METABOLIC ENGINEERING AND CHARACTERISATION OF THE MALOLACTIC WINE YEAST ML01

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ABSTRACT

Malolactic fermentation (MLF) is essential for deacidification of high acid grape must and the production of well-balanced wines. The bacterial MLF is unreliable and stuck MLFs often lead to spoilage of wines and the production of biogenic amines. A genetically stable industrial strain of *Saccharomyces cerevisiae* was constructed by integrating a linear cassette containing the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus œni* malolactic gene (*mleA*) under control of the *S. cerevisiae PGK1* promoter and terminator sequences into the *URA3* locus of an industrial wine yeast strain. The malolactic yeast strain, ML01, completes the MLF during the alcoholic fermentation in a variety of musts including a high acid Chardonnay must containing 9.2 g/L of malate. ML01 cannot appreciably decarboxylate L-malic acid to L-lactic acid when present at levels below 1% of the total inoculum. ML01 contains no antibiotic resistance marker genes or vector DNA sequences. Global gene expression patterns and analysis of the proteome showed that no metabolic pathway was affected by the introduction of the malolactic cassette. The presence of the malolactic cassette in the genome does not affect growth, ethanol production, fermentation kinetics or metabolism of ML01. Wines produced by the ML01 yeast have lower volatile acidity and improved color properties compared to wines produced with the parental yeast and a bacterial MLF. GC/MS analysis of volatile compounds revealed that wine produced by ML01 did not contain any compounds that were not detected in wine produced with the parental strain S92 or with S92 and malolactic bacteria. Moreover, ML01 reduces the processing time after alcoholic fermentation and produces wine that is judged highest in overall quality by trained tasters.

Analyses of the phenotype, DNA, RNA, and proteins demonstrate that the recombinant yeast ML01 is substantially equivalent to the parental strain S92. ML01 has been approved for use in Canada and has 'Generally Regarded As Safe' status with the US FDA. It is the first metabolically engineered yeast to be commercialised by the wine industry and is currently available in Canada, the USA and Moldova.

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LIST OF ABBREVIATIONS

aa	Amino acid
ADY	Active dry yeast
amu	Atomic mass unit
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
bp	Base pair
BC	British Columbia
BCE	Before the Common Era
°C	Degree Celsius
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CIELAB	Color model of the International Commission on Illumination
cm	Centimetre
Co.	Company
cRNA	Ribonucleic acid derived from cDNA
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Ethyl carbamate
EDTA	Ethylenediamine tetraacetic acid
FDA	Food and Drug Administraion
g	gram
g	Unit of acceleration (9.80665 m/s^2)

GC	Gas chromatography
GC/MS	Gas chromatography-mass spectrometry
GM	Genetically modified
GMO	Genetically modified organism
GO	Gene ontology
GRAS	Generally regarded as safe
hL	hectolitre
ID	Inner diameter
kbp	kilo base pair
kg	kilogram
L	Litre
LAB	Lactic acid bacteria
LC MS/MS	Liquid chromatography-tandem mass spectrometry
log	Logarithm
LSD	Least square difference
m	metre
M	Molarity
µL	microlitre
µm	micrometre
μ_{max}	Maximum specific growth rate
mg	milligram
min	minute
mL	millilitre

MLF	Malolactic fermentation
mM	millimolar
MMTS	Methyl methanethiosulphonate
MS	Mass spectrometer
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
ng	nanogram
nt	nucleotide
OD	Optical density
ORF	Open reading frame
PARC	Pacific Agri-Food Research Centre
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDM	Prise de Mousse
PEST	Protein region that consists of proline, glutamic acid, serine, threonine and to a lesser extent aspartic acid
PFGE	Pulsed Field Gel Electrophoresis
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase - polymerase chain reaction
s	second
SCX	Strong cation exchange
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGD	*Saccharomyces* genome database
sp.	Species
SPME	Solid phase microextraction
TA	Titratable acidity
TBE	Buffer consisting of Tris base, boric acid, EDTA and water
TCA	Tricarboxylic acid
TE	Buffer consisting of Tris base, EDTA and water
TOFMS	Time-of-flight mass spectrometry
UK	United Kingdom
USA	United States of America
V	volt
v/v	volume per volume
YANC	Yeast assimilable nitrogen concentration
YEG	Medium consisting of yeast extract and dextrose
YPD	Medium consisting of yeast extract, peptone, and dextrose

PREFACE

The following dissertation is prepared in the traditional format as described by the Faculty of Graduate Studies at the University of Brtitsh Columbia. It encompasses two different fields of study, oenology and molecular biology. Hence, some of the terms used by oenologists may not be familiar to molecular biologists and vice versa. In most cases the meaning of certain terms will be evident to the reader. The remaining terms, such as "must" may be confusing to non-oenologists and therefore I have provided a short list of definitions of such oenological terms.

Brix	A measurement of the dissolved solids (primarily sugars) in fruit juices at 20°C.
Lees	The sediment from an alcoholic fermentation (primarily composed of yeast).
Must	The unfermented or fermenting juice of grapes (or other fruits).
Racking	The process of removing wine off the lees to allow clarification and aid in stabilisation.

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† - deceased

1 INTRODUCTION

Approximately 27 billion litres of wine are produced annually worldwide (Pretorius and Bauer, 2002); the quality of wine depends on the grapes used in its production, the microorganisms involved in the alcoholic and malolactic fermentations, and the skills of the winemaker. The malolactic fermentation (MLF) is an indispensable tool for the deacidification of high acid grape must; it is also one of the most difficult steps to control in the winemaking process. *Oenococcus œni* and other lactic acid bacteria (LAB) deacidify wine by converting L-malic acid to L-lactic acid and CO_2, resulting in a decrease in titratable acidity and an increase in wine pH (Bousbouras and Kunkee, 1971). The decarboxylation of malate to lactate is catalyzed by the malolactic enzyme (L-malate:NAD^+ carboxy lyase) without the production of any free intermediates (Caspritz and Radler, 1983; Naouri et al., 1990; Spettoli et al., 1984). The reduction of acidity in wine is particularly important in cooler climates where L-malic acid can be present at concentrations up to 9 g/L.

In spite of the use of commercially available bacterial malolactic starter cultures, stuck and sluggish MLFs are common in wines because growth of LAB can be inhibited by many factors including sulphur dioxide, low temperature, pH, ethanol, low nutrient content of wine, the presence of fatty acids and interactions with other microorganisms (see reviews Davis et al., 1985; Henick-Kling, 1995; van Vuuren and Dicks, 1993; Wibowo et al., 1985). For the entire duration of the bacterial MLF, wine is at risk from microbial spoilage and oxidation since the addition of sulphur dioxide must be delayed and the temperature is often elevated in order to achieve a satisfactory MLF. Moreover, LAB can produce toxic substances such as biogenic amines and precursors of ethyl

carbamate that are of concern to consumers (Liu, 2002; Lonvaud-Funel, 2001; Marcobal et al., 2006).

This research was undertaken to construct and characterise an industrial wine yeast strain that can avoid the negative aspects of the bacterial MLF by completing the MLF during the alcoholic fermentation. The resulting wine can be immediately sulphited and processed; thereby reducing the effects of chemical oxidation and the probability of microbial spoilage and the production of off-flavours. The construction of a malolactic yeast strain that can complete the MLF during the alcoholic fermentation will be a significant addition to the repertoire of tools that winemakers can access to produce high-quality wines that should be free of toxic biogenic amines such as histamine and tyramine.

In this chapter the yeast *Saccharomyces cerevisiae* will be briefly introduced, followed by an overview of winemaking, a detailed description of the MLF and the history of the genetic engineering of *S. cerevisiae* to perform the MLF. Finally, a review of all the genetically engineered *S. cerevisiae* strains applicable to the wine industry will be presented.

1.1 *Saccharomyces cerevisiae*

The budding yeast *S. cerevisiae* is considered by many as a domesticated unicellular organism. For centuries, *S. cerevisiae* has been used by humans in the fermentation industries of baking, brewing, distilling and winemaking. In 1996, the laboratory strain of *S. cerevisiae* S288C, was the first eukaryotic organism to have its entire genome sequenced (Goffeau et al., 1996). S288C is largely derived from a strain,

EM93, that was originally isolated in 1938 from rotting figs (Mortimer and Johnston, 1986). The yeast genome contains approximately 5800 genes (~70% characterised as of September, 2006) (http://www.yeastgenome.org/) containing relatively few introns and little repetitive DNA. Haploid laboratory strains have approximately 12-13 Mb of DNA confined to 16 linear chromosomes (Olson, 1991).

Saccharomyces cerevisiae as haploid or diploid cells can reproduce vegetatively through budding. Haploids can be either "a" or "a" mating types; mating of opposite types will yield a diploid cell (Herskowitz, 1988). Diploid cells can undergo sporulation (meiosis), induced by growth in poor carbon sources or nitrogen starvation, to produce four haploid spores formed within an ascus (Herskowitz, 1988). The recovery of the meiotic products allows advanced genetic analyses to be performed that are not possible in most eukaryotic organisms. *S. cerevisiae* can also be heterothallic or homothallic. Heterothallic strains have a fixed mating type and homothallic strains are able to switch mating types (Herskowitz, 1988).

Most laboratory strains of *S. cerevisiae* are haploid or diploid, heterothallic, sporulate efficiently when diploid and contain multiple nutritional auxotrophic mutations. In contrast, industrial strains are predominantly diploid or anueuploid and occasionally polyploid, homothallic, prototrophic and have low sporulation efficiency combined with poor spore viability (Codon et al., 1998; Snow, 1983). Industrial strains are grown under commercial manufacturing conditions to be used as inocula in their respective industries, such as baking and winemaking. Wild isolates are mostly homothallic diploids that are often heterozygous (Bisson, 2004).

Although many natural isolates of *S. cerevisiae* have been obtained from grapes, the actual origin of wine strains is the subject of some controversy. Some researchers claim that the principal source of wine yeast is the vineyard (Mortimer and Polsinelli, 1999; Torok et al., 1996); others believe that modern wine strains are the result of an association with artificial environments such as wineries (Martini, 1993; Vaughan-Martini and Martini, 1995). The controversy is due to the fact that it is relatively difficult to find *S. cerevisiae* on the surface of healthy, undamaged grapes (Martini, 1993), although it can be isolated in berries damaged by birds or insects which represent about 1 in 100 grapes (Landry et al., 2006). The population size of *S. cerevisiae* within any damaged fruit can range from 10^4 to 10^5 cells (Mortimer and Polsinelli, 1999). However, a recent investigation on the survival and development of an inoculated *S. cerevisiae* wine strain showed that this particular wine strain failed to colonise and was unable to out compete the epiphytic yeast present on damaged and undamaged grapes (Comitini and Ciani, 2006). Other environments that *S. cerevisiae* has been isolated from include soil associated with oak trees (Sniegowski et al., 2002), the Danube River (Slavikova and Vadkertiova, 1997) and occasionally from immunocompromised humans (Malgoire et al., 2005; Sethi and Mandell, 1988). Evidence also exists that insects and birds are important vectors for the dispersal of yeasts (Mortimer and Polsinelli, 1999; Phaff and Starmer, 1987).

1.2 Overview of winemaking

Several lines of archaeological evidence suggest that wine was made as early as the seventh century BCE (Robinson, 1994). Molecular evidence for fermentation by *S.*

cerevisiae in wine has been obtained from wine jars discovered in Egypt dating back to 3150 BCE (Cavalieri et al., 2003). Alcoholic fermentation represents the oldest form of biotechnological application of microorganisms and from the early days of winemaking to the present, the basic principles have changed very little. Following the harvest of grapes (which can be performed manually or by machine), the berries are delivered to the winery where they are crushed and the stems removed. At this point, modern winemakers generally add pectolytic enzymes to increase the volume of free juice and to assist with clarification (Moreno-Arribas and Polo, 2005). After crushing, the grape must can be used directly for fermentation (for red wines) or pressed to separate the juice from the skins (for white wines).

The must may be fermented with selected wine yeast strains or left to be fermented by the resident microflora found on the surfaces of grapes and in the winery. The inoculation of commercial yeast starter strains into must was started in the 1960s and by the mid-1980s became common practice in most of the world's wine regions (Moreno-Arribas and Polo, 2005; Reed and Nagodawithana, 1988). Approximately 150 different strains are now commercially available; these strains produce high quality wines with reproducible characteristics including a complete and rapid fermentation (Schuller and Casal, 2005). In uninoculated "spontaneous" fermentations there is a sequential growth pattern of indigenous yeasts. Yeast of the genera *Kloeckera*, *Hanseniaspora* and *Candida* generally predominate in the early stages, followed by *Metschnikowia* and *Pichia* when ethanol concentrations reach 3-4% (Fleet and Heard, 1993). The final stages are ultimately dominated by the ethanol-tolerant *S. cerevisiae*, which rapidly ferments the present sugars (18-40%, depending on style and geography of wine being

produced) to produce ethanol and carbon dioxide (Querol et al., 2003). The high levels of ethanol, the low pH, osmotic stress and anaerobic conditions essentially eliminate the other less tolerant microorganisms.

During the fermentation of red wines, the skins and other insoluble material will form a cap on the surface that must be submerged or stirred in order to extract the skin cell components (Bisson, 2004). The cap can be submerged by either "punching down" or must from the bottom of the tank can be "pumped over" the cap. Red wines are also fermented at higher temperatures, 18-30 °C, and whites are commonly fermented at 12-18 °C. After the alcoholic fermentation, certain styles of wines are immediately treated with SO_2 to prevent chemical oxidation and inhibit microbial activity. Most red wines and certain white wines are not sulphited until after they have undergone a MLF. After the MLF, the wine is clarified by racking, fining, centrifuging or filtering (Boulton, 1996). The winemaker may also choose to delay the clarification in order to extract desired flavours from the lees (yeast sediment) (Ough, 1992). During wine processing and storage, the SO_2 levels are adjusted to prevent oxidation of wine and proliferation of spoilage microorganisms; protective SO_2 levels are then monitored and maintained until bottling. After clarification, wines can be stored, at low temperatures with no air contact, in inert containers or oak barrels. Prior to the final step in the winemaking process, bottling, wines may be blended and tested for physical instabilities, such as the potential to form precipitates (Boulton, 1996).

1.3 The malolactic fermentation

The MLF refers to the biological conversion of the dicarboxylic L-malic acid, into the monocarboxylic acid L-lactic acid, and CO_2. In 1858, Pasteur's "*Memoire sur la fermentation lactique*" revealed that the MLF was caused by living organisms; Muller-Thurgau, in 1891, showed that the organisms were bacteria (Bartowsky, 2005; Paul, 1996). In 1901, the equation for the conversion of L-malic acid to L-lactic acid and CO_2 was independently revealed by Moslinger and Seifert (Bartowsky, 2005). In 1928, using Moslinger and Seifert's equation, Ferré showed that the bacteria transformed 1.0 g of malic acid into 0.671 g of lactic acid and 0.329 g of CO_2 (Paul, 1996). Since this time, MLF has continued to attract considerable attention from researchers around the globe.

The bacterial MLF is an important secondary fermentation that typically occurs after the alcoholic fermentation has been completed. The MLF deacidifies wine and is favoured in cool-climate regions (such as northern Europe, eastern United States, New Zealand and Canada) where the grapes at harvest tend to have naturally higher acid at harvest. Conversely, in warmer regions of the world, grapes usually have lower levels of acidity and the MLF is less desired (Beelman and Gallander, 1979). The difference in total fixed acids between cool and warm climates is partly the result of respiratory catabolism of L-malic acid by the grape that is enhanced by warmer temperature during the ripening (Jackson and Schuster, 1987).

1.3.1 Wine acidity

Grape juice contains a variety of organic acids, the dominant acids being tartaric, malic and citric acids. Tartaric and malic acids represent, on average, 90% of the

titratable acids prior to the fermentation (Boulton, 1996; Radler, 1993; Ribereau-Gayon et al., 2000a). Near maturity in warmer climates, tartaric acid is the predominant acid in grapes, accounting for 2.0-8.0 g/L, with malic acid accounting for 10-40% of the total acid fraction (Boulton, 1996; Ough, 1992). In cooler climates or in grapes picked at early maturity, the amounts of malic acid can exceed those of tartaric acid and may constitute as much as 60% of the organic acid fraction (Boulton, 1996; Ough, 1992). Malic acid is usually present in grapes at concentrations ranging from 2.0-6.0 g/L (Boulton, 1996), but can reach 9 g/L in cool viticultural areas. L-Malic acid is an essential compound, with important cellular functions in metabolic pathways such as the tricarboxylic acid (TCA) cycle, glyoxylate cycle, and malate-aspartate shuttle; in grapes it is synthesized from glucose via pyruvate (Mathews and van Holde, 1990). Smaller amounts of citric acid and trace amounts of other acids of the citric acid cycle are also present in the juice.

1.3.2 *Oenococcus œni* and other lactic acid bacteria of wine

Lactic acid bacteria (LAB) responsible for the MLF in wine are from the bacterial genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Dicks et al., 1995; London, 1976; van Vuuren and Dicks, 1993). The LAB can be isolated from the skins of healthy grape berries in low concentrations ($<10^3$ colony forming units/g) (Fleet, 1998; Lonvaud-Funel, 1999; Wibowo et al., 1985) and can also be found in the winery (Boulton, 1996). In grape must the LAB population varies from 10^2 cfu/mL to 10^4 cfu/mL, however, during alcoholic fermentation the LAB population will decrease to a few cells per mL (Lonvaud-Funel, 1999; van Vuuren and Dicks, 1993). Moreover, the original diversity of the species diminishes and *O. œni* typically remains as the

predominant LAB at the end of alcoholic fermentation (Lonvaud-Funel, 1999; Tracey and Britz, 1989; van Vuuren and Dicks, 1993).

In 1967, *O. œni* was first characterised and classified as *Leuconostoc oenos*, to differentiate it from other less acid-tolerant *Leuconostoc* species (Garvie, 1967). *Leuconostoc oenos* was later re-classified into the new genus, *Oenococcus*, of which *O. œni* is the only species (Dicks et al., 1995). The genomes of two *O. œni* strains, IOEB8413 and PSU1, have recently been sequenced (Klaenhammer et al., 2002; Mills et al., 2005), although neither of the sequences are currently accessible by the public. The reported size of the *O. œni* genomes are 1.78 and 1.75 Mb for PSU1 and IOEB8413, respectively (Mills et al., 2005). The use of multilocus sequence typing and phylogenetic analyses revealed that *O. œni* seems to have a high level of genetic heterogeneity and may also have a panmictic (highly variable) population structure (de las Rivas et al., 2004). A number of *O. œni* genes have also been studied at the molecular level including genes associated with the MLF (Denayrolles et al., 1994; Labarre et al., 1996a; Labarre et al., 1996b; Volschenk et al., 1997b), genes involved with diacetyl metabolism (Garmyn et al., 1996), genes associated with amino acid metabolism (Coton et al., 1998a; Divol et al., 2003; Marcobal et al., 2004; Tonon et al., 2001), and stress-related genes (Bourdineaud et al., 2003; Bourdineaud et al., 2004; Fortier et al., 2003; Jobin et al., 1999a; Jobin et al., 1999b; Jobin et al., 1997). A putative glucosyltransferase gene, *dps*, was also detected in *O. œni* isolated from a highly viscous wine (Walling et al., 2005).

The MLF begins during the early stages of *O. œni* growth (or the growth of other LAB) and a significant rate of malate degradation is usually not observed until cell densities reach a concentration of 10^6 cfu/mL or more (Costello et al., 1983; Lafon-

Lafourcade et al., 1983; Wibowo et al., 1985). LAB cannot grow with L-malic acid as a sole carbon source and they rely on residual sugars not fermented by the yeast (Liu et al., 1995a) or amino acids such as arginine to allow cell growth (Liu and Pilone, 1998). Although, *O. œni* cannot grow on L-malic acid as a sole carbon source, the malolactic conversion confers an energetic advantage to the cell. The MLF provides cells with additional metabolic energy from the increased intracellular pH which produces an increased proton motive force (? p) across the cytoplasmic membrane (Cox and Henick-Kling, 1989; Cox and Henick-Kling, 1995). The proton gradient is created across the cell membrane after one molecule of lactate (after malate decarboxylation) leaves the cell accompanied by one H^+ (Cox and Henick-Kling, 1989). The resulting increase in the proton gradient can be used to drive transport processes and to produce ATP via the membrane-bound ATPase (Cox and Henick-Kling, 1989; Henick-Kling, 1995; Olsen et al., 1991). At a pH of <4.5 and with limited amounts of sugar (as prevalent in wine conditions), the additional ATP can allow for increased growth yields (Garcia et al., 1992; Henick-Kling, 1993; Renault et al., 1988). This theory helps to explain a stimulatory effect observed during the early stages of growth (Kunkee, 1991). In addition to indirect generation of ATP, the MLF may allow *O. œni* to take up nutrients by the chemiosmotic mechanism and help to maintain a suitable pH for enzymatic activity and cell growth (Cox and Henick-Kling, 1989).

Once all of the malic acid is decarboxylated, wine is sulphited and further activity from *O. œni* is inhibited (as well as activity from other SO_2-sensitive bacteria and yeast). After the MLF, the risk of further microbial activity in the wine is considerably reduced

since the wine is essentially depleted of essential nutrients and fermentable substrates (Davis et al., 1988).

1.3.3 Biochemistry of the malolactic fermentation

Decarboxylation of L-malic acid to L-lactic acid is catalyzed by L-malate:NAD^+ carboxy lyase without the production of any free intermediates (Caspritz and Radler, 1983; Naouri et al., 1990; Spettoli et al., 1984). This enzyme has been termed the malolactic enzyme and it requires the cofactors Mn^{2+} and NAD^+ (Caspritz and Radler, 1983; Naouri et al., 1990; Spettoli et al., 1984). The malolactic enzyme was first purified from *Lactobacillus plantarum* (Lonvaud, 1975; Schutz and Radler, 1973). Characteristics of the malolactic enzyme from the various malolactic bacteria (*L. plantarum*, *L. casei*, *L. murinas, L. mesenteroides,* and *O. œni*) show significant differences in regards to the enzyme affinity constants for L-malic acid, NAD^+ and Mn^{2+}, but they are quite similar in molecular weight (ranging form 60-70 kDa) (Battermann and Radler, 1990; Caspritz and Radler, 1983; Lonvaud-Funel and Desaad, 1982; Naouri et al., 1990; Schutz and Radler, 1973; Spettoli et al., 1984; Strasser de Saad et al., 1984). The malolactic enzyme is hypothesized to be active as a homodimer or homotetramer (Battermann and Radler, 1990; Labarre et al., 1996b) and it is inducible by L-malic acid in the presence of fermentable sugars (Nathan, 1961; Renault et al., 1989).

1.3.4 The effect of the malolactic fermentation on wine and the winemaking process

The main effect of the MLF on wine is the reduction of the total acidity and the increase in pH as a result of the decarboxylation of L-malate to L-lactate and CO_2. The

consequences of an elevated pH can leave some wines susceptible to the growth of less fastidious spoilage microorganisms whose growth is normally inhibited by low pH (Davis et al., 1988). Bacteriological stability in low-acid wines can be accomplished by the addition of acidulating agents (tartaric acid, citric acid, lactic acid or D(+)-malic acid) after the MLF (Boulton, 1996; Rankine, 1977). Another disadvantage of pH increase after the MLF is the relative loss of colour (up to 30%) in red wine (Kunkee, 1967). As wine pH increases, anthocyanin pigments undergo structural changes to uncoloured forms (Jackson, 1994; Lonvaud-Funel, 1999; Ribereau-Gayon et al., 2000b).

The most notable sensory difference after a MLF is the disappearance of the taste of malic acid ('tart') and the appearance of lactic acid ('soft'). The next most evident sensory characteristic of a MLF is the production of diacetyl, an aroma compound with a 'buttery' or 'butterscotch' flavour/aroma (Martineau et al., 1995a; Martineau et al., 1995b). Diacetyl is produced from the oxidative decarboxylation of a-acetolactate, an unstable intermediary compound formed during the reductive decarboxylation of pyruvic acid to 2,3-butanediol (Cogan, 1987; Ramos et al., 1995). Pyruvic acid is derived from the metabolism of citrate and sugar. Yeast cells are also able to synthesise diacetyl as an intermediary compound but it is generally further reduced to acetoin and 2,3-butanediol during the alcoholic fermentation (Martineau and Henick-Kling, 1995). *O. œni* and other LAB in certain conditions are also capable of further metabolising diacetyl to acetoin and 2,3 butanediol, thereby reducing the buttery sensory characteristics (Ramos et al., 1995). Diacetyl in wine usually tends to be found at a concentration of 5-10 mg/l (Lonvaud-Funel, 1999).

The impact of the MLF on the organoleptic qualities of wine beyond the primary role of deacidification and the production of diacetyl is not completely defined. Numerous studies have concluded that the MLF affects wine aroma, however, few compounds or mechanisms have been implicated in those changes. The MLF has been implicated in an increase of the fruity aroma of wine (probably due to the production of esters by wine LAB), a decrease in vegetative/grassy aromas (likely due to the catabolism of acetaldehyde) and it may improve the body, mouthfeel and after taste of wine (potentially through the production of polyols and polysaccharides) (Henick-Kling, 1993; Liu, 2002; Liu and Pilone, 2000). These observations suggest benefits to the winemaking process beyond the deacidification that results from the MLF.

However, not all sensory changes attributed to the MLF can be considered advantageous (Davis et al., 1985). A substantial increase in acetic acid (the main component of volatile acidity) can accompany a MLF due to the metabolism of sugars by LAB, especially if the yeast did not completely ferment the sugars or the LAB started to multiply prior to the completion of the alcoholic fermentation (Wibowo et al., 1985). *O. œni* and other heterofermentative LAB (*Leuconostoc* sp. and certain *Lactobacillus* sp.) ferment hexoses via the phosphoketolase pathway to lactate, ethanol, CO_2 and ATP (Liu, 2002). Acetic acid (and additional ATP) is generated during hexose fermentation by converting acetyl phosphate to acetate (instead of ethanol) (Liu, 2002; Pilone et al., 1991). The heterofermentative LAB can also use oxygen and pyruvate as electron acceptors which further results in the production of more acetate and ATP (Liu, 2002). Homofermentative species of *Lactobacillus* and *Pediococcus* produce D-lactic acid and ATP through the Embden-Meyerhof-Parnas pathway (Liu, 2002; Sponholz, 1993).

