML01 proteins, a custom database was constructed which included the sequences for the mleAp, mae1p, and the three putative ORFs (5′ *ura3* truncated ORF, and the two 5′ *adp1* truncated ORFs). The fourth putative ORF could not be tested using this method since it contained only homologous *URA3* sequences. The mleAp was present in ML01 (confidence level >99%) but absent in S92. The membrane bound mae1p and the three putative proteins that might be encoded by the novel ORFs

created by cloning, were not identified at a confidence level as low as 50%.

4. Discussion

Moderate consumption of wine protects consumers against cardiovascular disease, dietary cancers, ischemic stroke, peripheral vascular disease, diabetes, hypertension, peptic ulcers, kidney stones and macular degeneration (Bisson et al., 2002), and these health benefits have likely increased the popularity of wine in recent years. However, wine also contains compounds such as biogenic amines that exert negative effects on the central nervous and vascular systems of consumers. Naturally occurring lactic acid bacteria present in wine as well as *O. oeni*, the bacterium used in commercial starter cultures, produce these bioamines by decarboxylating naturally occurring amino acids in grape must to their corresponding bioamines. After consumption, biogenic amines are usually metabolized by amine oxidases, such as monoamine (MAO) and diamine oxidase (DAO) and histamine *N*-methyltransferase, to physiologically less active products (Santos, 1996). However, in consumers without sufficient bioamine detoxifying enzymes, these amines can be absorbed into the bloodstream causing negative health affects.

The production of high-quality wines that are enjoyable, healthful and produced by environmentally sustainable production methods, has become important in a globalized world where there has been a paradigm shift from a production-driven to a market-driven wine industry (Pretorius and Hoj, 2005). Consumers have become sensitized and concerned about food scandals (e.g., mad cow disease and dioxin in chocolates) and are increasingly demanding safe food and beverages. Genetic engineering of wine yeast can be used to prevent the formation of bioamines in wine. It has also been used to minimize the formation of ethyl carbamate, a well-known carcinogen in wines (van Vuuren, unpublished). Moreover, powerful genomic, transcriptomic, proteomic and metabolomic techniques are now available to demonstrate that recombinant yeasts are substantially equivalent to the parental strains.

We constructed a genetically stable commercial wine yeast strain, ML01, by expressing the *S. pombe* malate transporter gene (*mae1*) and the *O. œni* malolactic enzyme gene (*mleA*) under control of the *S. cerevisiae PGK1* promoter and terminator signals in a popular industrial wine yeast strain of *S. cerevisiae*. It is important to note that all these genetic donors are wine microorganisms (Garvie, 1967; Lodder, 1970; Barnett et al., 1990). The malolactic cassette integrated into the *URA3* locus of ML01 contains no vector sequences or antibiotic-resistance marker genes (Fig. 1). DNA sequencing confirmed that the integration site contains no DNA sequences other than those present in the isolated malolactic cassette. Results obtained from the hybridization of genomic DNA with various probes indicate that the malolactic cassette is correctly integrated into a *URA3* locus (Fig. 3). We found that ML01 was able to fully degrade 5.5 g/l of malate within 9 days at 13 °C; growth or ethanol production was not affected (Fig. 2). In contrast, *O. oeni* was unable to complete the MLF and after 4 months 2.61 g/l of malate remained in the wine. The malolactic yeast fermented the must to dryness and only 1.23 ± 0.05 g/l of sugars remained in the wine after fermentation. The residual sugar in wine fermented with the parental strain was 0.47 ± 0.02 g/l. Wine is considered to be dry when it contains less than 2 g/l of sugars (Bisson, 1999).

DNA microarray and iTRAQ analyses of the transcriptome and proteome of the yeast indicated that the introduction of the MLF cassette had little effect on global gene expression patterns (Tables 1 and 2) and protein levels. Based on these results as well as genetic and phenotypic characterization, we therefore conclude that the ML01 is substantially equivalent to the parental strain S92.

ML01 enjoys GRAS status with the FDA and is the first metabolically engineered yeast to be commercialized by the wine industry. *S. cerevisiae* ML01 conducts an efficient MLF that will solve the important issue of cellar capacity and prevent oxidation and microbial spoilage of wines that result in financial losses to wineries. Wines produced with ML01 should be free of toxic bioamines, providing great relief to wine lovers who were previously unable to consume many wines that underwent the bacterial malolactic fermentation.