Pentoses are thought to be metabolised by both heterofermentative and homofermentative wine LAB via the pentose phosphate pathway to produce ATP, lactate and acetate (Sponholz, 1993). Hence, the volatile acidity of wine can increase significantly after a bacterial MLF.

Other sensory faults attributed to LAB are acrolein formation, development of a 'mousy' off-flavour and an increase in viscosity known as 'ropiness'. Acrolein is produced by LAB degradation of glycerol and is associated with an unpleasant bitterness (Sponholz, 1993). Mousy off-flavours (2-acetyltetrahydropyridine, 2-ethyltetrahydropyridine and 2-acetyl-1-pyrroline) are also produced by LAB, possibly by the catabolism of glucose and fructose, and the amino acids ornithine and lysine, in the presence of ethanol (Costello and Henschke, 2002; Lonvaud-Funel, 1999). Ropiness is caused by LAB strains that can synthesize extracellular polysaccharides from residual sugars (Gindreau et al., 2001; Manca de Nadra and Strasser de Saad, 1995).

In addition to the production of spoilage chemicals and cosmetic problems, LAB can produce harmful compounds such as biogenic amines and ethyl carbamate. More than twenty amines have been found in wine (Lehtonen, 1996), the most notable being histamine, cadaverine, phenylethylamine, putrescine and tyramine (Lonvaud-Funel, 2001; Zee et al., 1983). Biogenic amines are produced from their respective precursor amino acids by specific amino acid decarboxylases. The characterisation of a histidine decarboxylase gene from an *O. œni* strain (Coton et al., 1998a; Coton et al., 1998b) and the tyrosine decarboxylase operon from a *Lactobacillus brevis* strain (Lucas et al., 2003; Moreno-Arribas and Lonvaud-Funel, 1999; Moreno-Arribas and Lonvaud-Funel, 2001) have been completed. The presence of biogenic amines in wine can be of great concern

for consumers since these molecules have been shown to produce undesirable physiological effects in susceptible individuals. For example, histamine is known to cause headaches and other allergenic symptoms such as hypotension, edema, palpitations, flushing, diarrhea, and vomiting (Santos, 1996; Soufleros et al., 1998; Wantle et al., 1994). Tyramine and phenylethylamine have been associated with migraines and hypertension (Soufleros et al., 1998). It is important to note that alcohol, acetaldehyde, antidepressant drugs and other biogenic amines such as cadaverine and putrescine can potentiate the toxic effect of histamine, tyramine and phenylethylamine (Straub et al., 1995; ten Brink et al., 1990). Given that wine consumers will be exposed to toxic biogenic amines through a fermented alcoholic beverage that likely contains acetaldehyde, suggests that the negative effects of the biogenic amines will in all cases be enhanced. 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). This may represent an additional risk for consumers.

Arginine metabolism by certain strains of *O. œni* and other LAB leads to the formation of ethyl carbamate (urethane) precursors (Liu and Pilone, 1998). Ethyl carbamate is a known animal carcinogen and potential human carcinogen (Ough, 1976; Ough, 1993). Ethyl carbamate precursors in wine are urea (produced by yeast), and citrulline and carbamyl phosphate (produced by LAB). Ethyl carbamate is formed through the chemical reaction of these carbamylic compounds and ethanol. The metabolism of arginine in LAB involves three enzymes: arginine deiminase, ornithine transcarbamylase and carbamate kinase (Liu et al., 1995b). The genes involved in the

arginine deiminase pathway have been characterised at the molecular level in *O. œni* (Tonon et al., 2001) and *L. hilgardii* (Arena et al., 2002). The legal limit for ethyl carbamate in table wine in Canada is 30 μg /L (Battaglia et al., 1990; Conacher et al., 1987), and in the USA there is a voluntary limit of 15 μg/L (Canas et al., 1994; Liu and Pilone, 1998). However, there are preliminary data to suggest that wines purchased in Canada may commonly exceed the legal limit for ethyl carbamate in this country (van Vuuren, personal communication, 2006). Given its potential role as a carcinogen in humans, ethyl carbamate in wine could represent an additional health risk for habitual wine consumers.

All LAB, to various degrees, are inhibited by ethanol, low pH, SO_2, low temperature, fatty acids produced by yeasts, decreased nutrient content, competitive interactions with yeast and other LAB, and bacteriophage infections (see reviews by Alexandre et al., 2004; Davis et al., 1985; Henick-Kling, 1993; van Vuuren and Dicks, 1993; Wibowo et al., 1985). Thus, malolactic bacteria often grow poorly and unpredictably in wine, especially Chardonnay wines, and thereby complicate the management of the winemaking process. The MLF can also occur in bottled wines, resulting in off-flavours and trapped carbon dioxide. Even under favourable conditions (optimal pH, no ethanol) the specific growth rate of *O. œni* is low, 0.01 – 0.04 h^{-1} on glucose and 0.06 – 0.10 h^{-1} on glucose and fructose (Maicas et al., 1999; Salou et al., 1994; Zhang and Lovitt, 2005). To overcome some of the difficulties associated with a MLF, selected starter cultures have been developed and commercialized (Maicas et al., 1999; Nielsen et al., 1996; Rodriguez et al., 1990). In some cases the use of starter cultures will reduce the time required to complete the MLF compared to a spontaneous

MLF. However, wine is still prone to oxidation and possible microbial spoilage because the malolactic starter culture is usually inoculated only after the conclusion of the alcoholic fermentation in order to avoid an increase in volatile acidity due to sugar metabolism by *O. œni*. Despite the use of malolactic starter cultures, wineries still experience many problems in ensuring an efficient MLF in high-acid white wines. Indeed, a 'stuck' or 'sluggish' MLF that may take weeks or months to complete is detrimental to wine quality because the addition of protective concentrations of SO_2 are delayed and wine is exposed to the negative effects of oxidation and the possibility of microbial spoilage.

To avoid the negative aspects of the bacterial MLF, winemakers can use blending, carbonate additions, precipitation of acids, dilution and carbonic maceration to reduce the acidity of wine or grape must. 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 *Schizosaccharomyces pombe* (Gallander, 1977; Silva et al., 2003), high density cell suspensions of yeasts (Gao and Fleet, 1995), and immobilization of *O. œni*, *Lactobacillus* sp., or the malolactic enzyme on a variety of matrices (see reviews by Kourkoutas et al., 2004; Maicas, 2001; Zhang and Lovitt, 2006). Unfortunately these methods often result in wine of inferior quality and they are not applicable to production of quality wine on a commercial scale.

1.4 Genetic engineering of *Saccharomyces cerevisiae* to perform the MLF

Wine microbiologists have been studying the problems associated with a bacterial MLF for many years; however, it was not until the advent of genetic engineering that a

possible solution to the MLF dilemma became available. Research concerning the construction of a malolactic wine yeast strain by cloning the malolactic gene of LAB and expressing it in *S. cerevisiae* has been progressing for over two decades. In 1984, a DNA fragment containing the malolactic enzyme from *Lactobacillus delbruekii* was first cloned into *Escherichia coli* (Williams et al., 1984). However, only a very low level of expression of the malolactic enzyme was obtained from the isolated clones (Williams et al., 1984). A DNA fragment containing the malolactic enzyme from *O. œni* was cloned into *E. coli* as well, but due to instability problems with the cloned DNA, no further research was conducted (Lautensach and Subden, 1984). The malolactic gene (*mleS*) from *Lactococcus lactis* was also cloned and characterised (Ansanay et al., 1993; Denayrolles et al., 1994). The open reading frame (ORF) of the *mleS* is 1620 nucleotides, encoding a putative protein of 540 amino acids (59 kDa) (Ansanay et al., 1993; Denayrolles et al., 1994). An alignment of the deduced protein sequence of the *mleS* with malic enzymes from different sources revealed highly conserved regions described as NAD-binding domains, a malate binding site, and other regions of unknown function (Denayrolles et al., 1994; Lonvaud-Funel, 1995). Expression of the *mleS* gene in *E. coli* and *S. cerevisiae* resulted in a weak MLF (Anasanay et al., 1993; Denayrolles et al., 1995). The first gene isolated and sequenced from the MLF system of *L. lactis* was the regulatory *mleR* gene (Renault et al., 1989). The product of this gene serves as a positive activator of the malolactic gene of *L. lactis* in the presence of L-malic acid.

The structural gene for the malolactic enzyme of *O. œni* (*mleA*) was eventually sequenced from a 3.4 kb fragment that also contained a gene for a malate carrier protein (*mleP*) (Labarre et al., 1996b). The *mleA* gene encodes a protein with a theoretical mass

of 59.1 kDa and the mleA amino acid sequence has a 66% homology to the mleS amino acid sequence (Labarre et al., 1996b). The heterologous expression of the *mleA* in *E. coli* and *S. cerevisiae* resulted in low MLF activity (Labarre et al., 1996b). In addition to the *mleA* and *mleP* genes, a third ORF transcribed in the opposite direction was found upstream of the apparent MLF operon and encoded for a protein belonging to the LysR-type regulatory protein family (Labarre et al., 1996a). This protein seems to be similar to the activator protein mleR found in *L. lactis* (Labarre et al., 1996a).

Commercial wine yeast strains of *S. cerevisiae* can metabolize malate to a very limited extent (10-20%) (Kuczynski and Radler, 1982). The basis of the inefficient malate degradation by *S. cerevisiae* is 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) which is also subject to catabolite repression (Redzepovic et al., 2003). The malic enzyme of *S. cerevisiae* has been isolated and requires Mn^{2+} and NAD^+ or $NADP^+$ as cofactors (Fuck et al., 1973; Osothsilp, 1987). Due to the absence of an active malate transport system in *S. cerevisiae*, previous attempts to construct recombinant yeast strains capable of MLF did not succeed. While *S. cerevisiae* cannot degrade malate efficiently, other K (-) yeasts like *Schizosaccharomyces pombe* can (Baranowski and Radler, 1984; Kuczynski and Radler, 1982; Rodriquez and Thornton, 1989). Yeast can be categorised into K(-) or K(+) groups depending on their ability to use L-malic acid and other TCA cycle intermediates as sole carbon or energy sources (Barnett and Kornberg, 1960; Barnett et al., 1990; Rodriguez and Thornton, 1990; Volschenk et al., 2003). The K(-) group can use the TCA cycle intermediates only in the presence of an assimilable carbon source

such as glucose; whereas the K(+) group can utilise TCA cycle intermediates directly as sole carbon or energy sources (Barnett and Kornberg, 1960; Volschenk et al., 2003). The efficient *S. pombe* malate metabolism depends on three enzymes: a malate permease, a malic enzyme, and a mitochondrial dehydrogenase enzyme (Osothsilp and Subden, 1986a). Malate transport in *S. pombe* is constitutive, active, and not subject to glucose repression (Grobler et al., 1995; Rodriguez and Thornton, 1990; Sousa et al., 1992; Sousa et al., 1995). The malate permease is also a general dicarboxylic acid transporter, responsible for the transport of L-malic acid, succinate and malonic acid (Grobler et al., 1995), and it functions as a proton dicarboxylate symporter (Sousa et al., 1992). The optimum pH of the transport of malate in *S. pombe* is 3.5 (Osothsilp and Subden, 1986b). Under fermentative conditions the cytosolic malic enzyme is solely involved in the degradation of L-malic acid to ethanol and CO_2, commonly known as the malo-ethanolic fermentation pathway (Magyar and Panyik, 1989; Mayer and Temperli, 1963; Taillandier and Strehaiano, 1991; Taillandier et al., 1988; Volschenk et al., 2003). Under aerobic conditions both the malic enzyme and malate dehydrogenase metabolise L-malic acid. However, the malate dehydrogenase only degrades approximately 10% of the malate in aerobic conditions (Osothsilp and Subden, 1986a; Subden et al., 1998).

The creation of *S. pombe* mutants unable to transport malate and the subsequent cloning of a *S. pombe Hind*III DNA fragment capable of complementing this mutation (Osothsilp and Subden, 1986a; Subden et al., 1998) was the first step that lead to the cloning of the malate transport gene. Grobler et al., (1995) used this *Hind*III DNA fragment to subclone, sequence and characterise the malate transport (*mae1*) gene. The structural gene of the malate permease encodes an ORF of 1314 bp that translates into a

protein of 438 amino acids with a theoretical weight of approximately 49 kDa (Grobler et al., 1995). The *mae1* gene is located on chromosome 1, is constitutively transcribed and is not subject to catabolite repression (Grobler et al., 1995). Analysis of the hydrophobic and hydrophilic composition of the amino acid sequence of the malate permease revealed typical motifs similar to other membrane transport-proteins. The mae1p contains a hydrophillic amino- and carboxy-terminal as well as ten putative membrane-spanning domains separated by hydrophilic linkers. Several conserved elements were identified in the mae1p, such as a leucine zipper motif, PEST region and several N-linked glycosylation and protein kinase C phosphorylation sites (Grobler et al., 1995). Volschenk *et al.* (1997), functionally co-expressed the *S. pombe mae1* gene and the *L. lactis mleS* gene under the regulation of the *S. cerevisiae* 3-phosphoglycerate kinase (*PGK1*) promoter and terminator sequences on multicopy plasmids in a laboratory strain of *S. cerevisiae*. The recombinant laboratory strain was able to efficiently decarboxylate 4.5 g/L L-malate to L-lactate and carbon dioxide in 4 days (Volschenk et al., 1997a; Volschenk et al., 1997b).

1.5 Genetically engineered *Saccharomyces cerevisiae* strains in the wine industry

The possible targets for the genetic improvement of wine yeast strains are presented in Table 1. Several strategies and methods can be used to obtain these desired properties in wine yeast. The classic methods include selection of clonal variants, mutagenesis and selection, and hybridisation via mating, rare-mating, cytoduction or spheroplast fusion (Bisson, 2004; Pretorius, 2000; Pretorius and Bauer, 2002). The recent application of recombinant DNA technologies have enabled a far more specific

and rational approach to improvements of industrial yeast strains. The capability to transform yeast based on chemical, electrical or biolistic methods, the development of a variety of vectors, and the publication of the yeast genome has led to major advances in the construction of wine yeast strains (Akada, 2002; Bisson, 2004; Pretorius, 2000; Pretorius and Bauer, 2002).

Table 1. Oenological targets for the genetic improvement of *S. cerevisiae* wine strains (adapted from Bisson, 2004; Giudici et al., 2005; Pretorius, 2000; Pretorius and Hoj, 2005)

Target
Fermentation performance
• Improve stress tolerance
• Improve fermentation rate
• Improve substrate utilisation
• Improve nitrogen assimilation
• Improve competitiveness
• Increase range of growth temperatures
• Reduce foam formation
Improve sensory attributes
• Biological adjustment of acidity
• Increase glycerol production
• Increase desirable esters
• Liberate grape terpenoids
• Optimised fusel oil production
• Increase autolysis flavour production
Reduce off-character production
• Decrease sulphur volatiles
• Decrease acetate, volatile acidity
• Decrease aldehydes
• Decrease higher alcohols
Reduce off-character production
• Decrease phenolic derivatives
Improve wine processing
• Optimise flocculation and sedimentation
• Improve protein and polysaccharide clarification
• Tannin reduction

Target
Improve biological control of wine spoilage microorganisms
• Increase production/tolerance to SO_2
• Express antimicrobial peptides or enzymes
Improve wholesomeness
• Decrease ethyl carbamate
• Decrease bioamine formation
• Increase production of resveratrol
• Increase vitamin production
• Increase pesticide and metal ion scavenging
• Decrease ethanol concentration

The current state of progress of genetically engineered wine yeast is illustrated in Table 2. Although significant advances have been accomplished in the last two decades only one genetically modified (GM) wine yeast is currently commercially available, it is the malolactic yeast reported in this dissertation. Furthermore, the urea-degrading yeast also produced by our group (Coulon et al., 2006) is ready for commercialisation. The majority of genetically engineered yeast strains reported in Table 2 are only at the 'proof of principle' stage and cannot be commercialised until several further requirements are met.

Table 2. Genetic engineering of wine yeast and stratagies used in their modification (adapted from Schuller and Casal, 2005)

Target	Protein	Gene(s)	Source	Promoter/ Terminator	Marker	Plasmid/ Integration	Host Strain*	Reference
Fermentation Performance								
-Stress tolerance	Glycogen synthase	*GSY2*	*S. cerevisiae*	Native	*URA3*	2μ	Laboratory	(Perez-Torrado et al., 2002)
-Killer factor synthesis	K1 killer toxin (Zymocin)	*KIL-K1*	*S. cerevisiae*	*ADH1/ADH1*	*LEU2 and* K1 immunity	CEN-based plasmid and integration	Laboratory	(Boone et al., 1990)
Sensory Attributes								
-Acidity adjustment	Malate permease/ Malolactic enzyme	*mae1/mleA*	*S. pombe/O. œni*	*PGK1/PGK1*	**None**	Integration	Industrial	(Husnik et al., 2006)
	Malate permease/ Malolactic enzyme	*mae1/mleS*	*S. pombe/L. lactis*	*PGK1/PGK1*	*URA3*	2μ	Laboratory	(Volschenk et al., 1997b)
	Malate permease/Malic enzyme	*mae1/mae2*	*S. pombe*	*PGK1/PGK1*	*SMR1-140*	Integration	Industrial	(Volschenk et al., 2001)
	Acetaldehyde dehydrogenase	*ALD6*	*S. cerevisiae*	(Deletion)	*kanMX* 4	Integration of marker	Laboratory	(Remize et al., 2000)
	Lactate dehydrogenase	*LDH*	*Lactobacillus casei*	*ADH1/ADH1*	Tn903 ($G418^r$)	2μ	Industrial	(Dequin et al., 1999)
-Increase glycerol production	Glycerol-3-phosphate dehydrogenase	*GPD1*	*S. cerevisiae*	*ADH1/ADH1*	Tn5*ble*	2μ	Laboratory and industrial	(Michnick et al., 1997; Remize et al., 1999)
-Liberate grape terpenoids	Endoglucanase	*egl1*	*Trichoderma longibrachiatum*	*ACT*	*CYH2*	2μ	Industrial	(Perez-Gonzalez et al., 1993)
	Arbinofuranosidase	*abfB*	*Aspergillus niger*	*ACT*	*CYH2*	2μ	Industrial	(Sanchez-Torres et al., 1996)

Target	Protein	Gene(s)	Source	Promoter/ Terminator	Marker	Plasmid/ Integration	Host Strain*	Reference
Sensory Attributes								
-Liberate grape terpenoids	Endoxylanase	*xlnA*	*Aspergillus nidulans*	*ACT*	*CYH2*	2μ	Industrial	(Ganga et al., 1999)
	Rhamnosidase	*rhaA*	*Aspergillus aculeatus*	*GPD/PGK*	*CYH2*	2μ	Industrial	(Manzanares et al., 2003)
-Acclerated autolysis	AAA ATPase	*csc1-1*	*S. cerevisiae*	*TDH3*	*URA3*	2μ	Laboratory	(Cebollero et al., 2005)
-Volatile phenol formation	Phenolic acid decarboxylase	*pdc*	*Lactobacillus plantarum*	*PGK1/PGK1*	*URA3 and SMR1-140*	2μ and integration of plasmid	Laboratory and industrial	(Smit et al., 2003)
-Optimise ester production	Alcohol acetyltransferase	*ATF1*	*S. cerevisiae*	*PGK1/PGK1*	*LEU2 and SMR1-140*	2μ and integration of plasmid	Laboratory and industrial	(Lilly et al., 2000)
	Alcohol acetyltransferase	*ATF2*	*S. cerevisiae*	*PGK1/PGK1*	*SMR1-140*	Integration of plasmid	Industrial	(Lilly et al., 2006)
	Isoamyl acetate-hydrolyzing esterase	*IAH1*	*S. cerevisiae*	*PGK1/PGK1*	*SMR1-140*	Integration of plasmid	Industrial	(Lilly et al., 2006)
	Ethanol hexanoyl transferase	*EHT1*	*S. cerevisiae*	*PGK1/PGK1*	*SMR1-140*	Integration of plasmid	Industrial	(Lilly et al., 2006)
-Decrease sulphur volatiles	Sulphite reductase	*MET10*	*S. cerevisiae*	*MET3*	*LEU2*	2μ	Laboratory	(Sutherland et al., 2003)
Wine Processing								
-Improve clarificaiton	Endopolygalacturonase	*PGU1*	*S. cerevisiae*	*PGK1/PGK1*	*kanMX*	2μ	Industrial	(Vilanova et al., 2000)
	Pectate lyase	*pelA*	*Fusarium solani*	*ACT*	*CYH*	2μ	Industrial	(Gonzalez-Candelas et al., 1995)

Target	Protein	Gene(s)	Source	Promoter/ Terminator	Marker	Plasmid/ Integration	Host Strain*	Reference
Microbial spoilage control								
-Production of antimicrobials	Pediocin	*pedA*	*Pediococcus acidilactici*	*ADH1/ADH1*	*URA3*	2μ	Laboratory	(Schoeman et al., 1999)
	Chitinase	*CTS1-2*	*S. cerevisiae*	*PGK1/PGK1*	*URA3*	2μ	Laboratory	(Carstens et al., 2003)
	Leucocin	*lcaB*	*Leuconostoc carnosum*	*ADH1/ADH1*	*URA3*	2μ	Laboratory	(Du Toit. and Pretorius, 2000)
	Glucose oxidase	*gox*	*Aspergillus niger*	*PGK1/PGK1*	*URA3*	Integration	Laboratory	(Malherbe et al., 2003)
Health Aspects								
-Ethyl carbamate reduction	Urea amidolyase	*DUR1,2*	*S. cerevisiae*	*PGK1/PGK1*	**None**	Integration	Industrial	(Coulon et al., 2006)
-Resveratrol production	? -Glucosidase	*bglN*	*Candida molischiana*	*ACT/ACT*	CYH2	2μ	Industrial	(Gonzalez-Candelas et al., 2000)
	Resveratrol synthase	*4CL216*	Hybrid poplar	*ADH2/ADH2*	*URA3*	2μ	Laboratory	(Becker et al., 2003)
	Coenzyme-A ligase	*Vst1*	*Vitis vinifera*	*ENO2/ENO2*	*LEU2*	2μ	Laboratory	(Becker et al., 2003)
-Reduction of ethanol	Glycerol-3-phosphate dehydrogenase	*GPD2*	*S. cerevisiae*	*ADH1* promoter	*SMR1-140*	2μ	Industrial	(de Barros Lopes et al., 2000)
	Glycerol-3-phosphate dehydrogenase and Acetaldehyde dehydrogenase	*GPD2/ALD6*	*S. cerevisiae*	*ADH1* promoter and *ALD6* deletion	*LEU2*	2μ	Laboratory	(Eglinton et al., 2002)

Target	Protein	Gene(s)	Source	Promoter/ Terminator	Marker	Plasmid/ Integration	Host Strain*	Reference
Health Aspects								
-Reduction of ethanol	Glycerol-3-phosphate dehydrogenase and Acetaldehyde dehydrogenase	*GPD1/ALD6*	*S. cerevisiae*	*ADH1/ADH1* and *ALD6* deletion	*kanMX*	Integration	Industrial	(Cambon et al., 2006)
	Hexose transporter 1 and 7	*Hxt1/Hxt7*	*S. cerevisiae*	*HXT7*prom/ *HXT1*term	*URA3*	Integration	Laboratory	(Henricsson et al., 2005)

*Industrial strains are defined here as wine yeast of the genus *Saccharomyces* that are used by the wine industry. Host strains that are derived from industrial yeast but have undergone significant alterations (multiple auxotrophies) in order to facilitate genetic modification are defined here as laboratory strains.

In general, GM wine yeast for commercial use should not contain any drug resistant markers such as *CYH2, Tn5ble, kanMX* (Tn903) or *SMR1-140*, conferring resistance to cycloheximide, phelomycin, geneticin (G418) and sulphometuron methyl (SMM), respectively; nor should they contain vectors or vector sequences with bacterial resistance markers such as the *E. coli* ampicilin resistance (*bla*) gene. Genetic modifications relying on plasmids should also be re-constructed with the genes/elements of interest stably integrated into the genome of the host strains. Moreover, all genetic material should be derived from the host species ("self-cloning") or from GRAS (Generally Regarded As Safe) organisms with a history of safe use in the food industry; the use of DNA sequences from pathogenic species or organisms known to produce allergens should be avoided. One of the main features of recombinant DNA technology versus classical genetics is the possibility to introduce a specific gene(s) into the host without loss of the phenotypic characteristics of the host strain. Apart from the introduced change, a commercially available GM wine yeast strain should produce wine that is not significantly different from the parent strain. Currently this last requirement may be very difficult for polygenic traits that are not well-characterised in wine yeast and only well-characterised traits requiring modifications to one or two genes have been successful (Coulon et al., 2006; Husnik et al., 2006).

A GM wine yeast strain for commercial use requires approval from the regulatory authorities of each country where the yeast will be sold. There are two major approaches to regulating GM products in the world. The first is exemplified by the United States and the other is the approach adopted by the European Union. The US system essentially evaluates the final product and is based on the principle of substantial equivalence.

Substantial equivalence means that if a food from a genetically modified organism (GMO) is "as safe as" the corresponding conventional food product then they should both be treated equally. A detailed knowledge of the genotype, phenotype and supporting transcriptomic, proteomic and metabolomic data are necessary for a successful evaluation. The US system works within existing regulations for the commercialisation of foods and does not require labelling unless an introduced gene encodes for a novel product that has never been part of any other food. Other countries like Canada, Australia and Japan are also based on the US system but with varying degrees of additional requirements and levels of stringency.

The EU system is considerably more complex. The EU approach, in addition to evaluating the final product, also takes into consideration the technique used to obtain the product and it is primarily based on the precautionary principle. The recently amended European Novel Food Regulations define the procedures for authorisation, labelling and traceability. The regulations for authorisation require the applicant to demonstrate that the product is safe to human and animal health, that it does not differ nutritionally from the conventionally produced product, it does not mislead consumers, and it is safe for the environment (Schuller and Casal, 2005). The government of the country introducing the proposed product must also inform all the commissions of the other members of the EU and the European Food Safety Authority in Brussels, Belgium (Ramon et al., 2005). In the EU, labelling is compulsory for GM food regardless of whether DNA or protein can be detected in the final product, although it is not required for foods with accidental or technologically unavoidable trace amounts of GMOs (<0.9%) (Schuller and Casal, 2005). The EU also requires that GMOs and products derived from GMOs must be traceable

through all of the stages of production and distribution (Schuller and Casal, 2005). However, GM 'processing aids', which are products used in the food and feed production process, do not fall under the strict EU regulations outlined above. GM yeast can be viewed as a processing aid in the EU: if less than 0.9% of the yeast is present in the final product, no labelling is required.

In addition to the demanding technical requirements to construct a suitable GM wine yeast strain and the regulatory hurdles that must be traversed, the largest challenge to a commercial GM wine yeast strain is consumer and winemaker acceptance. Several authors have suggested that the first GM wine products should demonstrate a direct benefit to consumers (Dequin, 2001; Pretorius and Bauer, 2002; Pretorius and Hoj, 2005; Ramon et al., 2005). Ideally, this benefit should be related to health, such as an increased concentration of a nutritional ingredient or the reduction in a naturally present toxic compound (Tables 1 and 2). Although the increase in a health-related compound such as resveratrol or vitamins could be viewed as a direct benefit to the consumer, an alcohol-based beverage may not be the most appropriate delivery method for bringing a health-related ingredient to the public. However, the reduction of a naturally occurring toxic or harmful compound already present in wine, such as biogenic amines and/or ethyl carbamate (Tables 1 and 2) would provide a direct benefit to wine consumers and the removal of such compounds should not affect the organoleptic qualities of the product. This direct benefit, in addition to the fact that the GM wine yeast would be removed by filtration, could increase the consumer acceptance of the first GM wine product. A GM yeast strain that provided a direct benefit to the winemaker, in addition to the consumer, is more likely to be commercially acceptable. In conclusion, a successful GM wine yeast

strain should provide a direct benefit to the consumer and to the winemaker without affecting the organoleptic qualities of the product.

1.6 Proposed Research

1.6.1 Significance of research

The research reported in this thesis addresses the need for a wine yeast strain capable of completing the MLF. ML01, the malolactic yeast described herein, is the first genetically engineered wine yeast to be commercialised. The development of a commercially-acceptable malolactic yeast strain is of great benefit to the wine industry. The application of ML01 will provide a direct advantage to the winemaker (by reducing processing time), improve wine sensory qualities (by reducing volatile acidity and preventing deterioration of colour by LAB), and possibly a direct health benefit to the wine consumer (by preventing the production of biogenic amines and ethyl carbamate by LAB). The preservation of wine immediately after alcoholic fermentation could also improve the organoleptic characteristics of the wine by preventing chemical oxidation and microbial spoilage often associated with a delayed bacterial MLF.

1.6.2 General hypotheses

The hypotheses that guided this research were that the construction of a genetically engineered wine yeast strain capable of MLF is possible and that this malolactic yeast could produce high quality wine.

1.6.3 Main objectives

The main objectives of this study are to:

1. Construct a genetically stable industrial wine yeast strain of *S. cerevisiae* that will be capable of performing an efficient MLF.
2. Characterise the genome, transcriptome and proteome of the malolactic yeast.
3. Characterise the phenotype of the malolactic yeast and evaluate the wine produced by the genetically engineered wine yeast.

2 MATERIALS AND METHODS

2.1 Strains and plasmids employed in the genetic construction and characterisation of ML01

The different strains and plasmids used in this study are listed in Tables 3 and 4, respectively.

Table 3. Different strains used in the metabolic engineering and characterisation of the malolactic yeast ML01.

Strains	Description	Reference
E. coli DH5a	F⁻ *end*A1 *gln*V44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *deo*R *nup*G F 80d*lacZ*? M15 ? (*lacZYA-argF*)U169 *hsd*R17(r_K^- m_K^+), ?⁻	(Hanahan, 1983)
O. œni	Viniflora® Oenos, a freeze dried pure culture of *O. œni*.	Chr. Hansen Ltd., Hoersholm, Denmark
S. cerevisiae 3597? 78-1	A hybrid industrial baker's yeast strain containing an integrated Tn*5ble* gene cassette at the *SUC2* locus.	Lesaffre Development, Marcq-en-Barœul, France
S. cerevisiae ML01	Industrial "Prise de Mousse" strain S92 containing the malolactic cassette integrated into the *URA3* locus.	(This study)
S. cerevisiae S92	Industrial "Prise de Mousse" strain originally isolated from the Champagne region in France.	Bio Springer, Maisons-Alfort, France (a division of Lesaffre International, Marcq-en-Barœul, France)

Table 4. Different plasmids used in the metabolic engineering and characterisation of malolactic clone ML01.

Plasmids	Description	Reference
pJH2	YEp352(? *Kpn*I) containing the *mae1* and the *mleA* expression cassettes cloned between *URA3* flanking sequences.	(Husnik, 2001)
pJH3	YCplac33-KanMX containing the malolactic cassette subcloned from pJH2.	(Husnik, 2001)
pUT332	*E. coli*/yeast episomal shuttle vector containing the Tn*5ble* dominant marker.	(Gatignol et al., 1987)
YCplac33-KanMX	*E. coli*/yeast centromeric shuttle vector containing the KanMX dominant marker.	Lesaffre Development, Marcq-en-Barœul, France

2.2 Culture conditions

Escherichia coli DH5a was used for plasmid propagation and cultured according to standard methods (Ausubel et al., 1995). All *S. cerevisiae* strains were cultured in YPD broth (Difco, Becton, Dickinson and Co., Sparks, USA) according to standard methods (Ausubel et al., 1995). Active dry yeast (ADY) were used either directly or re-hydrated at 40 °C in 7% synthetic must for 30 min (DNA microarray and proteomics studies) or re-hydrated at 37 °C in sterile distilled water for 15 min (post-fermentation and effect of residual ML01 concentrations on MLF studies). ADY was mixed intermittently during re-hydration and prior to inoculation.

The medium YEG (1% yeast extract, 2% dextrose, 1.5% Pastagar B [Bio-Rad, Hercules, USA]) supplemented with 100 µg/mL of phleomycin (Invivogen, San Diego, USA) was used for selecting co-transformed yeast colonies and testing for phleomycin sensitivity. Synthetic must was prepared by adding 1.0 mL/L of Tween 80 to synthetic

broth (Denayrolles et al., 1995). The pH of the synthetic must was adjusted to 3.5 for all studies except the DNA microarray, Real-Time RT-PCR and iTRAQ experiments. The tartaric acid concentration of the synthetic must for the growth kinetics, DNA microarray, Real-Time RT-PCR and iTRAQ experiments was adjusted to 4.5 g/L using L-tartaric acid. Pasteurized, commercially available Chardonnay grape must ("Vine Fresh", Wine Kitz, Vancouver, Canada; 22.65 Brix, pH 2.9, TA 4.39 g/L, 0.92 g/L malate, YANC 348.1 mg/L) was used to study growth kinetics and viability of ML01 post-fermentation. Malate concentration was adjusted to 4.5 g/L and the must was filter-sterilized (0.22 μm). Utilization of malate as a sole carbon source by ML01 was examined in modified YPD medium containing 10 g/L Yeast Extract (Difco, Becton, Dickinson and Co., Sparks, USA), 20 g/L Peptone (Difco, Becton, Dickinson and Co., Sparks, USA), 5 g/L Dextrose and 20 g/L L-malic acid. The pH was adjusted to 6.5 with KOH pellets and the medium was filter-sterilized (0.22 μm).

2.3 Genetic construction of malolactic wine yeast

2.3.1 Co-transformation of the malolactic cassette and pUT332

The malolactic cassette was isolated from plasmid pJH2 (Husnik, 2001) by digestion with *Srf*I and subsequent gel extraction and purification of an 8683 bp fragment containing the *mae1* and *mleA* expression cassettes flanked by homologous *ura3* sequences. *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 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 plates containing 100 µg/mL phleomycin and incubated at 30 °C for 3 days.

2.3.2 Screening of transformants for the integrated malolactic cassette

Co-transformed yeast colonies and the positive control strain, S92 transformed with pJH13 (YCplac33-KanMX with the malolactic cassette cloned into the *Xba*I site) (Husnik, 2001), were inoculated into sterile 96 round bottom micro-well plates containing 200 µL of synthetic must, wrapped in parafilm and 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. A 25 µL volume of the L-lactic acid dehydrogenase/NAD/ phenazine methosulfate/nitro blue tetrazolium reaction mixture (pH 8.3) (Subden et al., 1982) was added to each well containing 75 µL of the test sample and plates were incubated at 37 °C in the dark for 30 min. The reaction mixture causes wells containing L-lactic acid to show a purple/blue colour and yeast cells from the corresponding plate would be recovered by inoculation into 5 mL of YPD and incubated at 30 °C for 1-2 days. After recovery, malolactic clones were re-inoculated into 5 mL synthetic must and streaked onto YEG plates and incubated at 30 °C for 3-5 days. The degradation of malate and production of lactate in synthetic must was confirmed by enzymatic analysis (R-Biopharm, Darmstadt, Germany). Confirmation of integration was performed by PCR on genomic DNA (Promega, Madison, USA) from individual colonies from each clone identified in the screening. The PCR reaction was done according to manufacturer's recommendations (Invitrogen, Carlsbad, USA) using primers 5'-TTGTAATGTGACCA ATGAG-3' (inside the cassette, *PGK1* promoter) and 5'-CTCTTTATATTTACATGCTA

AAAATGG -3' (outside the cassette, 3'-end *URA3* flanking region). The 1095 bp PCR product was visualized by 0.8% agarose gel electrophoresis and ethidium bromide staining (Ausubel et al., 1995).

2.3.3 Loss of plasmid pUT322

Each positively identified clone was sub-cultured daily in non-selective YPD for one week to ensure loss of the co-transformed plasmid pUT332. Cultures were grown at 30 °C on a shaking platform (180 rpm) and sub-cultured by inoculating yeast from a 24 h culture into 5 mL of YPD to a final OD of 0.05. Malolactic clones, S92 (parental strain and negative control), and *S. cerevisiae* 3597? 78-1 (positive control containing phleomycin resistance gene Tn*5ble*) were plated onto YEG medium with and without 100 µg/mL of phleomycin to demonstrate sensitivity.

2.4 Functionality of malolactic wine yeast

2.4.1 Malate decarboxylation and residual sugar concentrations of wine produced by malolactic clones

The parental strain S92 and three individual colonies selected from each sub-cultured malolactic clone were grown in 250 mL Erlenmeyer flasks containing 50 mL of YPD at 30 °C with agitation (180 rpm) for two days. S92 transformed with pJH13 and YCplac33-KanMX were cultured in the same way as the parental strain and the malolactic clones except for the addition 200 µg/mL of G418. Yeast cells were harvested by centrifugation for 5 min at 3000 x *g* and re-suspended in 1 mL of sterile distilled water. All yeast cultures were inoculated into 500 mL sterile Erlenmeyer flasks

containing 500 mL synthetic must to a final cell density of 4 x 10^6 cells/mL. Synthetic must inoculated with S92 transformed with YCplac33-KanMX or pJH13 also contained 200 µg/mL of G418. Each fermentation flask was fitted with an autoclaved glass vapour lock. Fermentations were incubated at 20 °C with agitation (65 rpm) for 14 days. Malate was monitored for 14 days and residual sugar on day 14. L-Malic acid, D-glucose and D-fructose concentrations were determined by enzymatic analysis (R-Biopharm, Darmstadt, Germany).

2.4.2 Functionality of active dry wine yeast ML01

Malolactic yeast clone 4 (ML01) was produced as active dry yeast at the Lesaffre Development pilot plant facility (Lesaffre Development, Marcq-en-Baroeul, France) and tested for functionality in synthetic must. Active dry yeast strains ML01 and S92 were inoculated at a concentration of 0.2 g/L into 500 mL sterile Erlenmeyer flasks containing 500 mL synthetic must in triplicate. Each fermentation flask was fitted with an autoclaved glass vapour lock. Flasks were incubated at 20 °C with agitation (65 rpm) for 14 days and monitored for malate degradation, lactate production and residual sugar on day 14. L-Malic acid, L-lactic acid, D-glucose and D-fructose concentrations were determined by enzymatic analysis (R-Biopharm, Darmstadt, Germany).

2.5 Genetic characterisation of ML01

2.5.1 Chromosome karyotyping of ML01 and S92

Yeast chromosomes for pulsed field gel electrophoresis were prepared in low melting point agarose plugs (Schwartz and Cantor, 1984). Cells were harvested by

centrifugation and washed with 5 mM EDTA, pH 7.5. The cell pellet was re-suspended in 10 μL of 1 M Sorbitol, 0.1 mM EDTA (pH 8), 14 mM β-mercaptoethanol and 10 mg/mL of Driselase (Sigma-Aldrich, St. Louis, USA). Cell suspensions were mixed with low melting point agarose (Invitrogen, Carlsbad, USA) and placed into molds. Agarose plugs were incubated overnight at 37 °C in 0.5 M EDTA (pH 8), 10 mM Tris (pH 8) and 20 mM DTT. The plugs were subsequently incubated in 0.5 M EDTA (pH 8), 10 mM Tris (pH 8), 1% laurylsarcosine, 1 mg/mL proteinase K (Merck, Whitehouse Station, USA) for 6 h at 55 °C. After extensive washing in solutions of 50 mM EDTA (pH 7.5), TE, and 1X TBE the plugs were inserted into a 1% agarose-TBE gel. Yeast chromosomes were separated by Gene Navigator PFGE apparatus (Pharmacia Biotech, Uppsala, Sweden) in TBE buffer at 12 °C. Electrophoresis was performed at 165 V for 20 h with 90 s pulses, followed by 10 h with 110 s pulses, followed by 10 h with 125 s pulses and 4 hours with 30 s pulses. The gel was stained with ethidium bromide and photographed.

2.5.2 Southern blot analyses

Southern blotting, labelling of probes, hybridization, stringency washes and detection were completed as recommended by the ECL direct nucleic acid labelling and detection system (GE Healthcare, Buckinghamshire, UK). Genomic DNA was digested with *Eco*RV, *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, Basel, Switzerland) and fixed by heating at 80 °C for 1 h. Probes corresponding to the malolactic cassette were excised from pJH2 (Husnik, 2001) 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, *Nae*I/*Pme*I to produce the 721 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 (Appendix A). The 471 bp probe for the Tn*5ble* gene was prepared by PCR amplification using primers J20 (5'-AATGACCGAC CAAGCGACG-3') and J21 (5'-ATCCTGGGTGGTGAGCAG-3') and Pwo polymerase (Roche, Basel, Switzerland). A probe for the *bla* gene and 970 bp of non-*Saccharomyces* sequences of pUT332 was prepared by digesting pUT332 with *Ssp*I/*Cla*I resulting in a 1758 bp fragment (Appendix A).

2.5.3 Sequencing of the malolactic cassette integrated into the genome of ML01

Synthesis of primers (Table 5) and sequencing was completed at the Nucleic Acid Protein Service Unit (University of British Columbia, Vancouver, Canada). Synthesis was performed on a Perkin Elmer - Applied Biosystems synthesizer using solid support phosphoramidite chemistry (Applied Biosystems, Foster City, USA). Sequencing of the malolactic cassette from *S. cerevisiae* ML01 (both strands) was completed using an ABI PRISM 377 DNA sequencer and ABI's AmpliTaq FS DyeDeoxy Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster City, USA). A minimum of two sets of 13 unique templates spanning the entire malolactic cassette were obtained by PCR using *PfuTurbo* (Stratagene, La Jolla, USA) and primers listed in Table 5. 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. Primers used for sequencing are also listed in Table 5.

The two complete sequences were aligned and analyzed for differences using Discovery Studio Gene v1.1 software (Accelrys, San Diego, USA). If anomalies occurred, another round of PCR and sequencing of the region in doubt was run to determine the correct sequence. Genomic DNA for the PCR reactions was obtained using a Promega, Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). All PCR templates used for sequencing were unique to the malolactic cassette. A schematic representation of the sequencing strategy is presented in Appendix B.

Table 5. Primers used for sequencing of the malolactic cassette integrated into *S. cerevisiae* ML01

Primer name	Primer sequence
For19g	5'-AACTAATGAGATGGAATCGG-3'
For20g	5'-GAAGGTTAATGTGGCTGTGG-3'
For15B	5'-AAGGAACGTGCTGCTACTC-3'
For3p	5'-AGATCTCATATGCAAGACGC-3'
For4g	5'-TGGAGGATGGGCATCTTCG-3'
For5g	5'-TGAGAAAGCTGGTGGACCG-3'
For6g	5'-TGGCAAGCATGTCGATGAAC-3'
For7p	5'-AGTTCACCCATGTCGAATCG-3'
For18g	5'-TCTTGAGTTGAAGTCAGGAATC-3'
For21g	5'-TGATGCGTTCATGCCTGATC-3'
For8g	5'-TGGTACCGCGGCCGCAAG -3'
For9p	5'-ACTTCAAATCGTCGACGGC -3'
For10g	5'-TGGTCTTTCAGTATAACCAG -3'
For11p	5'-CCAGTTCCTTGAATATCATC -3'
For12g	5'-TGATATCGCGGCCATTAGC -3'
For13p	5'-ACTTACTGGATCTGTCATG -3'
For14p	5'-GCTTGCGGCCGCACAAAG -3'
For17B	5'-AGGTAGAGGGTGAACGTTAC -3'
Rev18g	5'-TTGTTCCGTTTGACTTGTCGC -3'
Rev2p	5'- ACCAGATTAGAGTACAAACGC -3'
Rev3bp	5'-TCGCAATGTCAACAGTACCC -3'
Rev19g	5'-ACTATAGTAGAGATAACGTC -3'
Rev20g	5'-ACAAGCAATCGAAGGTTCTG -3'
Rev4bg	5'-TTGTTGAACCGCAAGGTGC -3'
Rev5g	5'-TGCTAATGGCCGCGATATC -3'

Primer name	Primer sequence
Rev6g	5'-TTGCCGGCGTTCTTGGAG -3'
Rev7p	5'-AAGCCTTGATCGGTACTGG -3'
Rev8gb	5'-ACTAATGAGATCTCCTCGAG -3'
Rev9p	5'-AGCTTGCGGCCGCGGTAC -3'
Rev10g	5'-ATCTCTCGATTCGACATGGG -3'
Rev11g	5'-TGGATGGTGTGGGTCATTC -3'
Rev12g	5'-AACTCATCCGAGTATCTTGG -3'
Rev13p	5'-ACAGGTGTCCTTAACCCTAC -3'
Rev15p	5'-CTTGGTACCTACTTCTTCC -3'
Rev17B	5'-ATGAGTAGCAGCACGTTCC -3'
Rev21g	5'-TCCTTCTGCTCGGAGATTAC -3'

2.5.4 Sequence analysis

The assembled sequence of the malolactic cassette from ML01 was aligned to an assembled sequence containing previously published sequences. The published sequences for *URA3* and the *PGK1* promoter and terminator sequences were obtained from the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/), and for *mae1* and *mleA* from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The sequence of the malolactic cassette was also analysed for the presence of ORFs of more than 100 codons in length. All analyses were completed using Discovery Studio Gene v1.1 (Accelrys, San Diego, USA).

2.5.5 Genetic stability of the malolactic cassette in the genome of ML01

The ML01 strain produced as ADY (large-scale 'trade' fermentation) (Bio Springer, Maisons-Alfort, France) was inoculated into filter-sterilised Chardonnay grape must from fruit harvested in 2004 (23.75 Brix, pH 3.41, TA 8.78 g/L, malate 5.5 g/L, YANC 401.2 mg/L, 25 mg/L total SO_2) by Calona Vineyards, Okanagan Valley, BC.

ML01 was inoculated at a concentration of 0.05 g/L into a 500 mL sterile Erlenmeyer flask containing 500 mL grape must. The fermentation flask was fitted with a disinfected vapour lock and was incubated at 20 °C for 6 days. The flask was stirred once daily and samples for monitoring OD were obtained by puncturing the #6.5 rubber stopper (Fermenthaus, Victoria, Canada) with a sterile syringe and 18G x 3" needle (Air-Tite, Virginia Beach, USA). After absorbance readings, samples were centrifuged and the supernatant stored at –30 °C until chemical analyses were completed. L-Malic acid, and L-lactic acid concentrations were determined by enzymatic analysis (Megazyme, Wicklow, Ireland). On day 6 yeast cells were plated onto YPD medium and incubated at 30 °C for 4 days. Four hundred and four randomly selected colonies were inoculated into 200 µL of synthetic juice and incubated at 20 °C for 6 days. The presence/absence of L-lactic acid in each micro-vinification was determined by enzymatic analysis (Megazyme, Wicklow, Ireland).

2.5.6 Global gene expression analyses

One mL of re-hydrated ML01 (25 mg/mL) and one mL of S92 (25 mg/mL) were each inoculated in triplicate into 500 mL sterile flasks (with an additional 130 mL head space), containing 500 mL of synthetic must and a magnetic stir bar. Each fermentation flask was fitted with a disinfected vapour 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 OD were obtained by puncturing the #6.5 rubber stopper (Fermenthaus, Victoria, Canada) with a sterile syringe and 18G x 3" needle (Air-Tite,

Virginia Beach, USA). After absorbance readings, samples were centrifuged and the supernatant stored at –30 °C until chemical analyses were completed.

At 48 and 144 hours, five 30 mL volumes from each culture were centrifuged in 40 mL tubes for 3 min at 3500 x *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 x *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-labelled 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 RNeasy Midi kit (Qiagen, Hilden, Germany). Twelve YGS98 oligonucleotide arrays (Affymetrix, Santa Clara, 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, Santa Clara, USA). Absolute analysis was completed on triplicate data for ML01 and S92 at 48 hrs and 144 hrs. 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 $<$ 0.003 across all nine comparison were called 'increasers', probe sets with 'D' or 'MD' and a p value $\geq$ 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 the NetAffx analysis centre (Affymetrix,

Santa Clara, USA) (http://www.affymetrix.com/analysis/index.affx) and SGD (*Saccharomyces* Genome Database; http://www.yeastgenome.org/).

2.5.7 Confirmation of DNA microarray results by Real-Time PCR

Confirmation of the DNA microarray data was done by semi-quantitative reverse transcriptase Real-Time PCR. Total RNA isolated for DNA microarray assays was treated with DNase I as per manufacturer's instructions (RNeasy Mini kit, Qiagen, Hilden, Germany). After three applications of DNase I, 100 ng of total RNA was used as template in a PCR amplification (Fermentas, Burlington, Canada) to show the absence of genomic DNA using primers specific to *ACT1* (Table 6). Control genomic DNA was isolated (Ausubel *et al.*, 1995) and 50 ng was used for the PCR reaction (Fermentas, Burlington, Canada). The 100 bp PCR product for *ACT1* was visualized by 4% NuSieve GTG Agarose (Cambrex Corp., East Rutherford, USA) gel electrophoresis and ethidium bromide staining (Ausubel et al., 1995). One microgram of clean total RNA was used to synthesize cDNA using Superscript and a random hexamer primer mix as per manufacturer's instructions (Invitrogen, Carlsbad, USA). Semi-quantitative real-time PCR was conducted using SYBR Green PCR Master mix and an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, USA). PCR reactions were done in triplicate using 100 ng of cDNA as template. *ACT1* was used as the control gene. The genes and primer sets used are shown in Table 6.

Table 6. Primers used in semi-quantitative reverse transcriptase real-time PCR to confirm DNA microarray data.

Gene Symbol	Primer Direction	Primer Sequence
ACT1	Forward	5'-GTTTCCATCCAAGCCGTTTTG-3'
ACT1	Reverse	5'-GCGTAAATTGGAACGACGTGAG-3'
AQR1	Forward	5'-TCGAGCAAGACAAAGCTAACGG-3'
AQR1	Reverse	5'-GCTACGACGGCCAAGAAATTTT-3'
CTT1	Forward	5'-GAGAAAGAGTTCCGGAGCGTGT-3'
CTT1	Reverse	5'-ATTCTGGTATGGAGCGGCGTA-3'
DIP5	Forward	5'-TTTGTGGCTTGGCGTACATG-3'
DIP5	Reverse	5'-GGTGATCCAACTCAAGATTCCG-3'
ENA2	Forward	5'-CATTCGACTCAACTGTGAAGCG-3'
ENA2	Reverse	5'-GCAACAACTGATGATGCTTTCG-3'
PHO84	Forward	5'-GCCATTATTGCACAAACCGC-3'
PHO84	Reverse	5'-CGAAAATTTCCATGACGTGAGG-3'
PRR2	Forward	5'-GACTGCAGAACACGCCTATTCC-3'
PRR2	Reverse	5'-TTATTTAGCGCCTCACCCGTC-3'
PUT4	Forward	5'-CATCCACGGCAGACGTGTTTA-3'
PUT4	Reverse	5'-AGCCTTGCGGAATCTCAGGTAC-3'
SUE1	Forward	5'-TTGTTTGGTGAACGTGGCACT-3'
SUE1	Reverse	5'-CCACCAATTGAATGGCAACAG-3'
YPC1	Forward	5'-ACTGCTTGAACCACACGGATG-3'
YPC1	Reverse	5'-TGACGTTGAGCGTAATGACCC-3'
YML089C	Forward	5'-CAATGAAATGCAAGAGCGCA-3'
YML089C	Reverse	5'-GGAATTGTAAGGCACACCGAGT-3'

2.5.8 Transcription of *URA3* and transgenes *mae1* and *mleA*

Transcription of *URA3* and transgenes *mae1* and *mleA* was analysed by reverse transcriptase PCR (One-Step RT-PCR, Qiagen, Hilden, Germany). RNA isolation, DNase treatment and clean-up were completed as previously described and 1 μg of clean total RNA was used as a template for each RT-PCR reaction. *URA3* primers For15B and Rev3bp, *mae1* primers For4g and Rev11g, and *mleA* primers For9p and Rev6g are shown in Table 5. Primers ACTFor269 (5'-AAGAGAGGTATCTTGACTTTACG-3') and ACTRev1c (5'-ACAATACCAGTAGTTCTACCG-3') were used as internal controls.