Acknowledgments

This work was funded by Grants from the NSERC (203933) and Lesaffre to H.J.J. van Vuuren. We thank Russ Morris of the University of British Columbia Media Group for preparing the artwork.

References

Ansanay, V., Dequin, S., Blondin, B., Barre, P., 1993. Cloning, sequence and expression of the gene encoding the malolactic enzyme from *Lactococcus lactis*. FEBS Lett. 332, 74–80.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1995. Short Protocols in Molecular Biology. Wiley, Canada.

Barnett, J.A., Payne, R.W., Yarrow, D., 1990. Yeasts: Characteristics and Identification. Cambridge University Press, New York.

Beelman, R.B., Gallander, J.F., 1979. Wine deacidification. Adv. Food Res. 25, 1–53.

Bisson, L.F., 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. 50, 107–119.

Bisson, L.F., Waterhouse, A.L., Ebeler, S.E., Walker, M.A., Lapsley, J.T., 2002. The present and future of the international wine industry. Nature 418, 696–699.

Caspritz, G., Radler, F., 1983. Malolactic enzyme of *Lactobacillus plantarum*—purification, properties, and distribution among bacteria. J. Biol. Chem. 258, 4907–4910.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H., Fleet, G.H., 1985. Practical implications of malolactic fermentation—a review. Am. J. Enol. Vitic. 36, 290–301.

Denayrolles, M., Aigle, M., Lonvaud-Funel, A., 1995. Functional expression in *Saccharomyces cerevisiae* of the *Lactococcus lactis mleS* gene encoding the malolactic enzyme. FEMS Microbiol. Lett. 125, 37–43.

DeSouza, L., Diehl, G., Rodrigues, M.J., Guo, J., Romaschin, A.D., Colgan, T.J., Siu, K.W., 2005. Search for cancer markers from endometrial tissues using differentially labeled tags iTRAQ and cICAT with multidimensional liquid chromatography and tandem mass spectrometry. J. Proteome Res. 4, 377–386.

Dicks, L.M., Dellaglio, F., Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. Int. J. Syst. Bacteriol. 45, 395–397.

Erasmus, D.J., van der Merwe, G.K., van Vuuren, H.J.J., 2003. Genome-wide expression analyses: metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. FEMS Yeast Res. 3, 375–399.

Fuck, E., Stärk, G., Radler, F., 1973. Äpfelsäurestoff-wechsel bei *Saccharomyces II*. Anreicherung und eigenschaften eines malaten-zymes. Arch. Mikrobiol. 89, 223–231.

Gallander, J.F., 1977. Deacidification of eastern table wines with *Schizosaccharomyces pombe*. Am. J. Enol. Vitic. 28, 65–68.

Gao, C., Fleet, G.H., 1995. Degradation of malic and tartaric acids by high density cell suspensions of wine yeasts. Food Microbiol. 12, 65–71.

Garvie, E.I., 1967. *Leuconostoc oenos* sp. nov. J. Gen. Microbiol. 48, 431–438.

Gatignol, A., Baron, M., Tiraby, G., 1987. Phleomycin resistance encoded by the *ble* gene from transposon Tn 5 as a dominant selectable marker in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 207, 342–348.

Grobler, J., Bauer, F., Subden, R.E., van Vuuren, H.J.J., 1995. The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C4 dicarboxylic acids. Yeast 11, 1485–1491.

Henick-Kling, T., 1995. Control of malolactic fermentation in wine: energetics, flavor modification and methods of starter culture preparation. J. Appl. Bacteriol. 79, S29–S37.

Husnik, J.I., 2001. Genetic construction of malolactic wine yeasts. M.Sc. Thesis, Department of Food Science, University of Guelph, Guelph, p. 138.

Labarre, C., Guzzo, J., Cavin, J.F., Divies, C., 1996. Cloning and characterization of the genes encoding the malolactic enzyme and the malate permease of *Leuconostoc oenos*. Appl. Environ. Microbiol. 62, 1274–1282.

Lautensach, A., Subden, R.E., 1984. Cloning of malic acid assimilating activity from *Leuconostoc oenos* in *E. coli*. Microbios 39, 29–39.

Lehtonen, P., 1996. Determination of amines and amino acids in wine: a review. Am. J. Enol. Vitic. 47, 127–133.

Lodder, J., 1970. The Yeasts: A Taxonomic Study. North-Holland, Amsterdam.