Control genomic DNA was isolated (Ausubel *et al.*, 1995) and 50 ng was used for each RT-PCR reaction (One-Step RT-PCR, Qiagen, Hilden, Germany). The 269 bp, 783 bp, 807 bp and 499 bp PCR products for *ACT1*, *mae1*, *mleA*, and *URA3* respectively, were visualized by 1.5% agarose gel electrophoresis and ethidium bromide staining (Ausubel et al., 1995).

2.5.9 Analysis of the proteome of ML01

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 8 (Glen Mills, Clifton, USA) with intermittent vortexing. After lysis the samples were centrifuged at 18000 x *g* for 2 min at 2 °C. The supernatant was transferred to clean tubes and centrifuged at 18000 x *g* for 10 min at 2 °C. Trichloroacetic acid 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 18000 x *g* for 30 min at 2 °C. Precipitated proteins were washed twice with 100% acetone (-20 °C) and centrifuged at 2 °C for 30 min and 20 min. Pellets were dried at room temperature until all visible liquid was evaporated and then stored at -80 °C. Proteins were examined by one dimensional SDS-PAGE (Ausubel et al., 1995) and total protein determination was completed using a commercial Bradford assay reagent (BioRad, Hercules, USA).

Protein pellets were shipped on dry ice to Genome BC Proteomics Centre for iTRAQ analysis (University of Victoria, Victoria, Canada). Denaturation, blocking of

cysteines, digestion with trypsin and labelling with iTRAQ tags were done according to the iTRAQ protocol (Applied Biosystems, Foster City, USA). Protein samples were labelled 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 min. 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 (Dionex/LC Packings, Sunnyvale, USA). 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 (v. 1.0) (Applied Biosystems, 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) (Celera, Rockville, USA) was used for the protein searches. Methyl methanethiosulphonate (MMTS) modification of cysteines was used as a fixed modification. All results were written to a Microsoft Access database (Microsoft, Redmond, USA). In order to reduce protein redundancy, experimental software, ProGroup viewer (Applied Biosystems, 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. Weighted average ratios were calculated for protein ratios with a p-value < 0.05 across all comparisons.

2.6 Phenotypic characterisation of ML01

2.6.1 Growth kinetics of ML01

A Bioscreen C™ Automated Microbiology Growth Curve Analysis System (Thermo Electron Co., Waltham, USA) was used to examine the growth kinetics of the ML01 or S92 yeasts in YPD and commercially available Chardonnay must. A single colony from stock culture plates was inoculated into 5 mL of YPD broth which was incubated at 30 °C until the culture reached stationary phase. One µL of the culture was

inoculated into one well of a microtiter plate (Thermo Electron Co., Waltham, USA) containing 99 μL of YPD test media or Chardonnay must. A total of nine replicates and one blank control were inoculated per type of test media. The plate was incubated at 30 °C for 64 h with continuous shaking at high intensity. Optical densities for all wells were measured every 10 min using the wide band measurement filter (OD_{600nm}). Maximum specific growth rate (μ_{max}) was calculated by converting the OD readings to natural log values and the maximum slope during the exponential growth phase was determined for each replicate. Generation time was calculated as described by (Reed and Nagodawithana, 1991). Data was analyzed using Excel 2000 (Microsoft, Redmond, USA).

2.6.2 Utilization of malate as sole carbon source by ML01 and S92

Single colonies from stock culture plates of the parental strain S92 or the ML01 strain were transferred to 5 mL YPD broth and were incubated at 30 °C until the cultures reached stationary phase. Four 1-L Erlenmeyer flasks were filled with 150 mL of modified YPD containing 2% L-malic acid and were closed with cotton plugs. Two of the flasks were inoculated with ML01 to achieve an initial OD_{600nm} of 0.01 and two additional flasks were similarly inoculated with S92. The flasks were incubated at 30 °C under constant agitation at 180 rpm. Samples were removed at each sampling time and optical densities were measured at abs 600 nm. The samples were then centrifuged in a micro-centrifuge at 18,000 x *g* for 10 min (Eppendorf, Hamburg, Germany), and supernatants were frozen at -30 °C for later analysis. The OD_{600nm} readings from the two ML01 flasks and the two S92 flasks were averaged and growth curves were generated

using Microsoft Excel 2000 (Microsoft, Redmond, USA). L-Malic concentrations were determined in triplicate for each sample at 350 h by enzymatic analysis (Roche, Basel, Switzerland).

2.6.3 Winemaking with ML01

Wines were made from Chardonnay and Cabernet Sauvignon grapes. Chardonnay grape must from fruit harvested in the year 2000 (22.5 Brix, pH 3.18, TA 13.45 g/L, 9.2 g/L malate, YANC 285.7 mg/L, 60 mg/L total SO_2) was obtained from Quails' Gate Estate Winery, Okanagan Valley, BC, Canada. Two carboys (11.7 L capacity) and two flasks (3 L capacity) of Chardonnay grape must were directly inoculated with 0.2 g/L of ML01 and four carboys and four flasks were directly inoculated with 0.2 g/L of S92. After the alcoholic and MLF fermentations were completed by the malolactic yeast ML01, the wines were racked, topped-up, provided with sulphite (40 mg/L total SO_2), and aged at 7 °C for nine months. The alcoholic fermentations were considered complete once the wines reached a specific gravity of 0.990 – 0.996. After alcoholic fermentation with the parental strain S92 was completed, two carboys were racked, topped-up, sulphited (40 mg/L total SO_2), and aged at 7 °C for nine months. Wines in the remaining two carboys fermented by S92 were racked, topped-up, and inoculated with a freeze dried preparation of *O. œni* as per manufacturer's recommendations (Vinoflora Oenos, Chr. Hansen, Hoersholm, Denmark) and placed at 20 °C. The two carboys inoculated with *O. œni* were re-inoculated one week later with *O. œni* (recommended concentrations) and again two weeks later with a double inoculum of *O. œni* and placed at 25 °C. Six months after the initial inoculation with *O. œni*, the

MLF was stuck at 0.25 g malate/L in one carboy and at 2.98 g/L in the second carboy. The wine was racked, sulphited (40 mg/L total SO_2) and aged at 7 °C for 3 months. After ageing, all wines were racked and bottled (total SO_2 adjusted to 40 mg/L). Sensory analyses were performed after four months and again after four years of bottle ageing at 14 °C. Physical, chemical and sensory analyses were performed on at least three bottle replicates from one carboy. Analyses for wines inoculated with S92 and *O. œni* were conducted on the carboy with a residual of 0.25 g malate/L.

In order to obtain sufficient biological replicates to analyse the volatile compounds in wine by GC/MS, the fermentations with ML01, S92 and S92 plus *O. œni* were repeated in Chardonnay grape must from fruit harvested in 2004 (23.75 Brix, pH 3.41, TA 8.78 g/L, malate 5.5 g/L, YANC 401.2 mg/L, 25 mg/L total SO_2) by Calona Vineyards, Okanagan Valley, BC, Canada. Eight 500-mL flasks, two carboys and two 2-L flasks were directly inoculated with 0.05 g/L of ML01. Sixteen 500-mL flasks, four carboys and four, 2-L flasks were directly inoculated with 0.05 g/L of S92. 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 the alcoholic and MLF fermentations were completed by the malolactic yeast ML01, the wines were racked, topped-up, sulphite levels adjusted to 0.8 mg/L molecular SO_2), and kept at 4 °C for 11 months. After alcoholic fermentation with the parental strain S92 was completed, half the wines were racked, topped-up, sulphite levels adjusted to 0.8 mg/L molecular SO_2, and aged at 4 °C for 11 months. The remaining S92 produced wine was racked, topped-up, and inoculated with *O. œni* as per manufacturer's recommendations (Vinoflora Oenos, Chr. Hansen, Hoersholm, Denmark) and placed at 20 °C. The S92 wine inoculated with *O. œni* was re-

inoculated 2 weeks later with a double inoculum of *O. œni*. Nine days after the double inoculum the temperature was increased to 25 °C. One month after the temperature increase, 1.5 L of wine undergoing MLF was mixed with wine not showing active MLF. Two months later a double inoculum of *O. œni* and 50 mg/L of Leucofood (Gusmer Enterprises, Mountainside, USA) were added. Nine months from the initial inoculation with *O. œni*, the bacterial MLF was completed. The wine was racked, sulphite adjusted to 0.8 mg/L molecular SO_2 and aged at 4 °C for two months. After ageing, all wines were racked and bottled. Flasks for chemical analyses (500 mL fermentations) were stirred once before daily sampling in order to obtain a homogenous sample. Sampling was done anaerobically as described for the transcriptome/proteome study (Section 2.5.6). Physical, chemical and GC/MS analyses were performed on three biological replicates.

Cabernet Sauvignon must (22.9 Brix, pH 3.72, TA 7.41 g/L, malate, 6.2 g/L, YANC 325.9 mg/L, 30 mg/L total SO_2) was obtained from Hawthorne Mountain Vineyards, Okanagan, BC in the 2000 vintage. The grapes were crushed and must was vinified without skin contact at the Pacific Agri-Food Research Centre (PARC) using standard winemaking procedures. Fermentations were conducted using the same procedures as described for the Chardonnay 2000 must. The two carboys inoculated with *O. œni* (Vinoflora Oenos, Chr. Hansen, Hoersholm, Denmark) after the completion of the alcoholic fermentation were re-inoculated two weeks later with *O. œni* (recommended concentrations). After completion of the MLF the wine was racked, sulphited (80 mg/L total SO_2) and stored at 7 °C for eight months. After ageing, all wines were racked and

molecular SO_2 adjusted to 0.4 mg/L before bottling. Physical and chemical analyses were performed on at least three bottle replicates.

2.6.4 Analyses of must and wine

Microbiological populations in grape musts were determined in samples diluted with 0.1% peptone that were spread onto Modified Rogosa Agar (Pilone and Kunkee, 1976) and Dichloran Rose Bengal Agar (Difco, Becton, Dickinson and Co., Sparks, USA). Plates were incubated for two and seven days at 25 °C to enumerate yeast and lactic acid bacteria, respectively. Plating was done in duplicate. Soluble solids (Brix) in wines were determined by specific gravity and with a Reichert ABBE Mark II Refractometer (Reichert Analytical Instruments, Depew, USA). Titratable acidity was determined according to AOAC method number 926.12 and by using a Metrohm 686 Titroprocessor (Metrohm, Herisau, Switzerland) (tartaric acid as reference). The pH was determined using a Corning 455 pH/ion analyzer (Corning, Corning, USA) and a Metrohm 686 Titroprocessor (Metrohm, Herisau, Switzerland). The viscosity of the wine was determined with a Brookfield viscometer Model DV-II (Brookfield Engineering Labs, Stoughton, USA) equipped with a LV spindle. The viscometer was set at 60 rpm at 25 °C. Colour according to CIELAB tristimulus scales was measured in Chardonnay 2000 wines and Cabernet Sauvignon after four months of bottle ageing at 14 °C in the dark. The wines were analyzed with a Beckman DU640B scanning spectrophotometer (Beckman Coulter, Fullerton, CA) and $A_{420nm+520nm}$ with an Ultrospec 3000 (GE Healthcare, Chalfont St. Giles, UK). All colour analyses were completed without dilutions. Malate, lactate, glucose, fructose, acetate and ethanol determinations

were done by enzymatic analysis (Megazyme, Wicklow, Ireland). Physical and chemical analyses were performed on at least three biological replicates or at least three bottle replicates.

2.6.5 Sensory analysis

Chardonnay wines produced in 2000 were evaluated in duplicate after four years of bottle ageing at 14 °C for colour, aroma, flavour-by-mouth and overall quality, by thirteen trained judges. The methodology of the sensory study was approved by the Clinical Research Ethics Board at the University of British Columbia (Appendix C). All tasters provided written informed consent to participate in the study (Appendix D). Prior to the sensory evaluation, five experienced wine judges bench tested the samples to select attributes that would characterise the wines. The bench testers were also instructed to screen out any defect wines. The attributes selected for aroma were fruity and buttery; the attributes selected for flavour-by-mouth/taste were fruity, sweet, buttery, acidity and body (Table 7).

Thirteen judges (six females and seven males) evaluated the wines in duplicate using a completely randomized design. All judges were experienced wine tasters. Judges participated in a training session to become familiarized with the tasting protocol and unstructured line scale. The tasting/rinsing protocol required judges to swirl and sniff the glass for the aroma assessment, and sip and swirl the wine in their mouth for the flavour/ quality assessment. Judges were instructed to rinse well with water between assessments. All aroma, flavour-by-mouth and quality assessments were conducted in individual tasting booths. A 30 mL wine sample was presented at room temperature in 250 mL

IANO- ISO glasses. Glasses were labelled with a three-digit random number and covered with plastic Petri dishes. The colour assessment was conducted on 20 mL samples in 25 mL plastic Petri dishes. Evaluations were conducted against a white background and were evaluated under natural light. Different random codes were used for the colour, aroma, flavour-by-mouth and quality evaluations to prevent bias among assessments. Evaluations took place on two successive afternoons. Judges scored each attribute on a 10 cm unstructured line scale, anchored at 1 cm and 9 cm with low and high (or light and dark for colour). Data were quantified by measuring the distance of the judge's mark from the origin.

Table 7. Definition of sensory attributes for visual, olfactory and gustatory evaluations

Attributes	Definitions
Visual	
Colour	Relative degree of colour intensity from light yellow to dark yellow
Olfactory	
Fruity Aroma	Intensity of fruity aromas (generic) from low to high
Buttery Aroma	Intensity of diacetyl and/or lactic qualities from low to high
Gustatory	
Fruity	Intensity of fruity tastes (generic) from low to high
Sweet	Intensity of sweetness from low to high
Buttery Taste	Intensity of diacetyl and/or lactic qualities from low to high
Acidity	Intensity of sour taste from low to high
Body	Tactile sensation (mouth coating) differentiating low-ethanol (thin) from high-ethanol (full-bodied) wines within the context of these wines
Overall Quality	A composite response of all sensations (Visual, aroma, flavor and aftertaste) from low to high quality

2.6.6 Analysis of volatile compounds in wine by gas chromatography/mass spectrometry

Volatile compounds in Chardonnay wines were analyzed by GC/MS headspace analysis as described by Danzer et al., (1999) except that no SPME was used. A 10 mL sample of wine was placed into a 20 mL headspace vial containing 3 g of NaCl and then positioned into the headspace auto sampler. Sample equilibration was done at 85 °C for 10 min with agitation set on high. 3-octanol (100 μL of 0.565 mg/L) was used as the internal standard.

Volatile compounds were analyzed and quantified using an Agilent 6890N GC (Agilent Technologies, Palo Alto, USA) interfaced to a 5973N Mass Selective Detector. A 60 m x 0.25 mm ID, 0.25 μm thickness DBWAX fused silica open tubular column (J&W Scientific, Folstom, USA) was used. The carrier gas was ultra high purity helium at a constant flow of 1.3 mL/min. The headspace sample valve and transfer line temperatures were set at 100 °C and 110 °C, respectively. The GC oven temperature was initially set at 40°C for 5 min, then raised to 100 °C at 5 °C/min, then raised to 200 °C at 3 °C/min, held for 1 min, and then raised to 240 °C at 20 °C/min. The injection volume was 1 mL and the injection mode was split with a ratio of 10:1. The MS was operated in scan mode (35-400). The analysis was completed in triplicate, data were analyzed using Enhanced Chemstation software (MSD Chemstation Build75, Agilent Technologies, Palo Alto, USA), and compounds found were matched with the Wiley275 library (Wiley and Sons, Hoboken, USA).

2.6.7 Quantification of ethyl carbamate in wine produced by ML01

Determination of ethyl carbamate concentration in wine was done by solid phase microextraction and GC/MS as described by Coulon et al., (2006). Chardonnay wines were heated at 70 °C for 48 h to maximize ethyl carbamate production.

2.6.8 Effect of residual ML01 populations on MLF in wine fermented primarily with the parental strain S92

The effect of different concentrations of ML01 on the decarboxylation of malic acid to lactic acid was examined in 500 mL fermentation flasks containing 500 mL of synthetic must inoculated with S92 and ML01. S92 and ML01 were inoculated in the following ratios: 50:0.005, 50:0.05, 50:0.5, 50:5 and 50:50 mg/L. Control fermentations were inoculated to a final concentration of 50 mg/L, 55 mg/L, and 100 mg/L with S92 or ML01 strains alone. The flasks were incubated at 20 °C without shaking and OD_{600nm} was measured in two samples from each flask at each sampling time. After measurement of OD, individual samples were centrifuged (18000 x *g*, 10 min) and the supernatants were stored at -30 °C. L-Malic and L-lactic acid concentrations were determined by enzymatic analysis as described earlier. The OD_{600nm} readings and the L-malic or L-lactic acid concentrations from each replicate were averaged and plots were generated using Microsoft Excel 2000 (Microsoft, Redmond, USA). Fermentations were conducted in duplicate.

2.6.9 Post-fermentation viability of ML01

Fermentation flasks (250 mL capacity) containing 200 mL Chardonnay grape must were inoculated with 100 mg/L of re-hydrated ML01 or S92 yeast. Each fermentation flask was fitted with an ethanol-disinfected vapour lock. The flasks were incubated at 20 °C without agitation for 269 days. Samples were removed from stirred flasks after 0, 6, 20, 50, 81, 115, 170, and 269 days. Samples were vigorously vortexed, serially diluted in 0.1% peptone water (with vortexing between serial dilutions) and cultured on YPD agar at 30 °C to estimate viable cell populations. All fermentations were conducted in duplicate. Results were expressed as mean colony forming units (CFU)/mL.

2.7 Statistical analyses

A three-way analysis of variance (ANOVA) was used to examine the main effects of judge, wine and replication for each of the sensory attributes. All two-way interactions were calculated (judge x wine, judge x replicate, and wine x replicate), in order to track the panel consistency, judge reproducibility and sample-to-sample variation, respectively. Mean scores and least significant differences (Fisher LSD $p < 0.05$) were calculated. The mean scores with significant differences among wines were plotted using a cobweb diagram (Figure 17). A correlation principal component analysis (PCA) was performed on the mean sensory scores (n=13) from each of the replications (n=2). This allowed the location of both replicates to be on the diagram (Figure 18). ANOVAs were used to pre-select the most relevant attributes prior to PCA analysis. The term 'overall quality' was

not included in the PCA in order to clearly delineate the objective flavour profile analysis (attribute intensities) from the more subjective quality assessment.

One-way ANOVAs were used to evaluate the variation in physico-chemical properties and volatile compounds among the wines. Duncan's post-hoc tests were performed to determine which treatment means were statistically different for each of the measurements ($p < 0.05$). Differences in generation times between ML01 and S92 as well as differences in sugar concentrations of wines produced by ML01 and S92 were evaluated using a two-tailed independent samples t tests ($p < 0.05$). All statistical calculations were performed using Minitab 14 (Minitab, State College, USA), SPSS v. 11.5 (SPSS, Chicago, USA) and Excel 2002 (Microsoft, Redmond, USA).

3 RESULTS

3.1 Integration of the malolactic cassette into the genome of S92

After screening approximately 2000 yeast transformants for integration of the malolactic cassette (Figure 1), five clones were found that produced lactic acid from malic acid using the screening method developed for the production of L-lactic acid (Figure 2). After recovery of the malolactic clones in YPD and subsequent inoculation into synthetic must, clone 2 was removed since it no longer continued to produce lactic acid. The PCR with primers specific to *PGK1*p and downstream of the 3'-end integration site were used to show that the four remaining clones had the malolactic cassette integrated into the *URA3* locus (Appendix E).

Figure 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 that encodes resistance to phleomycin.

3.2 Functionality of malolactic wine yeast

Three individual colonies from each malolactic clone were inoculated into synthetic juice containing 4.5 g/L of malate. Each of the three selected colonies from clones 1, 3 and 4, and two colonies from clone 5 was able to degrade the 4.5 g/L of malate in the synthetic must to = 0.05 g/L by day six (Table 15, Appendix F). All three of the selected colonies from clone 4 were also capable of completing the alcoholic fermentation within 14 days (Tables 16 and 17, Appendix F). Clones 1, 3 and 5 were

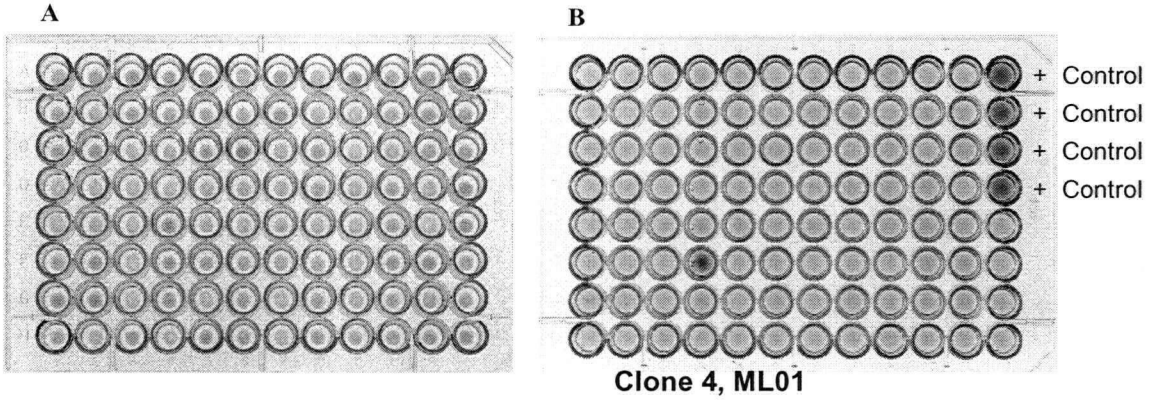

Figure 2. Test for L-lactic acid production in S92 cells transformed with the malolactic cassette and pUT332. (A) S92 industrial wine yeast transformants growing in a micro-well plate containing 200 µL of synthetic must per well (4 days at 30 °C). (B) Micro-well plate containing 75 µL of the L-LDH/NAD/phenazine methosulfate/nitro blue tetrazolium reaction mixture per well and 25 µL of supernatant from micro-wells in panel A. Wells containing L-lactic acid were a purple/blue colour. The positive controls were S92 yeast transformed with pJH13 (a CEN-based plasmid containing the malolactic cassette and KanMX marker) (Husnik, 2001).

either unable to complete the alcoholic fermentation by day 14 or did not ferment as rapidly as clone 4 by day 7 (Appendix F). The residual sugar (glucose and fructose) in the synthetic must after 14 days was = 1.3 g/L for all of the colonies selected from clone 4 (Appendix F). Based on these results, malolactic clone 4 (ML01) was selected for pilot-scale ADY production. ML01 produced as ADY was capable of decarboxylating 4.46 ± 0.02 g/L of malate (99.1%) within the first three days and producing equimolar amounts of lactic acid (3.01 ± 0.07 g/L) by day 14. In contrast the parental strain S92 consumed 0.89 ± 0.1 g/L of malate and only 0.02 g/L of lactate was detected in the media after 14 days of fermentation. Residual sugar (glucose and fructose) was 1.71 ± 0.99 g/L and 3.79 ± 0.95 g/L for ML01 and S92, respectively. The final pH of the fermentations was 3.31 ± 0.01 and 3.15 ± 0.01 for ML01 and S92, respectively.

3.3 Genetic characteristics of ML01

3.3.1 Confirmation of the identity of the parental strain

Chromosome separation by pulsed field gel electrophoresis (Figure 3) and PCR amplification patterns of genomic DNA regions in between d elements of the Ty1 retrotransposon (Appendix G) confirmed that the parent strain of ML01 was S92.

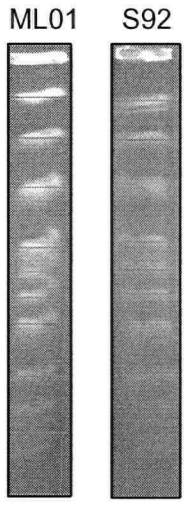

Figure 3. ML01 and S92 chromosomes as separated by pulsed field gel electrophoresis. Chromosomal patterns for ML01 and the parent S92 were identical. In addition, PCR profiles of amplified DNA between Ty1 retrotransposon d sequences were also identical for both strains (Appendix G).

3.3.2 Correct integration of the malolactic cassette into the genome 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 kbp and 2.8 kbp DNA fragments, and one signal was detected for S92 corresponding to a 1.7 kbp DNA fragment (Figure 4). 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 (Figures 5 and 6), and the *PGK1* promoter (Appendix H). Blots with the *mae1, mleA* and *PGK1* promoter probes confirmed the presence of these genes in the ML01 strain and correct integration into only the *URA3* locus.

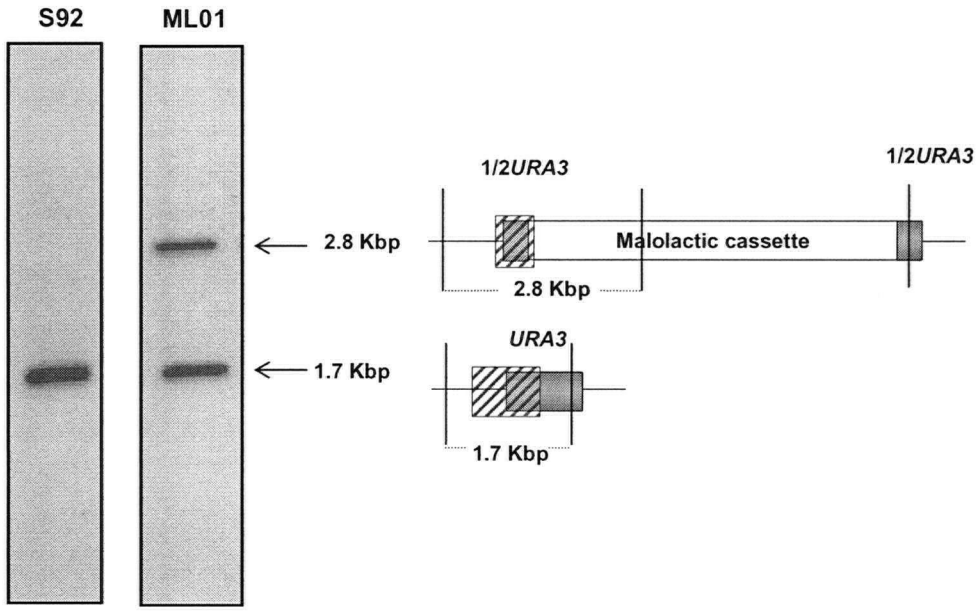

Figure 4. Integration of the malolactic cassette into the *URA3* locus of S92 was confirmed by Southern blot analysis using a *URA3* probe. The schematic representation illustrates the *Nsi*I restriction sites with vertical lines and the *URA3* probe (area with hatched boxes in panel on the right).

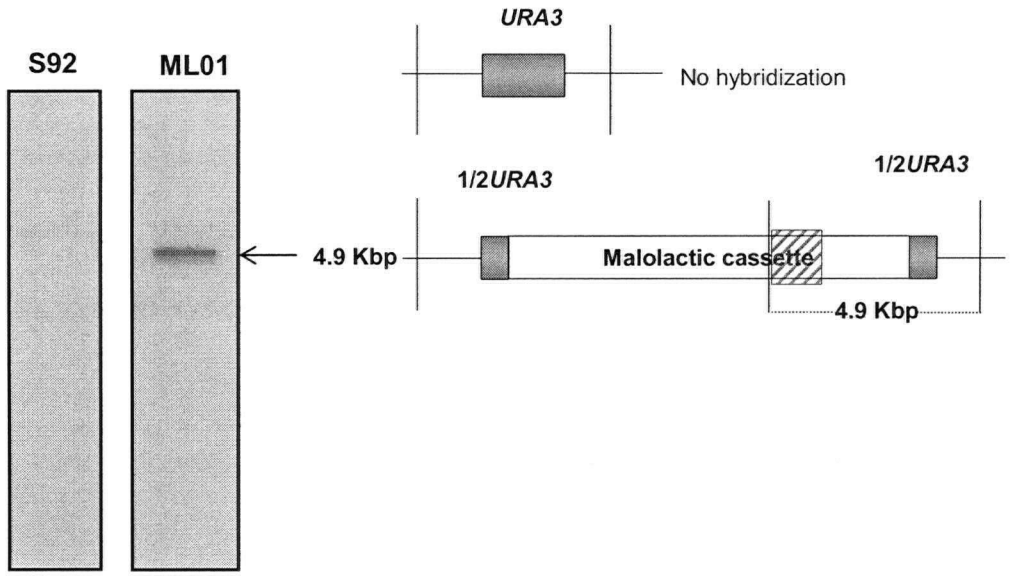

Figure 5. Integration of the malolactic cassette into the *URA3* locus of S92 was confirmed by Southern blot using a *mleA* probe. The schematic representation illustrates the *Pvu*II restriction sites with vertical lines and the *mleA* probe hybridization site is depicted as a hatched box in the panel on the right.

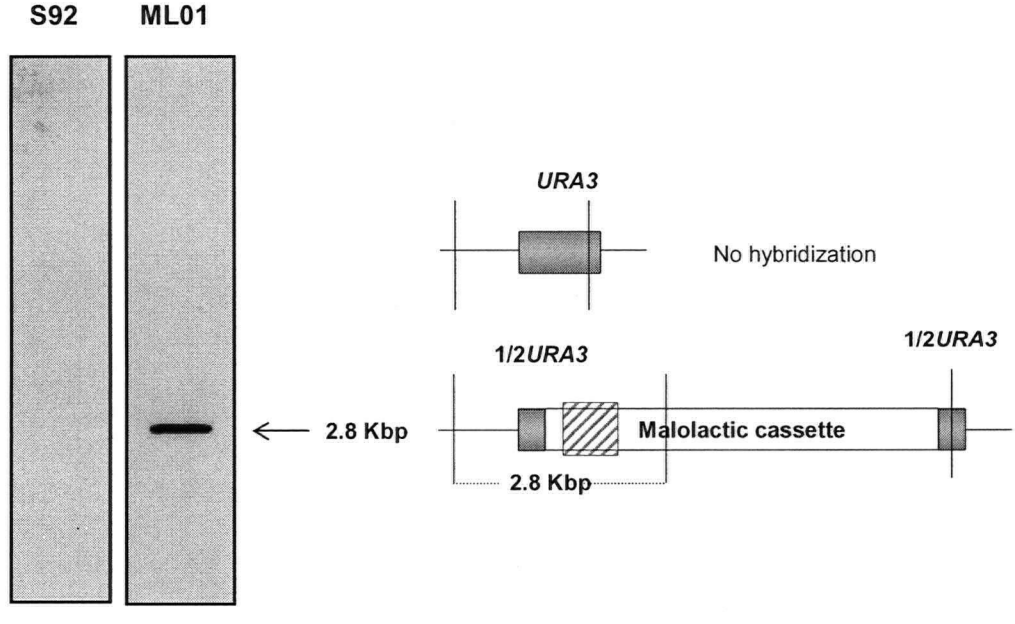

Figure 6. Integration of the malolactic cassette into the *URA3* locus of S92 was confirmed by Southern blot using a *mae1* probe. The schematic representation illustrates the *Nsi*I restriction sites with vertical lines and the *mae1* probe hybridization site is depicted as a hatched box in the panel on the right.

Both S92 and ML01 form ascospores (Appendix I). 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.

3.3.3 Genetic stability of the malolactic cassette in the genome of ML01

After large-scale ADY production and completion of MLF in Chardonnay grape must, 401/404 randomly chosen ML01 colonies tested positive for the MLF phenotype (99.3 ± 1.0 %, $p < 0.05$). The integration of the malolactic cassette in the *URA3* locus of S92 strain is therefore sufficiently stable for ADY production and the subsequent MLF during the winemaking process.

3.3.4 ML01 does not contain *bla* and Tn*5ble* antibiotic markers

After transformation, ML01 was cultured successively for seven 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 (Figure 7). A Southern blot using a probe specific to the Tn*5ble* genes of pUT332 revealed that this gene was absent in ML01 (Figure 7). Southern blot analyses also verified that ML01 does not contain the *bla* gene (nor an additional 970 bp of bacterial derived pUT322 sequences) (Figure 8).

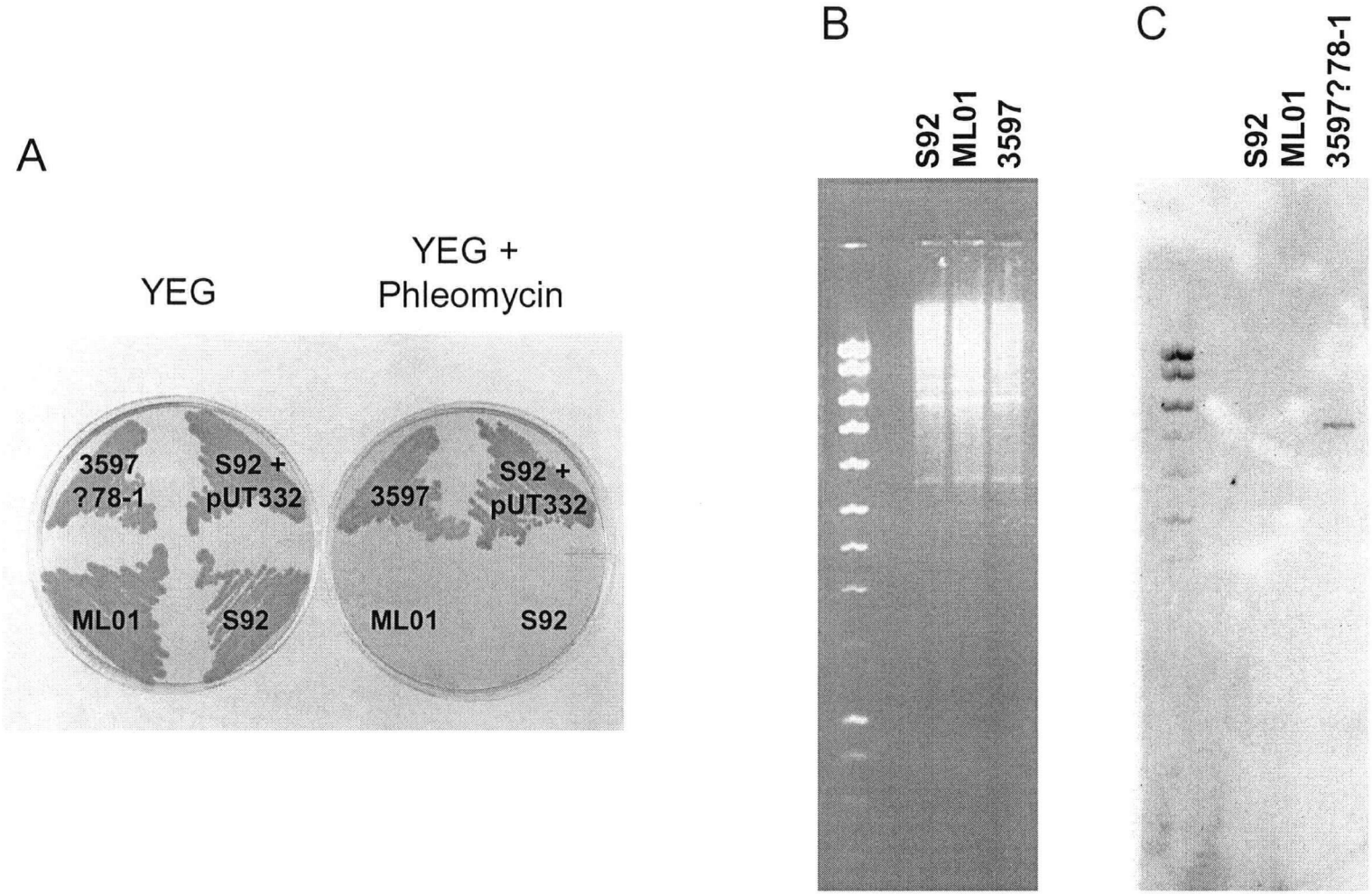

Figure 7. The phleomycin resistance gene is absent in the genome of ML01. (A) Growth of ML01, S92, S92 transformed with pUT332 and *S. cerevisiae* 3597 ? 78-1 (integrated Tn*5ble* gene) yeast strains on YEG media with or without 100 µg/ml phleomycin. (B) Agarose gel showing fractionated genomic DNA of S92, ML01 and *S. cerevisiae* 3597 ? 78-1 digested with *Nco*I restriction enzyme. (C) Southern blot analysis of the genomes of S92, ML01 and *S. cerevisiae* 3597 ? 78-1 probing for the presence of the Tn*5ble* gene.

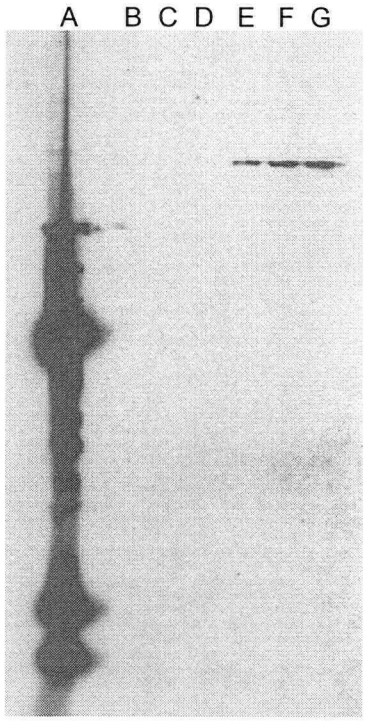

Figure 8. The ampicillin resistance gene and 970 bp of pUT332 non-*Saccharomyces* vector are absent in the genome of ML01. Lane A represents a DNA Ladder, lane B contains 3 µg of *Eco*RV digested S92 genomic DNA, lanes C and D each contain 3 µg of ML01 genomic DNA, lanes E to G contain increasing concentrations of *Eco*RV digested DNA from S92 transformed with pUT332 (0.3 µg, 0.75µg and 1.5 µg). A description of the probe used is given in Appendix A.

3.3.5 Sequence of the malolactic cassette integrated into the genome of ML01

The sequence of the malolactic cassette integrated at the *URA3* locus as well as 35 bp upstream and 184 bp downstream of the genomic flanking sequences were verified by sequencing (Figure 9). A detailed description of the sequence is given in Appendix J.

Alignment of DNA sequences: ML01 and Native *URA3* locus

Upper line: ML01, from 1 to 8901
Lower line: Native *URA3* locus, from 1 to 2081

```
1      CAGCAATTAATACTTGATAAGAAGAGTATTGAGAAGGGCAACGGTTCATCATCTCATGGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1      CAGCAATTAATACTTGATAAGAAGAGTATTGAGAAGGGCAACGGTTCATCATCTCATGGA

61     TCTGCACATGAACAAACACCAGAGTCAAACGACGTTGAAATTGAGGCTACTGCGCCAATT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61     TCTGCACATGAACAAACACCAGAGTCAAACGACGTTGAAATTGAGGCTACTGCGCCAATT
       ............................................................
       .....continuation of malolactic cassette.....
       ............................................................

8641   CGCTGCCTTGGGACAAGGCTTGGGCCGATAAGGTGTACTGGCGTATATATATCTAATTAT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1821   CGCTGCCTTGGGACAAGGCTTGGGCCGATAAGGTGTACTGGCGTATATATATCTAATTAT

8701   GTATCTCTGGTGTAGCCCATTTTTAGCATGTAAATATAAAGAGAAACCATATCTAATCTA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1881   GTATCTCTGGTGTAGCCCATTTTTAGCATGTAAATATAAAGAGAAACCATATCTAATCTA

8761   ACCAAATCCAAACAAAATTCAATAGTTACTATCGCTTTTTTCTTTCTGTATCGCAAATAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1941   ACCAAATCCAAACAAAATTCAATAGTTACTATCGCTTTTTTCTTTCTGTATCGCAAATAA

8821   GTGAAAATTAAAAAAGAAAGATTAAATTGGAAGTTGGATATGGGCTGGAACAGCAGCAGT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2001   GTGAAAATTAAAAAAGAAAGATTAAATTGGAAGTTGGATATGGGCTGGAACAGCAGCAGT

8881   AATCGGTATCGGGTTCGCCAC
       ||||||||||||||||||||
2061   AATCGGTATCGGGTTCGCCAC
```

Figure 9. The upstream and downstream sequences flanking the malolactic cassette in *S. cerevisiae* ML01 are 100% identical. The sequence obtained from ML01 is shown in the upper line and the native sequences are shown in the lower line. Only a partial sequence of the malolactic cassette is displayed. The *SrfI* half sites (integration sites) are indicated in bold and underlined.

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 (Appendix K). 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 (Appendix K). The first difference resulted in a change from aspartic acid to glutamic acid (aa 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 new ORFs were created during construction of the malolactic cassette; these ORFs were primarily composed of *Saccharomyces* sequences (Figure 10). In addition to the *S. cerevisiae* sequences, three ORFs contained one or two restriction endonuclease sites that were required for construction of the cassette. Novel ORF 1 (435 bp) contains one *Kpn*I (6 bp) restriction endonuclease site; novel ORF 2 (663 bp) contains a *Kpn*I and a *Not*I (8 bp) site; novel ORF 3 (636 bp) contains a *Not*I site; novel ORF 4 (360 bp) is entirely composed of *S. cerevisiae* sequences.

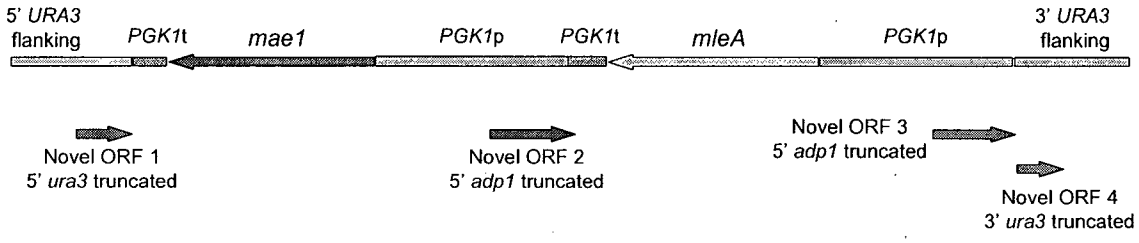

Figure 10. A schematic representation of new ORFs of more than 100 codons generated during construction of the malolactic cassette; four new ORFs primarily composed of *S. cerevisiae* sequences, were created.

3.3.6 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 hours and 144 hours, corresponding to the middle of the MLF and completion of the MLF (Figure 11). The 48 h and 144 h time points also corresponded to log phase and stationary phase, respectively (Figure 12). At 48 h the ML01 strain had consumed 1.73 ± 0.04 g/L of malate and produced 0.92 ± 0.04 g/L of lactate. In contrast, the parental strain S92 had consumed 0.28 ± 0.16 g/L of malate and only 0.05 ± 0.01 g/L of lactate was detected in the media. Equal amounts of glucose/fructose remained; 169.9 ± 4.0 g/L and 166.7 ± 7.3 g/L for ML01 and S92, respectively (difference not significant at $p < 0.05$). Transcription of 19 genes was affected = two-fold in ML01 after 48 hours; 11 genes were expressed at higher levels and eight genes were expressed at lower levels (Table 8).

At 144 h the transcription of six genes was affected = two-fold; three genes were expressed at higher levels and three genes were expressed at lower levels (Table 9). After 144 hours of fermenting, the ML01 yeast had consumed all of the malate (4.47 ± 0.002 g/L) and produced 3.05 ± 0.14 g/L of lactate, whereas the control strain consumed 0.51 ± 0.05 g/L of malate and negligible amounts of lactate (0.06 ± g/L) was detected in the medium (Figure 11). The fermentation rate (monitored as ethanol production and CO_2 loss) was similar to that of the parental yeast S92 (Figure 13) and the sugar concentrations (glucose/fructose) at this time point were 37.09 ± 2.79 g/L and 36.5 ± 1.74 g/L for ML01 and S92, respectively (difference not significant at $p < 0.05$).

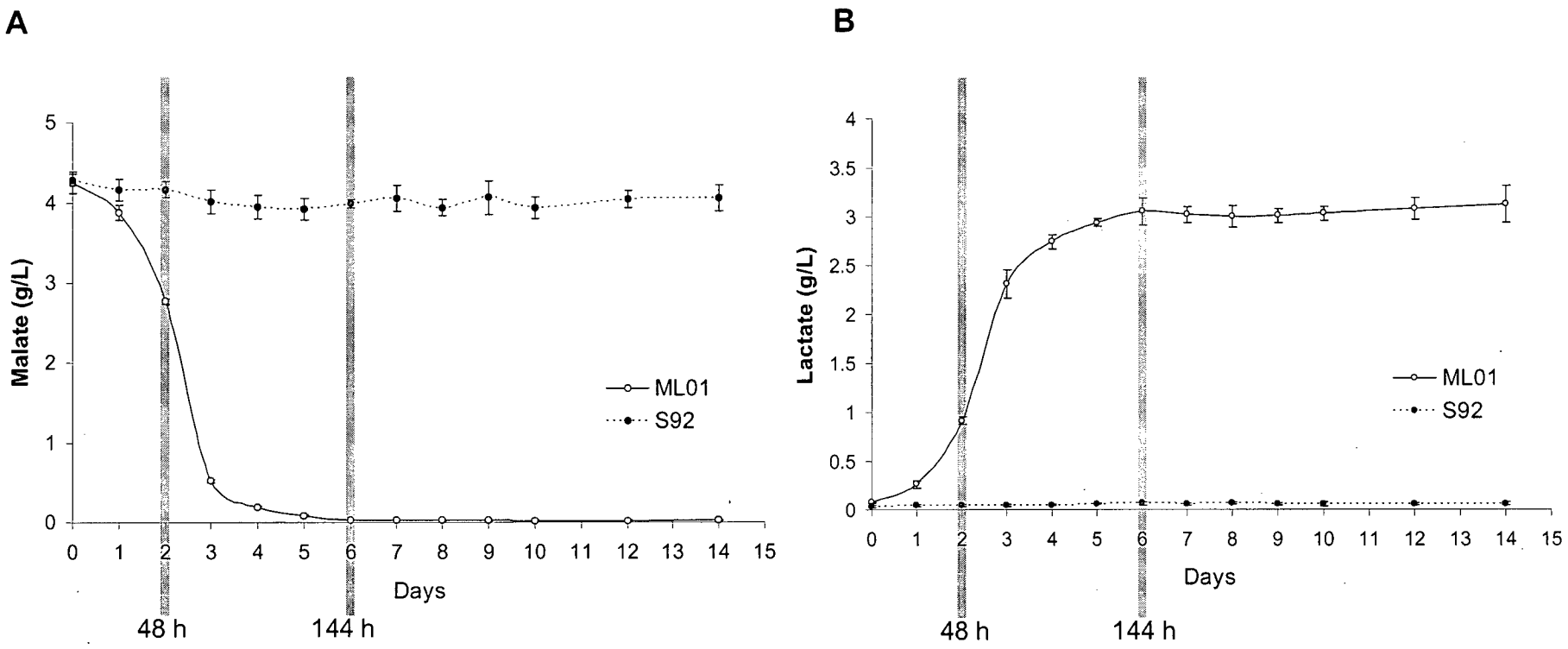

Figure 11. Malate degradation and lactate production by ML01 and S92 yeast strains in synthetic must (n=3). (A) Malate degradation by yeast strains ML01 and S92; at 144 h ML01 and S92 degraded 4.47 g/L and 0.51 g/L of malate, respectively. (B) Lactate production by ML01 and S92; at 144 h ML01 had produced 3.05 g/L of lactate (negligible amounts of lactate could be detected in S92 fermentations). Yeast were harvested at 48 h (during MLF by ML01) and 144 h (completion of MLF by ML01) for DNA microarray analysis (represented schematically as vertical lines).

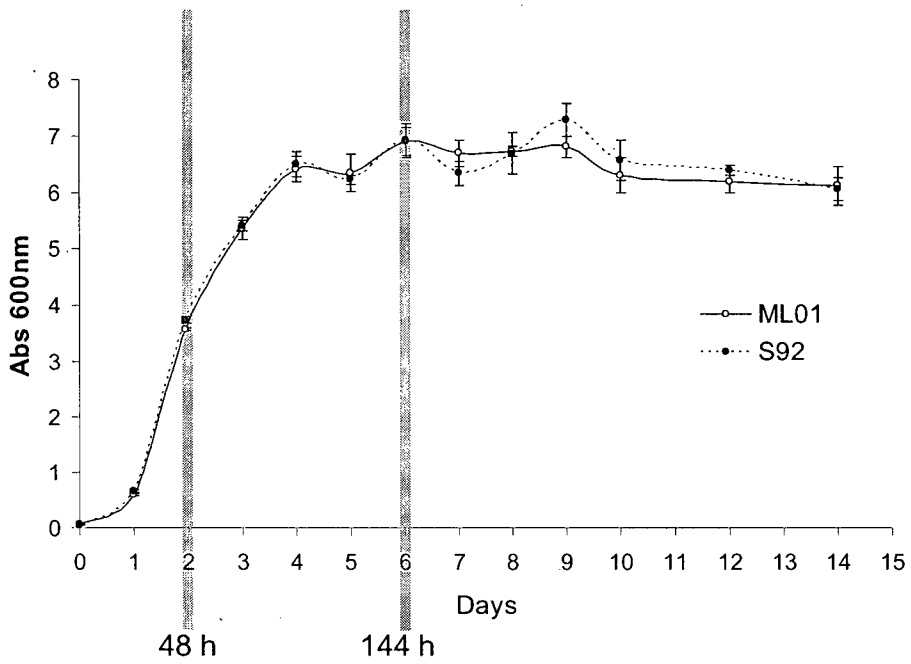

Figure 12. Growth of ML01 and S92 yeast strains in synthetic must (n=3). Daily absorbance readings showed no difference in growth. Yeast were harvested at 48 h (log phase) and 144 h (stationary phase) for DNA microarray analysis (represented schematically as vertical lines).

The introduction of the malolactic cassette into *S. cerevisiae* S92 thus had a minimal effect on the transcription of the 5773 ORFs (*Saccharomyces* Genome Database; June 1, 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. Only one gene, *AQR1*, was found to be expressed differently at the two time points; -3.23 and 1.77 fold change at 144 hours and 48 hours, respectively.

Table 8. Effect of the integrated the malolactic cassette in the genome of S92 on global gene expression patterns in *S. cerevisiae* ML01 at 48 hours (≥ 2 fold change).

Genes Expressed at Higher Levels in ML01		
Gene Symbol	**Fold Change**	**Biological Process**
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 9. Effect of the integrated malolactic cassette in the genome of S92 on global gene expression patterns in *S. cerevisiae* ML01 at 144 hours (≥ 2 fold change).