Lonvaud-Funel, A., 1995. Microbiology of the malolactic fermentation—molecular aspects. FEMS Microbiol. Lett. 126, 209–214.

Lonvaud-Funel, A., 2001. Biogenic amines in wines: role of lactic acid bacteria. FEMS Microbiol. Lett. 199, 9–13.

Maicas, S., 2001. The use of alternative technologies to develop malolactic fermentation in wine. Appl. Microbiol. Biotechnol. 56, 35–39.

Medina, M.A., Quesada, A.R., de Castro, I.N., Sanchez-Jimenez, F., 1999. Histamine, polyamines and cancer. Biochem. Pharmacol. 57, 1341–1344.

Naouri, P., Chagnaud, P., Arnaud, A., Galzy, P., 1990. Purification and properties of a malolactic enzyme from *Leuconostoc oenos* ATCC 23278. J. Basic Microbiol. 30, 577–585.

Pretorius, I.S., Hoj, P.B., 2005. Grape and wine biotechnology: challenges, opportunities and potential benefits. Aust. J. Grape Wine Res. 11, 83–108.

Pryme, I.F., Bardocz, S., 2001. Anti-cancer therapy: diversion of polyamines in the gut. Eur. J. Gastroenterol. Hepatol. 13, 1041–1046.

Redzepovic, S., Orlic, S., Majdak, A., Kozina, B., Volschenk, H., Viljoen-Bloom, M., 2003. Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. Int. J. Food Microbiol. 83, 49–61.

Santos, M.H.S., 1996. Biogenic amines: their importance in foods. Int. J. Food Microbiol. 29, 213–231.

Shalaby, A.R., 1996. Significance of biogenic amines to food safety and human health. Food Res. Int. 29, 675–690.

Soufleros, E., Barrios, M.L., Bertrand, A., 1998. Correlation between the content of biogenic amines and other wine compounds. Am. J. Enol. Vitic. 49, 266–278.

Spettoli, P., Nuti, M.P., Zamorani, A., 1984. Properties of malolactic activity purified from *Leuconostoc oenos* ML34 by affinity chromatography. Appl. Environ. Microbiol. 48, 900–901.

Straub, B.W., Kicherer, M., Schilcher, S.M., Hammes, W.P., 1995. The formation of biogenic amines by fermentation organisms. Z. Lebensm. Unters. Forsch. 201, 79–82.

Subden, R.E., Lin, W.L., Lautensach, A., Meiering, A.G., 1982. An L-lactic acid dehydrogenase based method for detecting microbial colonies performing a malo-lactic fermentation. Can. J. Microbiol. 28, 883–886.

ten Brink, B., Damink, C., Joosten, H.M.L.J., Tveld, J.H.J.H.I., 1990. Occurrence and formation of biologically active amines in foods. Int. J. Food Microbiol. 11, 73–84.

van Vuuren, H.J.J., Dicks, L.M.T., 1993. *Leuconostoc oenos*: a review. Am. J. Enol. Vitic. 44, 99–112.

Volschenk, H., Viljoen, M., Grobler, J., Bauer, F., Lonvaud Funel, A., Denayrolles, M., Subden, R.E., van Vuuren, H.J.J., 1997a. Malolactic fermentation in grape musts by a genetically engineered strain of *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. 48, 193–197.

Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R.E., Young, R.A., Lonvaud, A., Denayrolles, M., van Vuuren, H.J.J., 1997b. Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nat. Biotechnol. 15, 253–257.

Wallace, H.M., Caslake, R., 2001. Polyamines and colon cancer. Eur. J. Gastroenterol. Hepatol. 13, 1033–1039.

Wantle, F., Gotz, M., Jarisch, R., 1994. The red wine provocation test: intolerance to histamine as a model for food intolerance. Allergy Proc. 15, 27–32.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H., Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine—a review. Am. J. Enol. Vitic. 36, 302–313.

Williams, S., Hodges, R.A., Strike, T.L., Snow, R., Kunkee, R.E., 1984. Cloning the gene for the malolactic fermentation of wine from *Lactobacillus delbruekii* in *Escherichia coli* and yeasts. Appl. Microbiol. Biotechnol. 47, 288–293.

Zee, J.A., Simard, R.E., Lheureux, L., Tremblay, J., 1983. Biogenic-amines in wines. Am. J. Enol. Vitic. 34, 6–9.