Genes Expressed at Higher Levels in ML01		
Gene Symbol	**Fold Change**	**Biological Process**
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	-3.23	Monocarboxylic acid transport and drug transport
YML089C	-3.14	Unknown
YIL152W	-2.46	Unknown

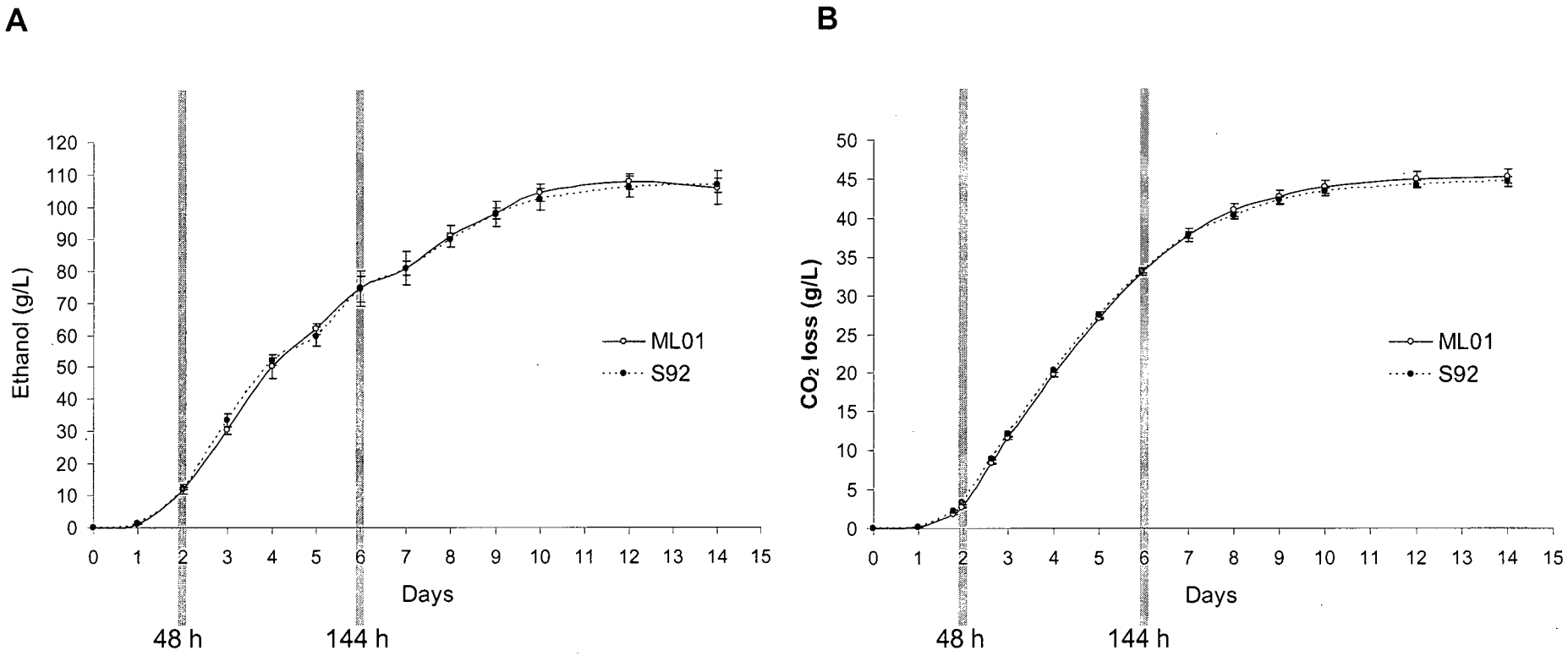

Figure 13. Ethanol production and CO_2 loss of ML01 and S92 yeast strains in synthetic must (n=3). (A) Ethanol production by ML01 and S92 in synthetic must; no difference was detected. (B) CO_2 loss in fermentations by ML01 and S92 in synthetic must; no difference could be detected. Yeast cells were harvested at 48 h and 144 h (represented schematically as vertical lines) for DNA microarray analysis.

The DNA microarray data was verified by semi-quantitative reverse transcriptase PCR of 10 genes shown to have a = 2 fold change in the microarray experiments (Table 6); similar levels of expression for nine genes were obtained (one gene, *ENA2* was below the threshold for the real-time PCR experiment) (Appendix L). Transcripts of the *mae1*, *mleA* and *URA3* were also detected by reverse-transcriptase PCR at 48 and 144 hours (Appendix M).

3.3.7 Effect of the integrated malolactic cassette on the proteome of ML01

The proteomes of ML01 and S92 were analyzed after 48 hours using iTRAQ (DeSouza et al., 2005) and multidimensional 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 (Erg11p) 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 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 new ORFs (5' *ura3*

truncated ORF, and the two 5' adp1 truncated ORFs). The fourth new 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 ORF's created by cloning were not identified at a confidence level as low as 50%.

3.4 Phenotypic properties of ML01

3.4.1 Growth kinetics

In YPD, there was a slight difference in μ_{max} between ML01 ($0.54 \pm 0.01\ h^{-1}$) and S92 ($0.55 \pm 0.005\ h^{-1}$); the corresponding generation times were 1.28 ± 0.02 h and 1.26 ± 0.01 h for ML01 and S92, respectively ($p < 0.05$, n=9). In Chardonnay must no statistical difference was observed between the μ_{max} for ML01 ($0.37 \pm 0.03\ h^{-1}$) and the parental S92 ($0.37 \pm 0.02\ h^{-1}$); the corresponding generation times were 1.88 ± 0.13 h and 1.86 ± 0.08 h for ML01 and S92, respectively (n=9). Growth during the commercial production of ML01 ADY was not affected by introduction of the malolactic cassette into the *URA3* locus of S92 (Didier Colavizza, Lesaffre Development, rue Gabrie Péri 137, F-59700 Marcq-en-Baroeul, France, personal communication).

3.4.2 Utilization of malate as sole carbon source by ML01 and S92

The ML01 and parental strains were unable to consume malate as a sole carbon source. When grown aerobically in modified YPD media containing only 5 g/L of glucose (to trigger the *PGK1* promoter) and 20 g/L of malate, the strains had similar growth kinetics (Figure 14) and no malate was consumed by ML01 or S92. After 350

hours, malate concentrations in the media inoculated with ML01 and S92 were 20.4 ± 0.99 g/L and 20.2 ± 1.03 g/L of malate, respectively.

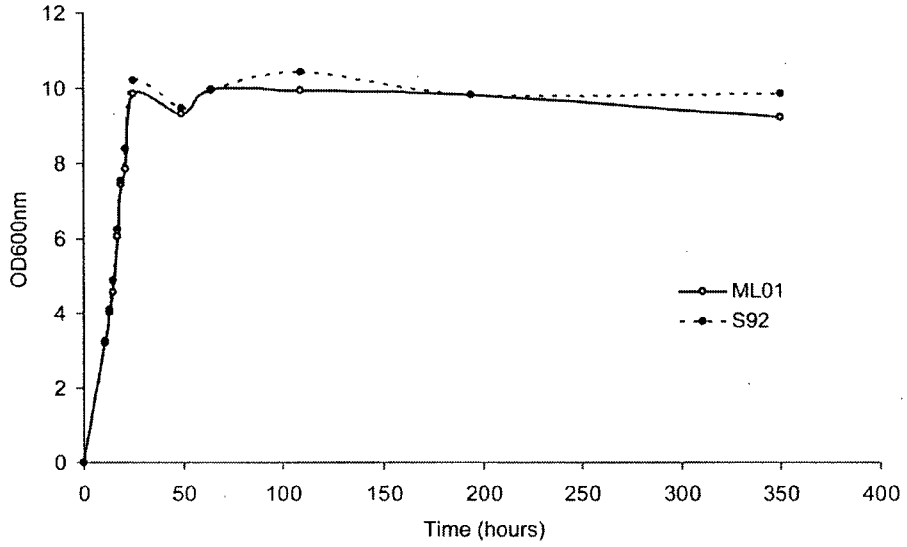

Figure 14. ML01 and S92 cannot consume L-malate as a sole carbon source. ML01 (?) and S92 (?) strains were inoculated (0.01 OD_{600nm} final concentration) into modified YPD medium containing 20 g/L of malate and 5 g/L of glucose and grown aerobically for 350 hours (n=2). Malate analyses after 350 h showed no reduction in malate levels in the medium; 20.4 ± 0.99 g/L and 20.2 ± 1.03 g/L of malate remained in media inoculated by ML01 and S92, respectively.

3.4.3 Malolactic fermentation in Chardonnay and Cabernet Sauvignon musts by ML01

The sulphited and cold stabilized Chardonnay must (from fruit harvested in 2000) contained 105 CFU/mL of yeast and 40 CFU/mL of lactic acid bacteria prior to inoculation with ML01 or S92 ADY. The ML01 and S92 strain both attained a final specific gravity of 0.996 in the high-acid Chardonnay wine at the end of the alcoholic

fermentation. The ML01 strain completed the alcoholic fermentation in 22 days and the S92 strain in 32 days (Figure 15). The ML01 strain consumed 9.04 ± 0.03 g/L of malate (n=2) within the first five days of the alcoholic fermentation (Figure 16a) and produced an approximately equimolar amount of 6.0 ± 0.1 g/L of lactate in the must by day seven (Figure 16b). In contrast the S92 strain consumed only 0.93 ± 0.26 g/L of malate (n=4) and no lactate was produced in the Chardonnay wine at the end of the alcoholic fermentation. *O. œni* required 171 days after alcoholic fermentation to consume 5.29 g/L and 8.02 g/L of malate (n=1) (Figure 16a) and produce 3.96 g/L and 5.42 g/L of lactate (n=1) (Figure 16b), respectively in wine fermented with S92. No further decarboxylation of malic acid was observed (Figure 16b). Analysis of titratable acidity, acetate, pH, viscosity and colour properties of the Chardonnay wines produced by ML01, S92 and S92 plus *O. œni* are shown in Table 10.

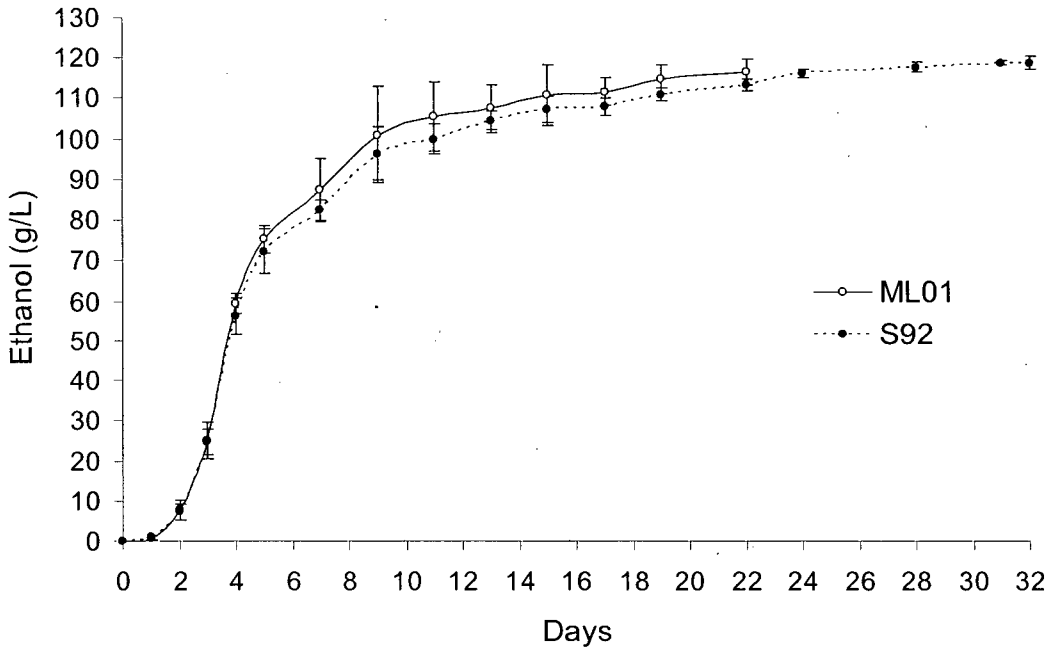

Figure 15. Ethanol production by ML01 (?) and S92 (?) in high-acid Chardonnay grape must fermented at 20 °C was positively affected by introduction of the malolactic cassette into a *URA3* locus in the industrial wine yeast S92. ML01 completed the alcoholic fermentation to a specific gravity of 0.996 in 22 days and the parental strain in 32 days. Duplicate biological replications were analysed in triplicate.

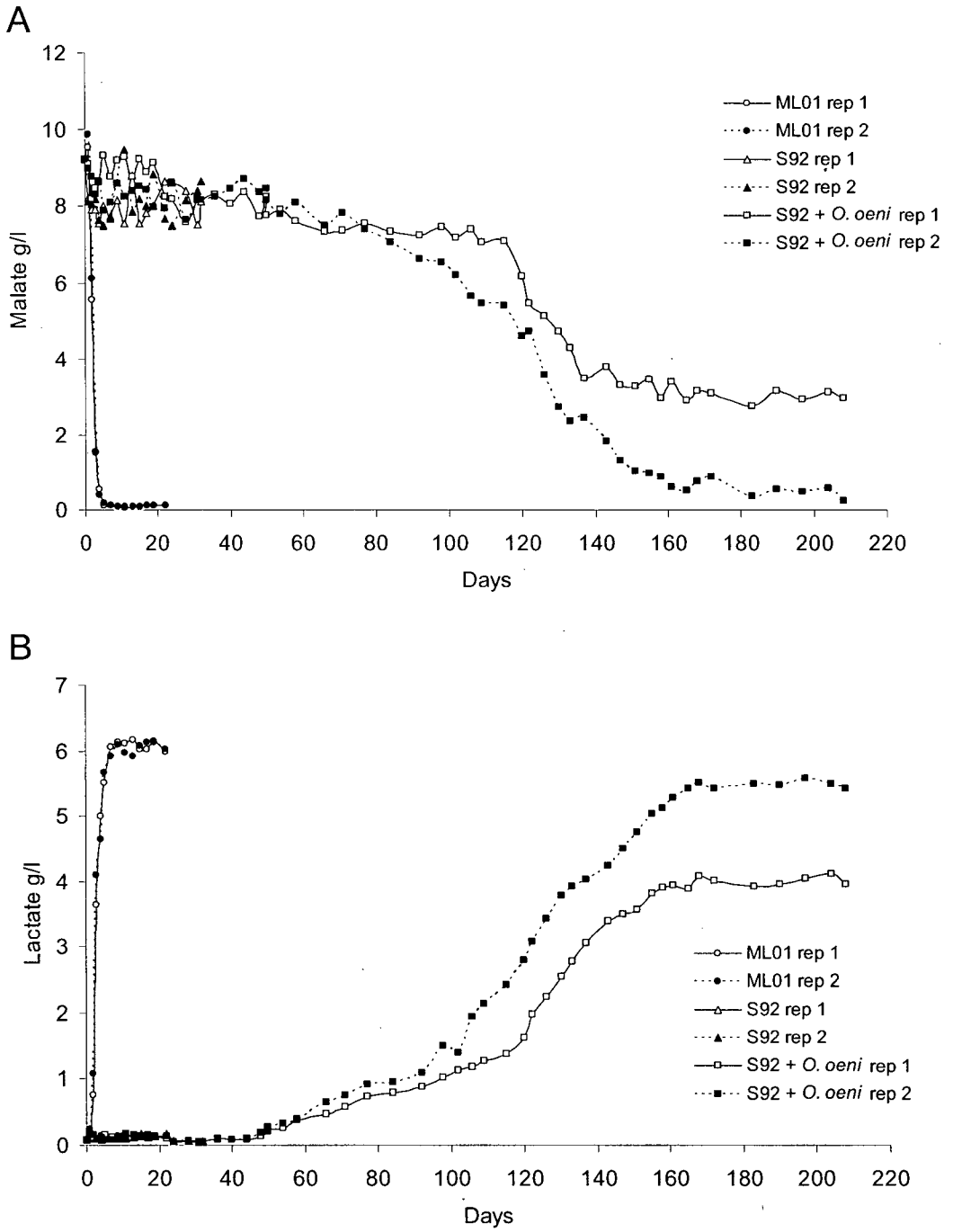

Figure 16. MLF by ML01 is completed in the first five days of the alcoholic fermentation in high-acid Chardonnay grape must (9.2 g/L). (A) Malate degradation and (B) lactate production by ML01 (? /?) and S92 (? /?) and S92 + *O. œni* (? /¦). The ML01 strain fully and efficiently degraded 9.08 g/L of malate and produced equimolar amounts of lactate (6.07 g/L of lactate). Wine inoculated with *O. œni* required 171 days post-alcoholic fermentation to complete the MLF in carboy replicate 2 (0.25 g/L residual malate). A stuck MLF was observed for S92 + *O. œni* in carboy replicate 1; 2.98 g/L of malate remained despite four inoculums of *O. œni*. The parental strain consumed approximately 10% of the malate during the alcoholic fermentation.

Table 10. Physicochemical and colour measurements of high-acid Chardonnay wines (2000 harvest) produced by ML01, S92 and S92 plus *O. œni*[1].

	ML01	**S92**	**S92 + *O. œni***	***p*[a]**
Titratable acidity (g/L)	7.7 a[b]	10.9 b	7.4 c	***
Acetate (g/L)	0.452 a	0.399 b	0.5 c	***
pH	3.22 a	3.09 b	3.24 c	***
Viscosity (mPa.s)	1.64 a	1.62 ab	1.60 b	*
Colour measurements				
L (degree of lightness)	99.07 a	98.72 b	99.06 a	***
a (greenness)	-0.44 a	-0.53 b	-0.82 c	***
b (yellowness)	4.77 a	5.50 b	5.84 c	***
$A_{420nm} + A_{520nm}$	0.151	0.158	0.174	ns

[1]The mean values for bottle replicates are given for all quantities (n=3)
[a]*, **, ***, ns: significant at $p < 0.05$, 0.01, 0.001, or not significant
[b]Means separated at $p < 0.05$ by Duncan's post-hoc test

In Chardonnay grape must from fruit harvested in 2004 the ML01 and S92 strains both attained a final specific gravity of 0.990 at the end of the alcoholic fermentation. This specific gravity corresponded to a residual glucose/fructose concentration of 1.23 ± 0.05 g/L and 0.47 ± 0.02g/L for the ML01 and S92 produced wines, respectively. The ML01 and S92 strains completed the alcoholic fermentation in 21 days (Appendix N). The ML01 strain consumed 5.43 ± 0.002 g/L of malate (98.7% complete) and produced approximately equimolar amount of 3.69 ± 0.03 g/L of lactate by the end of the alcoholic fermentation; 97.7% of the malate was consumed within the first nine days predominantly at a temperature of 13 °C (Appendix N). The S92 strain consumed only 0.50 ± 0.1 g/L of malate and no lactate was produced in the Chardonnay wine at the end of the alcoholic fermentation (Appendix N). *O. œni* required 9 months at 20 and 25 °C after the alcoholic fermentation to complete the MLF. Analysis of titratable acidity,

acetate, pH and colour properties of the Chardonnay wines produced by ML01, S92 and S92 plus *O. œni* are shown in (Appendix O).

The sulphited Cabernet Sauvignon must (without skin contact) contained 7.2 x 10^3 CFU/mL of yeast and no lactic acid bacteria prior to inoculation with ML01 or S92 ADY. The ML01 and S92 strains completed the alcoholic fermentation in 16 days. The ML01 and S92 strains both attained a final specific gravity of 0.990 in Cabernet Sauvignon wines at the end of the fermentation. The ML01 strain consumed 6.13 ± 0.02 g/L of malate (n=2) within the first four days of the alcoholic fermentation. In contrast, the S92 strain consumed 1.87 ± 0.07 g/L of malate (n=2) by the end of the alcoholic fermentation. *O. œni* required 42 days after alcoholic fermentation to consume 6.13 ± 0.03 g/L of malate (n=2) in wine fermented with S92. Analysis of titratable acidity, acetate, pH, and colour properties of the Cabernet Sauvignon wines produced by ML01, S92 and S92 plus *O. œni* are shown in Table 11.

Table 11. Physicochemical and colour measurements of Cabernet Sauvignon wines produced by ML01, S92 and S92 plus *O. œni*[1].

	ML01	**S92**	**S92 + *O. œni***	p^a
Titratable acidity (g/L)	4.39 a[b]	6.38 b	4.27 a	***
Acetate (g/L)	0.324 a	0.237 b	0.355 c	***
pH	3.98 a	3.80 b	4.05 c	***
Colour measurements				
L (degree of lightness)	84.60 a	84.56 a	88.05 b	***
a (redness)	12.67 a	14.32 b	8.01 c	***
b (yellowness)	24.34 a	22.05 b	24.78 a	***
$A_{420nm} + A_{520nm}$	1.00 a	1.14 b	0.84 c	***

[1]The mean values of bottle replicates are given for all quantities (n=3) in the title [a] relates only to pH
[a]*, **, ***, ns: significant at $p < 0.05$, 0.01, 0.001, or not significant
[b]Means separated at $p < 0.05$ by Duncan's post-hoc test

3.4.4 Sensory profile of Chardonnay wines produced by ML01

The ANOVA results of the sensory attribute ratings for wines produced by ML01, S92 and S92 with MLF are summarized in Table 12. Significant differences among wines were observed for seven sensory attributes (colour, fruity aroma, fruity taste, sweetness, acidity, body and overall quality) of the nine which were measured. Judge variation was significant for all attributes, except for acidity (Table 12). This was expected due to individual physiological and scoring differences. Panel inconsistencies as indicated by significant judge x wine interactions (Table 12) were present for two of the seven significantly different attributes observed for wines (fruity aroma and overall quality). Therefore F-values were re-calculated for these two attributes using a more conservative random effects model ($MS_{treatment}/MS_{j*w}$) (Goniak and Noble, 1987). While the new F-value for fruity aroma (2.29) was not significant, the new F-value for overall quality (7.99) was statistically significant. This indicates that the panel inconsistencies were relatively minor compared to the magnitude of the overall quality effects. Judge x replication and wine x replication interactions are shown in Table 12.

The significantly different mean sensory attributes were plotted on a cobweb diagram (Figure 17) and a PCA plot (Figure 18). Wine produced by ML01 was significantly highest in overall quality, body, and perception of sweetness and lowest in acidity when compared to wines produced by S92 with and without a MLF. Wine produced by ML01 was also significantly highest for fruity taste when compared to wines produced by S92 with a bacterial MLF. The main characteristics of wine produced by S92 without a MLF were its darker colour and high acidity. Wines produced by S92 with

bacterial MLF were judged to be more acidic, less sweet, have less body, less fruity taste and lower in quality than wines produced with ML01.

Table 12. F-values from analysis of variance of Chardonnay wines for sensory attributes (three wines, 13 judges, two replications)

Descriptor	Wine	Judge	Rep	Judge X Wine	Judge X Rep	Wine X Rep
Yellow colour	16.02***[a]	9.81***	0.19	1.48	1.10	4.85*
Fruity aroma	6.36*	11.71***	0.01	2.78*	1.44	1.44
Buttery aroma	1.14	4.73**	0.00	1.74	0.86	1.27
Fruity taste	16.71***	7.68***	1.72	1.27	2.01	0.74
Buttery taste	1.55	7.16***	1.39	4.50***	2.57*	0.95
Sweet	49.46***	8.46***	0.92	1.40	2.14	0.92
Acidity	23.05***	1.81	4.01	1.24	1.03	1.12
Body	12.01***	3.37*	0.09	1.45	1.85	0.67
Overall quality	16.51***	6.71***	0.29	2.02*	1.16	1.29

[a]*, **, ***: significant at $p < 0.05, 0.01, 0.001$

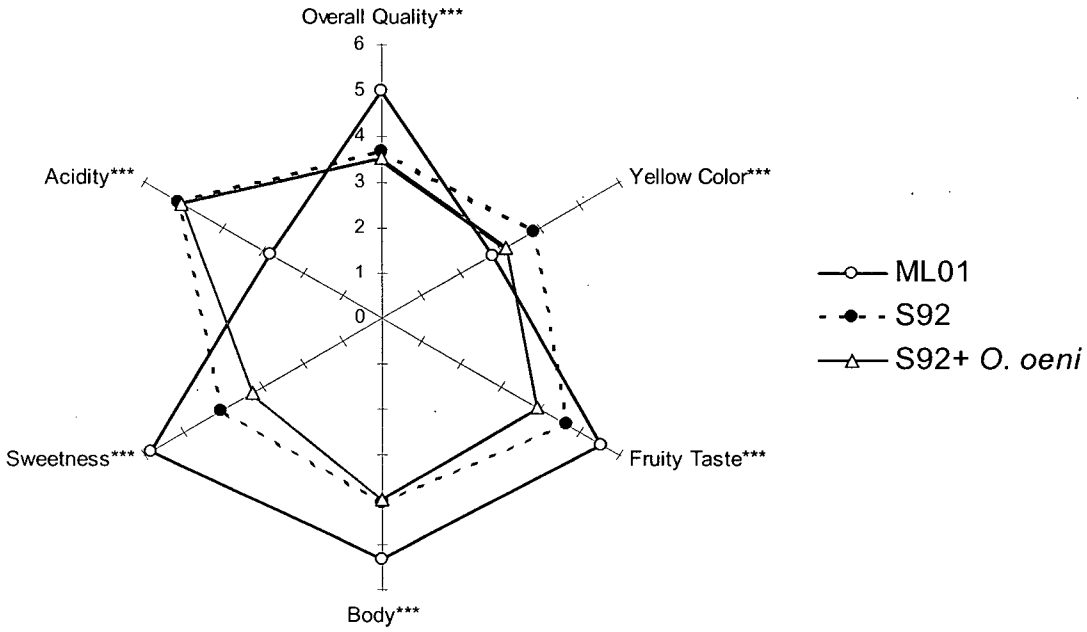

Figure 17. Cobweb diagram showing the significantly different mean sensory attributes of Chardonnay wines produced by ML01, S92, and S92 plus *O. œni* (n=26).
*** $p < .001$.

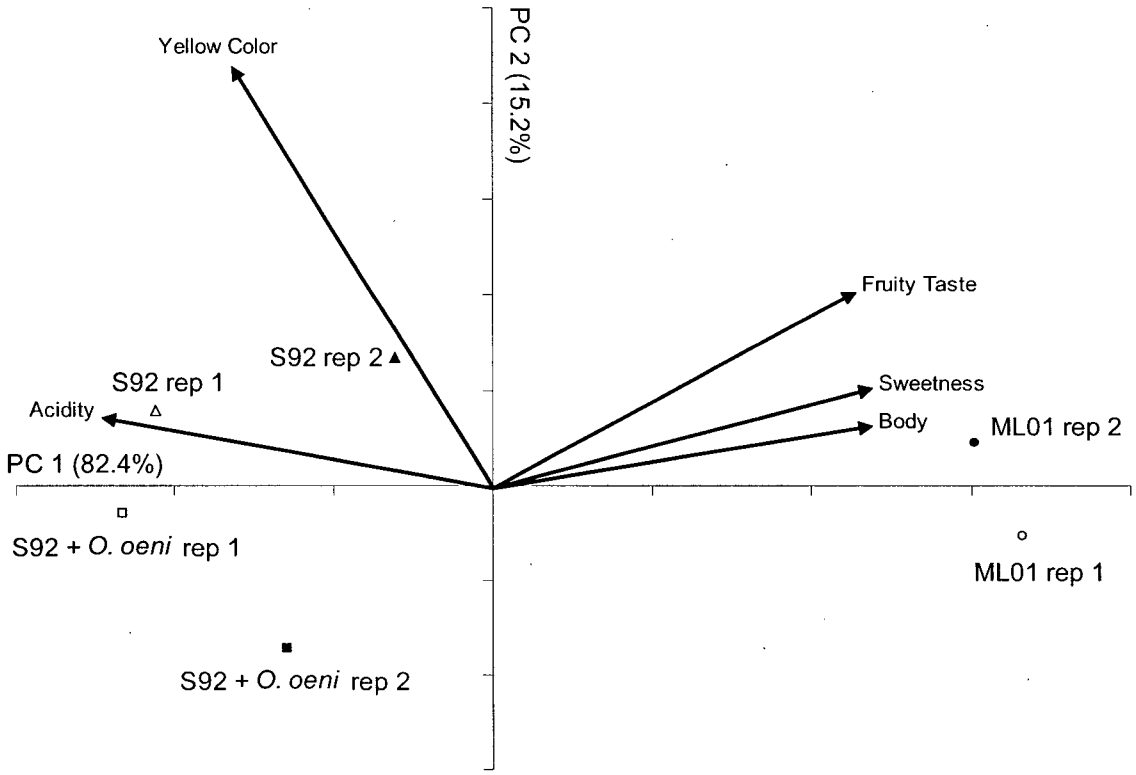

Figure 18. A principal component analysis plot showing the significantly different mean sensory attributes for Chardonnay wines produced by ML01, S92, and S92 plus *O. œni*. PCA factors 1 and 2 explain 82.4% and 15.2% of the variability, respectively (n=13).

The first two factors of the PCA mean sensory scores accounted for 97.7% of the variance among the wines (Figure 18). The attributes fruity taste, body, sweetness and acidity were most heavily loaded on PC1 accounting for 82.4% of the variability. Colour was most heavily loaded on PC2 accounting for 15.2% of the variability. The PCA plot showed that body was negatively correlated with acidity, as indicated by the 180 degree angles between the vectors. Body was positively correlated with sweetness and fruity taste, as indicated by the small angles. Overall quality (not shown in the PCA plot of objective aroma/flavour assessments) was also strongly correlated with body (Appendix

P). In contrast, yellow colour and fruity taste were uncorrelated, as shown by their 90 degree orientation. The wines from ML01, located on the right, were characterized by their full body, sweet taste and fruity flavour. In contrast, S92 and S92 + *O. œni* wines, located to the left, were higher in acidity and lower in body, sweetness and fruitiness. S92 wines located slightly higher in the plot were distinguished by their darker yellow colour. These traits are consistent with characteristics identified from the cobweb diagram (Figure 17).

3.4.5 Volatile compounds in wine produced by ML01

GC/MS headspace analysis (Table 13) revealed that no additional compounds were detected in wine produced by ML01 when compated to wine produced with the parental strain S92 or wine produced with S92 and malolactic bacteria. One compound, ethyl 2-methylbutanoate, was detected in wine produced with S92 plus *O. œni* that was not present in wines produced with ML01 and S92 without a MLF. Wines produced with ML01 and S92 without a MLF contained several compounds, acetal, 2,4,5-trimethyl-1,3-dioxolane, 1,1-diethoxyisopentane, n-hexanal, and benzaldehyde that were not detected in wines produced with S92 plus *O. œni*.

Table 13. Concentration of volatile compounds in Chardonnay wines produced with ML01, S92, S92 plus *O. œni*. Wines were analyzed by GC/MS headspace assay[1].

Compounds	ML01 (mg/L)	S92 (mg/L)	S92 + *O. œni* (mg/L)	p[a]
acetaldehyde	83.71	78.33	43.52	ns
dimethylsulfide	0.04 a[b]	0.05 a	0.43 b	**
ethyl formate	0.2	0.28	0.28	ns
methyl acetate	0.32	0.33	0.35	ns
ethyl acetate	180.21 a	173.18 a	277.08 b	*
isobutyl acetate	0.008	0.008	0.007	ns
ethyl butanoate	8.16	8.04	12.24	ns
propanol	71.93	64.02	91.54	ns
ethyl isovalerate	0.003 a	0.005 a	0.014 b	*
isobutyl alcohol	229.09 a	262.89 a	393.11 b	*
isoamyl acetate	1.24	1.24	2.33	ns
n-butanol	1.29	0.91	1.40	ns
2-methyl-1-butanol	8.21	8.79	12.64	ns
3-methyl-1-butanol	94.66	102.34	150.7	ns
ethyl hexanoate	0.94	0.89	1.63	ns
1-hexyl acetate	0.16	0.16	0.15	ns
acetoin	5.7 ab	1.56 a	9.96 b	*
3-methyl-1-pentanol	0.06	0.05	0.04	ns
ethyl lactate	177.9 a	5.63 b	295.88 c	***
1-hexanol	21.69	20.27	30.92	ns
3-ethoxy-1-propanol	20.6	16.27	20.99	ns
3-octanol (IS)	0.21	0.21	0.21	-
ethyl octanoate	0.96 a	0.95 a	2.9 b	*
acetic acid	6.35 a	5.33 a	14.27 b	*
ethyl decanoate	0.46 a	0.46 a	1.3 b	**
diethyl succinate	0.21 a	0.35 a	1.22 b	***
phenylethyl acetate	0.33	0.22	0.2	ns
hexanoic acid	1.31	1.76	1.06	ns
phenylethyl alcohol	0.95	0.98	1.12	ns
octanoic acid	2.62	3.13	2.85	ns

[1]The mean values for biological replicates are given for all compounds (n=3)
[b]*, **, ***, ns: significant at $p < 0.05$, 0.01, 0.001, or not significant
[c]Means separated at $p < 0.05$ by Duncan's post-hoc test

No significant differences were found for acetaldehyde, ethyl formate, methyl acetate, isobutyl acetate, ethyl butanoate, propanol, isoamyl acetate, n-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl hexanoate, 1-hexyl acetate, 3-methyl-1-pentanol, 1-

hexanol, 3-ethoxy-1-propanol, phenylethyl acetate, hexanoic acid, phenylethyl alcohol and octanoic acid concentrations when the three wines were compared. The only significant difference between wines produced with ML01 and S92 without a MLF was that ethyl lactate was detected at a higher concentration in wine fermented with ML01 (177.9 mg/L) than S92 without a MLF (5.63 mg/L). Ethyl lactate concentration in wine produced with S92 plus *O. œni* was 295.88 mg/L. Wines produced with S92 plus *O. œni* also contained significantly higher concentrations of dimethyl sulfide, ethyl acetate, ethyl isovalerate, isobutyl alcohol, ethyl octanoate, acetic acid, ethyl decanoate, and diethyl succinate than wine produced with ML01 or S92 without a MLF. Acetoin was also significantly higher in wines produced with S92 plus *O. œni* than wines produced with S92 without a MLF. Physicochemical characteristics of Chardonnay wine produced for GC/MS analysis is given in Appendix O.

3.4.6 Ethyl carbamate in wine produced by ML01

Ethyl carbamate (EC) produced by the ML01 yeast and the S92 yeast with a bacterial MLF in Chardonnay grape musts is shown in Table 13. The S92 yeast and *O. œni* bacterium produced Chardonnay wine (2000 harvest) that had a maximum potential EC concentration of 71.32 µg/L and wine produced by ML01 had 50.89 µg/L, a reduction of 28.6%. In the Chardonnay (2004 harvest) wine, S92 and *O. œni* produced wine with 99.14 µg/L of EC and the ML01 yeast produced wine with 89.58 µg/L, a reduction of 9.6%. No significant difference in maximum potential EC concentration was determined between wines produced by ML01 and the parent strain S92 without a bacterial MLF.

Table 14. The production of ethyl carbamate in Chardonnay wines produced with ML01, S92 and S92 with a bacterial MLF.

Wines	ML01 (µg/L)	S92 (µg/L)	S92 + *O. œni* (µg/L)	p[a]
Chardonnay (2000 harvest)[b]	50.89 a[c]	44.92 a	71.32 b	***
Chardonnay (2004 harvest)[d]	89.58 a	84.24 a	99.14 b	***

[a]***, significant at $p < 0.001$
[b]The mean values for bottle replicates (n=3)
[c]Means separated at $p < 0.05$ by Duncan's post-hoc test
[d]The mean values of biological replicates (n=3)

3.4.7 Effect of increasing populations of ML01 on MLF in wine conducted with the parental yeast

To test the effect of different levels of ML01 cell populations on fermentations conducted with S92, fermentations in synthetic must were performed using mixed cultures of ML01 and S92 in different ratios. The MLF did not occur when the ML01 strain was present at < 1% of the total yeast cell population at the beginning of the alcoholic fermentation (Figures 19 a, b). Fermentations containing a 10% ML01 inoculum resulted in a partial (33.3 %) MLF (Figures 19 c, d). If 50% of the yeast population comprised ML01, an almost complete (95.3%) MLF was observed.

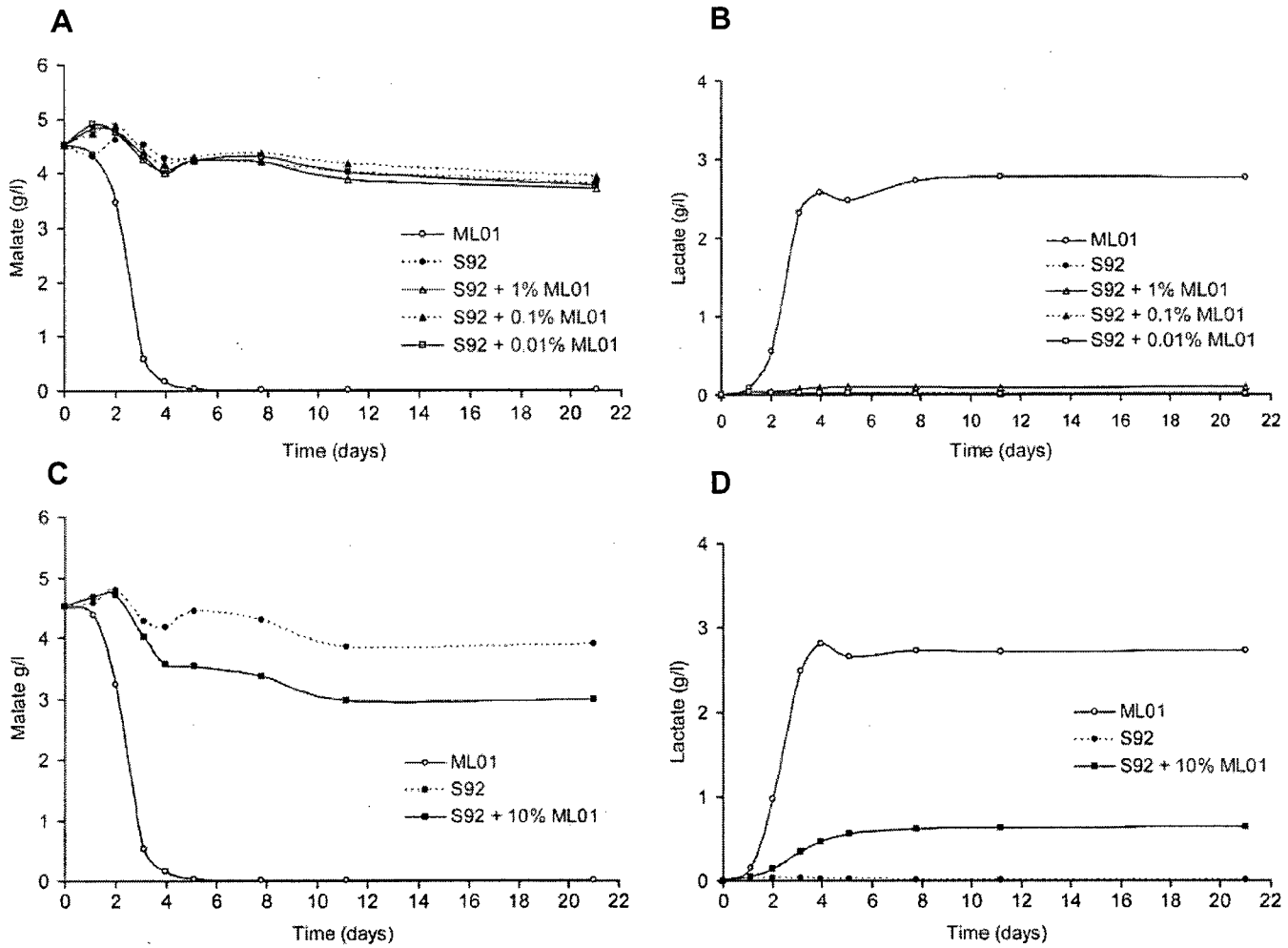

Figure 19. MLF was not detected in wines containing an inoculum < 1% of ML01 yeast. (A) Malate degradation and (B) lactate production by ML01 (?) and S92 (?), and S92 + 1% ML01 (?), S92 + 0.1% ML01 (?) and S92 + 0.01% ML01 (?) co-cultures in synthetic must containing 4.5 g/L of malate. (C) Malate degradation and (D) lactate production by a co-culture of S92 + 10% ML01 (¦); only 33% of the malate was consumed when ML01 was present at 10% of the total inoculum. The control ML01 culture completely decarboxylated malate within 5 days (n=2).

3.4.8 Post-fermentation viability of ML01

The viability of ML01 and S92 yeast cells post-fermentation was determined by plate counts on YPD for 269 days (Figure 20). The viability of ML01 and S92 cells post-fermentation declined at similar rates for ML01 and S92.

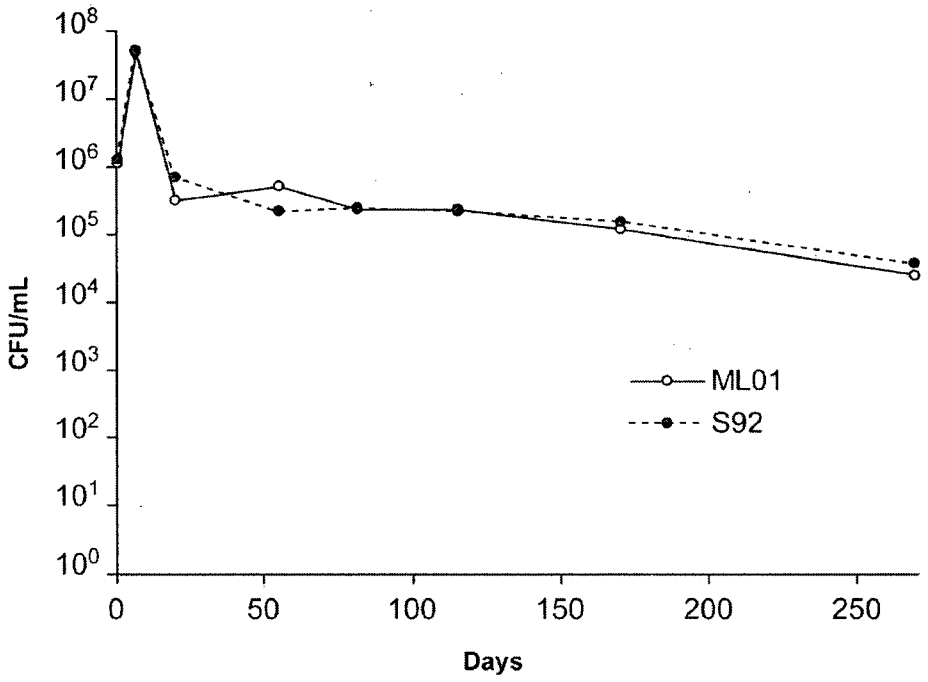

Figure 20. Post-fermentation viability of ML01 (?) is similar to that of S92 (?) in Chardonnay wine. Both strains were inoculated (100 mg/L) in duplicate into filter-sterilized Chardonnay must and viability of cells was determined by plate counts on YPD for 269 days (n=2).

4 DISCUSSION

The objective of this study was to genetically engineer and characterise a commercially acceptable *S. cerevisiae* wine yeast strain capable of decarboxylating extracellular L-malate (one of the major organic acids found in grape must) to L-lactate. The correct integration of the *S. pombe* malate transporter (*mae1*) and the *O. œni* malolactic enzyme (*mleA*) under the control of the *S. cerevisiae PGK1* promoter and terminator signals into the genome of the industrial wine yeast S92, yielded the malolactic yeast ML01. The genetically stable ML01 yeast strain does not contain any antibiotic marker sequences and can complete the MLF during the alcoholic fermentation in a variety of musts; ML01 cannot appreciably decarboxylate L-malic acid to L-lactic acid when present at levels below 1% of the total inoculum. Analysis of the transcriptome and the proteome showed that no metabolic pathway was affected by the introduction of the malolactic cassette. GC/MS analysis of the volatile compounds showed that wine produced by ML01 did not contain any compounds that were not detected in wine produced with the parental strain S92 or with S92 and *O. œni*. Wine produced by ML01 also revealed improved colour properties and lower volatile acidity than wines produced with a bacterial MLF. Moreover, processing time after alcoholic fermentation is reduced and wine produced by ML01 is judged highest in overall quality by trained tasters when compared to control wines.

4.1 Integration of the malolactic cassette into the genome of S92 yielded the functional malolactic yeast ML01

S. cerevisiae cannot effectively metabolise extracellular malate due to its 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). The successful integration of the malolactic cassette (Figure 1) into the *URA3* locus of the wine yeast S92 yielded the first malolactic wine yeast ML01. In addition to the *ura3* flanking sequences required for homologous recombination, the malolactic cassette contains the *S. pombe* malate transporter (*mae1*) gene (Grobler et al., 1995) and the *O. œni* malolactic enzyme (*mleA*) (Husnik, 2001); both genes are under control of the *S. cerevisiae PGK1* promoter and terminator sequences. It is important to note that the transgenes, *mae1* and *mleA* were acquired from wine microorganisms (Barnett et al., 1990; Garvie, 1967; Lodder, 1970). The *S. pombe* strain used in this study can trace its roots back to a strain isolated from sulphited grape must (Osterwalder, 1924). Moreover, *S. pombe* has recently been commercialised (Proenol, Industria Biotecnologica Lda., Portugal) as an alternative method for the deacidification of wines (Silva et al., 2003). *O. œni*, formerly designated *Leuconostoc œnos* (Dicks et al., 1995), has only been isolated from wines and related habitats such as wineries and vineyards (Williams et al., 1989). The *ura3* flanking sequences and the *PGK1* promoter and terminator sequences were acquired from *S. cerevisiae* strains GC210 (Cunningham and Cooper, 1991) and AB972 (Olson et al., 1986), respectively. The parental strain of ML01 is S92 and this was confirmed by electrophoretic karyotyping (Figure 3) and PCR amplification using d sequences of the

Ty1 retrotransposon (Appendix G). *S. cerevisiae* S92 is an isolate from the Champagne region in France and belongs to a family of very close or identical commercial strains designated as "Prise de Mousse" (PDM) strains. PDM strains are some of the most popular commercial strains and are found in every wine region in the world. The *S. cerevisiae* ML01 strain is the first genetically engineered wine yeast strain to be constructed without the integration of an antibiotic marker or *E. coli* vector sequences (Schuller and Casal, 2005).

Since the malolactic cassette did not have a selectable marker to detect transformants, a phenotypic screen was developed based on the previous work of Subden et al. (1982). This colorimetric method relies on the specific reaction between L-lactate present in the media and L-lactate dehydrogenase. The screening method was specific, economical, undemanding and effective (Figure 2).

Individual colonies from each of the identified malolactic clones were tested for functionality in synthetic must. All of the randomly chosen colonies of clone 4 were able to completely decarboxylate malate to = 0.05 g/L within the first five days of the alcoholic fermentation (Appendix F). The MLF is generally considered complete when the malate concentrations are = 0.05 to 0.2 g/L (Avedovech et al., 1992; du Plessis et al., 2002; Henick-Kling and Park, 1994). Individual colonies from clone 4 were also capable of consuming the glucose and fructose in the synthetic must to less than 2.0 g/L of residual sugar by day 14 of the alcoholic fermentation (Appendix F). The alcoholic fermentation is considered complete when the residual sugar concentration (glucose and fructose) is < 2.0 g/L (Bisson, 1999).

Based on these results malolactic clone 4 (ML01) was selected for pilot-scale ADY production. Originally, wine yeasts were commercialised as compressed cakes and as liquid cultures. In 1963, the first active dry wine yeast were produced and marketed to the wine industry (Reed and Chen, 1978). Since that time wine ADY have become widely accepted and are the preferred form of commercial yeast at wineries. ML01 was successfully produced as ADY and after direct inoculation into synthetic must (without rehydration) it was capable of completely decarboxylating the malate in the medium to equimolar amounts of lactic acid. ML01 was also capable of completing the alcoholic fermentation within 14 days (1.71 ± 0.99 g/L residual sugar), whereas the residual sugar for the parental strain under the same conditions was 3.79 ± 0.95 g/L. As expected, the final pH of the fermented synthetic must was 0.16 pH units higher than the fermentation with S92. The pH of wine that has undergone a MLF is generally 0.1 - 0.2 pH units higher than wine that has not gone through a MLF due to the decarboxylation of malate (Bartowsky, 2005; Beelman and Gallander, 1979; Boulton, 1996).

The successful construction and subsequent production of a functional malolactic yeast as ADY (pilot plant and eventual large scale manufacturing) was a major achievement towards the commercialisation of the first genetically modified wine yeast.

4.2 ML01 completes the MLF during alcoholic fermentation in Chardonnay and Cabernet Sauvignon musts

Most red and some white wine styles (such as Chardonnay) are subjected to the MLF. The MLF is especially favoured in cooler wine regions where the grapes at harvest tend to have naturally higher acidity. High-acid Chardonnay must is one of the most

difficult musts winemakers are confronted with to produce a well-balanced quality wine. After inoculation of the ML01 strain into a high-acid Chardonnay must (TA 13.45 g/L, pH 3.18), 98.3% (9.06 g/L) of the malate was consumed in the first five days of the alcoholic fermentation and an equimolar amount of lactate (6.0 g/L) was produced by day seven (Figure 16). The parental strain S92 consumed only 10.3% (0.95 g/L) of the malate in the media and no lactate was produced. The two carboys inoculated with *O. œni* after the alcoholic fermentation required 171 days, three additional inoculums and an increase in temperature (25°C) to consume 64.0% (5.29 g/L) and 97.0% (8.02 g/L) of malate in S92 produced wine (Figure 16). These vinification trials, conducted under ideal conditions at a research facility, confirm that the bacterial MLF relies on long and demanding protocols, which are not always in accordance with good winemaking practices. Indeed, wines had to be kept at a relatively high temperature that is conducive to the growth of *O. œni* for a long time; this prolonged fermentation at a higher temperature could alter wine aromatic volatile compounds and increase chances of spoilage by unwanted microorganisms and lead to oxidation of wines in wineries. In contrast, MLF by ML01 occurred rapidly, which will allow for early stabilization of wine in the cellar.

The rapid MLF in high-acid Chardonnay conducted by ML01 was also demonstrated in the Chardonnay must from fruit harvested in 2004 (Titratable acidity, 8.78 g/L, pH 3.41) and the Cabernet Sauvignon must (Titratable acidity, 7.41 g/L, pH 3.72). In Chardonnay must from fruit harvested in 2004, the ML01 strain efficiently decarboxylated 5.5 g/L of malate and produced equimolar amounts of lactate by day nine (Appendix N). In contrast, *O. œni* required nine months after alcoholic fermentation to

complete the MLF despite four additional inoculums, an increase in temperature (25°C) and an addition of LAB nutrients (Leucofood). In Cabernet Sauvignon must, the ML01 strain completed the MLF within the first four days of the alcoholic fermentation; *O. œni* required 42 days after alcoholic fermentation to complete the MLF.

The parental strain S92 required 10 additional days to complete the alcoholic fermentation in the high-acid chardonnay must compared to the ML01 strain (Figure 15). The low pH of Chardonnay must (pH 3.18) may have contributed to the slower and longer fermentation times for S92, whereas the ML01 was able to complete the fermentation more easily due to the slight pH increase resulting from malate degradation over the first five days (Table 10). In higher pH wines, the alcoholic fermentations by the two yeast strains were completed at the same time: 21 days in the Chardonnay from fruit harvested in 2004 (pH 3.41) and 16 days for the Cabernet Sauvignon must (pH 3.72) . These data indicate that the presence of the malolactic cassette in ML01 does not negatively affect ethanol production when compared to the parental strain S92.

4.3 Wines produced by ML01 have improved physicochemical and organoleptic properties

The main effect of the MLF is a decrease in titratable acidity due to the decarboxylation of L-malic acid to L-lactic acid. Titratable acidity of Chardonnay and Cabernet Sauvignon wines produced by ML01, S92 and S92 with a bacterial MLF are shown in Tables 10 and 11 and Appendix O. As expected, titratable acidity was considerably reduced in wines that had undergone a MLF. This deacidification is crucial for the production of less sour and balanced wines, particularly for higher-acid wines

from cool-climate regions. The decrease in titratable acidity after a MLF is also accompanied by an increase in pH which could affect microbiological stability in higher pH wines (Tables 10 and 11 and Appendix O). However, wines produced by the ML01 strain had lower pH than those produced with the parental strain and *O. œni* (Tables 10 and 11 and Appendix O). Therefore, winemakers conducting a MLF using LAB or the malolactic yeast in higher pH wines can adjust sulphur dioxide to appropriate concentrations (and acidity, if desired) to provide microbiological and chemical stability. The slightly lower pH of wines fermented by ML01 than S92 with a bacterial MLF (Tables 10 and 11, Appendix O) are probably due to the amount of L-lactic acid present in the wines. ML01 decarboxylates all of the malate present initially in the must to lactate, whereas *O. œni* starts the MLF with less malate in the wine since S92 has already consumed approximately 10 - 30% of the malate, probably via the malo-ethanolic pathway.

An increase in pH also plays a role in loss of colour in red wines. The substantial loss of colour after a bacterial MLF is generally attributed to the pH effects on anthocyanins (Boulton, 1996). In an acid medium, anthocyanins are red and as the pH increases their colour changes to blue and eventually fade to yellow (Ribereau-Gayon et al., 2000b). Interestingly, Cabernet Sauvignon wines produced by ML01 and S92 without a MLF, had a darker colour than wine produced with S92 with a bacterial MLF (Table 11, degree of lightness). As expected, the wine with the lowest pH produced by S92 without a MLF (pH 3.80) had the highest value for redness and colour intensity ($A_{420nm} + A_{520nm}$) and the lowest value for yellowness; wine produced by S92 with a bacterial MLF had the highest pH (pH 4.05) and was significantly lighter, the least red,

and the least intense (Table 11). Wine produced by ML01 (pH 3.98) had a similar degree of yellowness to wine produced by S92 with a MLF, but redness and intensity were significantly higher (Table 11). These data clearly indicate that the metabolic activity of *O. œni* impacts negatively on anthocyanins in red wine and the loss of colour cannot be attributed simply to an increase in pH.

Volatile acidity is another important component of wine acidity. Acetic acid, the main component of volatile acidity in wine, was found in significantly lower concentrations in wines produced with ML01 and S92 without a MLF than in wines produced with S92 and a bacterial MLF (Tables 10 and 11 and Appendix O). High-acid Chardonnay wine produced with ML01 had lower concentrations of acetate (0.452 g/L) than the wine produced with a bacterial MLF (0.5 g/L) but higher levels of acetate than wine fermented with S92 alone (0.399 g/L). The Chardonnay wine produced by ML01 from fruit harvested in 2004 had lower levels of acetate (0.25 g/L) than the wine produced with and without a bacterial MLF (0.424 g/L and 0.328 g/L, respectively). Acetate concentrations in Cabernet Sauvignon wines produced by ML01 was 0.324 g/L, by S92 with a bacterial MLF 0.355 g/L, and by S92 alone 0.237 g/L. The higher concentration of acetic acid in wines produced with *O. œni* can be attributed to the metabolism of remaining sugars and citric acid in the wine (Liu, 2002; Ribereau-Gayon et al., 2000a; van Vuuren and Dicks, 1993).

In addition to the acidity properties of wine, numerous other metabolites influence the organoleptic characteristics of wine. GC/MS headspace analysis of volatile compounds was conducted on 2004 Chardonnay wine since no biological replicates were available for the 2000 Chardonnay wines (only one carboy completed the bacterial MLF).

GC/MS analysis showed that no additional compounds were detected in wine produced with ML01 when compared to wine produced with the parental strain with or without a MLF. However, concentrations of certain compounds such as ethyl lactate, an aroma compound (buttery), that also gives the wine a 'broader', 'fuller' taste (Henick-Kling, 2002), varied greatly. Ethyl lactate concentrations were high in wines produced by ML01 (177.9 mg/L) and S92 with a bacterial MLF (295.88 mg/L) but were low in wine produced by the parental strain without a MLF (5.63 mg/L). The different ethyl lactate concentrations are most likely related to the lactate concentrations found in the wines. Concentrations of the following compounds were higher in wines produced with S92 and *O. œni* than wines produced by ML01 or S92 alone: dimethyl sulfide (quince, truffle odour), ethyl acetate (fruity at lower concentrations, acescence and unpleasant at higher concentrations), ethyl isovalerate (fruity, vinous odour), isobutyl alcohol (fruity), acetoin (milky and fatty odour), ethyl octanoate (soapy, candlewax odour), acetic acid (vinegar), ethyl decanoate (oily, fruity, floral), and diethyl succinate (faint, pleasant odour) (Table 13) (Clarke and Bakker, 2004; Ribereau-Gayon et al., 2000b). Although these volatile compounds were found in higher concentrations in wine produced with the S92 and *O. œni*, it is difficult to ascertain if the higher concentrations of several different chemicals in a complex matrix will detract from the quality of wine or improve it.

Sensory analysis of Chardonnay wines aged for four years showed that wine produced by ML01 was judged highest for overall quality (Figure 17). Overall quality was also strongly correlated with body and negatively correlated with acidity (Appendix P). Chardonnay wine produced by S92 without a MLF was judged to be significantly darker than wines produced by ML01 or S92 with MLF and this correlates well to the

degree of lightness measured by CIELAB colour measurements (Table 10). CIELAB colour measurements also indicated that Chardonnay wine produced with ML01 had the lowest amount of 'greenness' and 'yellowness' and S92 with a MLF had the highest amount of 'greenness' and 'yellowness' (Table 10). These colour subtleties were not perceived by the panellists. Body and perceived sweetness was highest for wines produced with ML01 and lowest for acidity. The complete degradation of malate by ML01 may have contributed to the perceived sweetness of wines produced by ML01. The main descriptive attributes that are associated with wine produced by ML01 are highest overall quality, fruity taste, sweetness (perceived because of a lack of acidity when compared to other wines) and body, whereas, high acidity is an attribute of wine produced with S92 with and without a MLF, and dark yellow colour is an attribute of wine produced with S92 without a MLF (Figures 17 and 18).

In addition to compounds that affect organoleptic properties of wine, lactic acid bacteria produce harmful compounds such as biogenic amines and ethyl carbamate (Lonvaud-Funel, 1999). Biogenic amines are produced from their respective precursor amino acids by specific amino acid decarboxylases. Histamine, the best studied biogenic amine, can cause headaches, hypotension, sneezing, flush, skin itch, shortness of breath and digestive problems (Soufleros et al., 1998; Wantle et al., 1994). The increase in biogenic amines after MLF is well documented (Marcobal et al., 2006) and could be dramatically reduced if wine were to be sulphited and processed immediately after alcoholic fermentation.

Arginine metabolism by certain strains of *O. œni* and other heterofermentative LAB leads to the formation of ethyl carbamate (urethane) precursors (Liu and Pilone,

1998). Ethyl carbamate is a potential human and known animal carcinogen found in wine (Ough, 1976; Ough, 1993). The main ethyl carbamate precursors in wine are urea (produced by yeast), and citrulline (produced by LAB). The utilisation of ML01 to conduct the MLF during alcoholic fermentation prevents the formation ethyl carbamate from citrulline. The concentration of ethyl carbamate in Chardonnay wines produced by ML01 and S92 without a bacterial MLF were significantly lower than wines produced with a bacterial MLF (Table 14). Complete reduction of ethyl carbamate is not possible with the ML01 yeast since other precursors such as urea and other carbamyl compounds are still available for EC production

4.4 Integration of the malolactic cassette in S92 does not confer a growth advantage to ML01 nor does it not affect the production of ADY or wine making

The MLF by ML01 coincides with the log growth phase of the yeast. In laboratory media (YPD) the maximum specific growth rate was slightly lower for the ML01 strain ($0.54 \pm 0.01\ h^{-1}$) than the parental strain S92 ($0.55 \pm 0.005\ h^{-1}$). However, the slightly longer doubling time observed in YPD media did not affect the growth of ML01 on molasses during the production of ADY (Didier Colavizza, Lesaffre Development, rue Gabrie Péri 137, F-59700 Marcq-en-Baroeul, France, personal communication). Moreover, the ML01 strain and the parental strain, S92, have the same maximum specific growth rate in Chardonnay must ($0.37\ h^{-1}$) and die at a similar rate (Figure 20) indicating that the introduction of the malolactic cassette should not affect wine making.

The ML01 strain, like the parental strain S92, is unable to consume malate as a sole carbon source (Figure 14) showing that the malolactic cassette does not confer any competitive advantage to the ML01 strain in this respect. Furthermore, although the ML01 yeast can efficiently decarboxylate malate to lactate in a variety of musts, it cannot appreciably decarboxylate malic acid to lactic acid when present at levels below 1% of the total inoculum (Figures 19 a,b). Even at inoculum levels of 10% ML01, the decarboxylation of malate to lactate was limited (33.3%) and the MLF did not continue after the first few days (Figures 19 c,d). These data indicate that the MLF mainly coincided with the growth phase of the ML01 strain and that the MLF was not active during the later stages of the fermentation. Hence, cross-contamination of ML01 yeast in must not destined for deacidification in wineries should not be a concern under commercial conditions.

4.5 The malolactic cassette without any antibiotic resistance markers is integrated correctly and stably into the genome of ML01

Screening for the integration of the malolactic cassette in the genome of S92 was completed with the assistance of a co-transforming plasmid pUT332. The purpose of pUT332 was to reduce the number of transformants to be screened by the colorimetric method. The linear malolactic cassette was combined in a 10:1 molar ratio with pUT332 in order to select yeast cells that were permeable to DNA. After successive subculturing in non-selective media the plasmid was lost (Figure 7). Southern blots probing for Tn5*ble* (phleomycin resistance) (Figure 7) and the *bla* gene (ampicillin resistance) (Figure 8) confirmed absence of these antibiotic genes. The probe for the *bla* gene also

contained 970 bp of pUT332 non-*Saccharomyces* sequences comprising 67.1% of all the pUC19 sequences distributed throughout the sequence of pUT322 (Appendix A). Southern analyses confirmed that pUT332 or components of the plasmid (antibiotic markers and *E. coli* vector sequences) are not present in ML01 or S92. ML01 is therefore the first genetically engineered wine yeast strain to be constructed without the integration of an antibiotic marker or *E. coli* vector sequences (Schuller and Casal, 2005).

The screening method and the colony PCR results (Appendix E) demonstrated that at least one copy of the malolactic cassette was integrated into one copy of the *URA3* locus. Southern blots were performed in order to confirm the absence of concatamers and illegitimate integrations elsewhere in the genome (Figures 4-6 and Appendix H). Utilising different restriction enzyme digests (*Eco*RV, *Nsi*I and *Pvu*II) and four different probes corresponding to the *mae1*, *mleA*, and *URA3* genes, and the *PGK1* promoter, it was confirmed that a single copy of the *URA3* gene was disrupted by the malolactic cassette without the formation of concatamers; no additional copies of the malolactic cassette were intergrated elsewhere in the genome. Furthermore, analysis of ML01 ascospores confirmed that one copy of the *URA3* gene was disrupted by the malolactic cassette since yeast cells from two spores were auxotrophic for uracil but positive for MLF and cells from the remaining two ascospores were prototrophic for uracil and negative for MLF.

The targeted integration of the malolactic cassette to the *URA3* locus (YEL021W) was completed using flanking sequences that are 925 bp (5' end) and 933 bp (3' end) long. Although it has been previously shown that flanking sequences of 30 bp are sufficient for homologous recombination in lab strains of *Saccharomyces* sp. (Baudin et

al., 1993; Manivasaskam et al., 1995), the efficiency largely depends upon perfect homology at both ends of the transforming DNA to the target locus (Wach, 1996). Sequence polymorphism in different strain backgrounds reduces the efficiency of homologous recombination when short-flanking sequences are used. Transformation efficiencies can be increased approximately 30- to 50-fold using flanking sequences of several hundred base pairs in length (Wach, 1996).

Both strands of the integrated malolactic cassette were sequenced (Appendix B). Eight unexpected nucleotide differences were found after comparing the sequence of the integrated malolactic cassette to previously published sequences (Appendix K). Six differences were found in either non-coding regions (*PGK1* promoter) or in the non-functional disrupted *URA3* ORF (Appendix K). Two changes were found in the *mleA* sequence, one corresponding to a silent mutation, the other involving the exchange of one amino acid for another amino acid of the same family (Appendix K). These anomalies could be due to genetic polymorphisms or amplification errors during the construction of the cassette. Alternatively, the differences may be attributed to errors in the original published sequences. The two differences involving one of the *PGK1* promoter sequences have likely originated from polymerase errors during the amplification steps involved in the cloning of the malate expression cassette because the other *PGK1* promoter did not contain these two nucleotide differences. The two differences found in the coding sequence of the *mleA* gene could have originated from a mistake during the amplification steps or could be attributed to errors in the published sequence or a mutation within the strain isolate used for the cloning of *mleA*. Regardless, one change resulted in a silent mutation and the other change resulted in a switch between two acidic

amino acids, aspartic acid to glutamic acid and did not affect the functionality of the enzyme. No differences were found in the *mae1* sequence when compared to the published sequence which comes from the same *S. pombe* strain. These results indicate that the sequences composing the malolactic cassette that is integrated in ML01 are not significantly different than the original sequences isolated from the donor strains. Other than the eight nucleotide differences, the malolactic cassette contained only synthetic polylinkers and DNA from the two donor microorganisms (Appendices J and K).

The upstream and downstream sequences of the malolactic cassette were also sequenced and show the native half-*Srf*I sites and perfect homology to native sequences surrounding the integration sites at each end of the malolactic cassette (Figure 9). The blunt-end restriction enzyme *Srf*I was specifically used so that after digestion from pJH2 the remaining half sites of the linear malolactic cassette would be completely homologous to native sequences (Figure 9).

The integrated malolactic cassette must be stable for ADY yeast production and subsequent winemaking procedures. In general, yeast production starts with pure culture slants and then proceeds through several pure culture laboratory scale fermentations (0.2 – 25 kg of yeast), followed by several larger batch and fed-batch fermentations (25 – 15,000 kg of yeast), and then it concludes with the final fed-batch "trade" fermentation (15,000 - 100,000 kg of yeast) (Reed and Nagodawithana, 1991). Throughout yeast production and growth in grape must, *S. cerevisiae* cells divide mitotically by forming a bud that eventually leads to a daughter cell. Theoretically, if one diploid yeast cell (80 x 10^{-12} g) (Sherman, 1997) and its progeny continuously doubled (with each mother cell forming no more than 20 - 30 daughter cells) it would take 60 generations before

approximately 92,230 kg of yeast is produced. During commercial winemaking, yeast generally undergo a maximum of seven generations since large initial inoculums of 1.5 - 6 x 10^6 cells/mL (5 - 20 g/hL of ADY) are used and the cells multiply to a maximum of 5 - 15 x 10^7 cells/mL (Reed and Chen, 1978; Reed and Nagodawithana, 1991). Hence a relatively low number of generations (< 70) are required to produce wine using ADY. Over this length of time the majority of ML01 cells are expected to retain a functional malolactic cassette since the frequencies of molecular mechanisms that could affect stability of the malolactic cassette, and therefore the MLF phenotype, are relatively low. Spontaneous mutations (10^{-9} to 10^{-8} per generation in *S. cerevisiae*) (Magni and von Borstel, 1962), loss of the malolactic cassette via mitotic gene conversion or crossing over (10^{-6} to 10^{-5} per generation) (Petes et al., 1991; Puig et al., 2000) or loss of a portion of the malolactic cassette (either the *mae1* or *mleA* expression cassettes) due to recombination at the direct repeats (the two *PGK1* promoter or terminator sequences) (10^{-4} to 10^{-3} per generation) (Estruch and Prieto, 2003; Wach et al., 1994) are infrequent and will have a minimal effect on the MLF performed by the majority of functional ML01 yeast cells. To date, several wine fermentations with ML01 ADY (produced at a pilot plant and large-scale fermentation facility) have been done and all have achieved a complete MLF. Even wine fermentations conducted with a 1:1 ratio of ML01 and S92 cells are able to accomplish an almost complete MLF (95.3% of malate degraded, data not shown). Moreover, after growth and completion of MLF in Chardonnay must by ML01 ADY (produced at a large-scale ADY production facility, Lesaffre, Turkey), 99.3 ± 1.0 % of ML01 colonies tested positive for a MLF phenotype.

4.6 The malolactic cassette has a minimal effect on the transcriptome and proteome of ML01

Global gene expression patterns of ML01 and S92 were analysed at 48 hours and 144 hours. These two time points were selected because at 48 hours the ML01 yeast cells perform the MLF and at 144 hours the MLF is complete (Figure 11). The two time points also correspond to log and stationary growth phase of the yeast (Figure 12). At 48 hours the transcription of 19 genes were affected = two-fold, and at 144 hours, the transcription of six genes were affected = two-fold (Table 9). With only 25 genes having a = two-fold change at both time points it is clear that the introduction of the malolactic cassette into *S. cerevisiae* S92 had a minimal effect on the transcription of the 5773 ORFs (4286 verified amd 1487 uncharacterised, *Saccharomyces* Genome Database, June 1, 2005) in the yeast cell. Moreover, the data suggests that no metabolic pathway was affected by the presence of the malolactic expression cassette integrated into the genome of ML01. The difference in the number of genes being affected at 48 hours (19 genes) and 144 hours (6 genes) is likely due to the MLF that is proceeding within ML01 cells at 48 hours.

Only one gene, *AQR1*, was found to be expressed differently at both time points. At 48 hours, *AQR1* is expressed 1.77 fold higher in ML01 than S92 and at 144 hours *AQR1* is expressed -3.23 fold lower. *AQR1* is a gene involved in the excretion of short-chain (C_2-C_6) monocarboxylic acids (such as lactate), quinidine, and excess amino acids (Tenreiro et al., 2002; Velasco et al., 2004). At 48 hours, lactic acid is present within ML01 yeast cells and it is likely that Aqr1p is involved in the transport of lactic acid out of the yeast. Interestingly, an Aqr1p-GFP fusion protein was shown to be localized to

multiple internal membrane structures and appears to cycle between these components and the cell surface (Velasco et al., 2004). According to this model, it is possible that Aqr1p catalyzes transport of lactic acid into vesicles that subsequently release the lactic acid into the external medium by exocytosis. Excretion of lactic acid via exocytosis may also explain the observation in the lag of lactic acid accumulation in the external medium during MLF. At 144 hrs there was a -3.23 fold decrease in expression of *AQR1* in the ML01 strain (Table 9). At 144 hrs, the malolactic fermentation was complete with the production of equimolar amounts of lactic acid from malic acid and no further increases in lactic acid were observed. This indicates that the yeast has transported all of the lactic acid into the surrounding media and no longer requires higher levels of Aqr1p to remove the intracellular lactic acid.

AQR1 is also involved in the secretion of excess amino acids (Velasco et al., 2004). The amino acids reported to be present in the highest concentrations in the cytosol of cells growing in glucose-ammonium medium (such as the synthetic must) are glutamate, aspartate and alanine, followed by asparagine, glutamine, serine and glycine (Messenguy et al., 1980). Other amino acids present at high levels are predominately found in the vacuole. The higher levels of *AQR1* during MLF could also result in the excretion of the main cytosolic amino acids, in addition to exporting lactic acid out of the cell. This depletion of amino acids may explain the increase in expression of *DIP5* (2.81 fold, Table 8) at 48 hours. *DIP5* is a dicarboxylic amino acid permease that mediates high affinity and high-capacity transport of glutamate and aspartate (Regenberg et al., 1998). DIP5p also transports alanine, glutamine, asparagine, serine and glycine; the same

amino acids potentially excreted by the over expression of *AQR1* (Regenberg et al., 1999).

Another gene of interest, *PHO84*, is a high-affinity inorganic phosphate transporter and a low-affinity manganese transporter involved in manganese homeostasis (Jensen et al., 2003). It is likely expressed at higher levels at 48 hours since the malolactic enzyme requires manganese as a cofactor to complete the decarboxylation of malate to lactate during the MLF. *PHO84* (2.18 fold, Table 8) may be required at higher levels in order to fulfill the manganese demand on the cell due to the presence of the transgenic mleAp.

Global gene expression patterns of ML01 indicate that no metabolic pathway was affected by the presence of the malolactic cassette integrated into its genome. The analysis also suggests that the genes *AQR1*, *DIP5* and *PHO84* are affected in order to remove intracellular lactic acid and maintain homeostasis.

The proteomes of ML01 and S92 were analysed after 48 hours of fermentation in synthetic must. The time point was selected because at 48 hours the ML01 yeast cells are performing the MLF (Figure 11) and the analysis of the transcriptome indicated that more genes are affected at this time point (Table 8). iTRAQ analysis identified only one protein, lanosterol 14-demethylase cytochrome P450 that was shown to have a weighted average ratio of 0.799 (±0.031) at a p-value < 0.05 across duplicate experiments. The protein corresponds to the gene *ERG11* and is one of the 19 enzymes involved in ergosterol biosynthesis. Since iTRAQ analysis identified 12 of 19 enzymes involved in the ergosterol pathway and only 1 protein showed a lower ratio, it is unlikely that the ergosterol pathway is affected by the malolactic cassette.

The analysis of the proteome of ML01 showed that 199 of the 559 detected proteins were 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, protein ratios for ML01 and S92 were identical.

The malolactic cassette encodes unique proteins such as the mleAp, mae1p, and possibly the three putative proteins created by cloning (Figure 10). The mleAp was identified in both ML01 duplicate samples (confidence >99%); the mae1p and the three putative proteins were not identified at the default parameters (confidence >94%, or at a confidence >50%). The difficulty in detecting the mae1p could be due to its membrane bound nature and possibly lower levels of protein due to degradation. The mae1p contains a PEST region (aa 421-434) at the C-terminal end that targets the protein for degradation (Grobler et al., 1995). Many proteins with short intracellular half-lives contain a PEST region that consists of proline, glutamic acid, serine, threonine and to a lesser extent aspartic acid (Rogers et al., 1986). Since the *mae1* transcript was detected by RT-PCR (Appendix M) and the ML01 yeast performs the malolactic fermentation efficiently; it is reasonable to suggest that the mae1p was present, even though the protein was not detected. The three putative proteins composed entirely of *Saccharomyces* sequences and one or two common restriction endonuclease sites (*Kpn*I and/or *Not*I), were not detected at a confidence level of >50%.

The introduction of the malolactic cassette into *S. cerevisiae* S92 affected the concentration of one protein out of 559 identified proteins; no metabolic pathway in the yeast cell was found to be affected. Therefore, the introduction of the malolactic cassette had minimal effect on global gene expression and protein levels.

4.7 Ethical considerations concerning use of the genetically modified yeast ML01

The issues surrounding the use of the malolactic yeast ML01 are the same as those facing every GMO designed for use in the food industry. Broadly speaking, these issues include possible effects on the health of consumers, potential environmental impact and social considerations. *S. cerevsiae* is an organism which has an extensive history of safe use. It has been used for millennia in fermentation processes such as bread leavening, and wine or beer production. The introduction of non-harmful, limited and well-characterised DNA from two other wine microorganisms should not adversely affect the safety of *S. cerevisiae* ML01. The *mleA* gene was isolated from *O. œni* which is not only found in wine but attains very high populations during MLF (10^6 to 10^8 cfu/mL) (Wibowo et al., 1985). Despite the two nucleotide differences discovered in the *mleA* gene integrated into ML01, the primary sequence of the malolactic enzyme is similar to the native malolactic enzyme since one difference corresponds to a silent mutation and the other involves the change of an acidic amino acid for another acidic amino acid. Moreover, the ML01 strain displays an efficient L-malate decarboxylating activity indicating that the secondary and tertiary structure of the enzyme is conserved. Sequencing of the inserted *mae1* gene of ML01 showed that no sequence discrepancy could be found between the *mae1* gene in ML01 and the native *mae1* gene from *S. pombe*. Therefore the primary structure of the malate permeases are the same and the fact that the malate permease is functional in ML01 indicates that the secondary and tertiary structures of the proteins are similar as well. Although *S. pombe* is a wine related microorganism and it may occasionally participate in spontaneous grape must fermentations (and was recently commercialised for use in the wine industry), this

organism is not as predominant in wine fermentations as *S. cerevisiae* and *O. œni*. Therefore, a literature search was carried out by Lesaffre's library department using Medline and Biosis databases to evaluate the allergenicity of *S. pombe*. Not a single paper implicating *S. pombe* in allergenic reactions was found (Didier Colavizza, Lesaffre Development, rue Gabrie Péri 137, F-59700 Marcq-en-Baroeul, France, personal communication). Moreover, it is likely that at the end of the fermentation very little malate permease will remain in ML01 yeast cells due to rapid intracellular degradation of the protein as a consequence of the presence of a PEST region in its C-terminus (Grobler et al., 1995). Storing the wine on lees (sediment composed primarily of yeast), if desired, would also further enhance this degradation of the malate permease. During storage on lees, cell proteins and nucleic material first undergo intracellular enzymatic degradation due to the liberation of intracellular proteases, aminopetidases, nucleases and phosphatases (Charpentier and Feuillat, 2002; Charpentier et al., 2005; Moreno-Arribas and Polo, 2005; Perrot et al., 2002). The yeast cell gradually loses its hydrolyzed contents as integrity of the cell wall is compromised. In the wine, further degradation can occur by proteases present in the extracellular media (Charpentier and Feuillat, 2002). Therefore the protein content of wines stored on ML01 lees or S92 lees should result in very similar hydrolysis products, namely small peptides and amino acids. Given the origin of the malate permease and the safe history of the presence of *S. pombe* strains in fermented beverages, as well as the absence of data concerning the allergenicity of this yeast, it can reasonably be concluded that if any small peptides hydrolyzed from the mae1p are present in wine they will not constitute a health safety issue.

Standard winemaking procedures also consist of various forms of clarification, wine stabilisation and filtration procedures that drastically reduce yeast cell numbers after alcoholic fermentation. It is a common practice to employ filtration prior to bottling to improve clarity and stabilisation of the wine. Various types of cellulose and membrane filters can reduce the viable yeast cell count from 50 cells/100 mL to <1 cell/100 mL, depending on the porosity of the individual filters (Ribereau-Gayon et al., 2000b). Although most wines (especially white wines) are filtered prior to bottling, some winemakers may rely only on clarification of their red wines with gelatins or egg white albumin and bottle without filtration. The use of ML01 is compatible with such a procedure although the final concentration of yeast in the bottle will be greater than filtered wine.

The thorough genetic characterisation of ML01 has confirmed that the integration site contains no DNA sequences other than those described in the malolactic cassette. The discovery that four new ORFs were created during construction of the malolactic cassette also does not affect the safety status of ML01. The three of the four putative proteins that could be checked by iTRAQ, composed entirely of *Saccharomyces* sequences and one or two common restriction endonuclease sites (*Kpn*I and/or *Not*I), were not detected. The fourth new ORF is entirely composed of *S. cerevisiae* sequences (truncated *URA3*, 3' end) and the iTRAQ method is incapable of differentiating it from the native URA3p. None of the putative proteins of these novel ORFs were detected by liquid chromatography and tandem mass spectrometry (iTRAQ) even at the 50% confidence limit.

The environmental impact of the ML01 strain should be no greater than the environmental impact of the industrial S92 strain. Commercial yeasts such as S92 are annually released in large quantities into the environment surrounding wineries. Recently, a large-scale three-year study of six different vineyards revealed that dissemination of commercial yeast in the vineyard is limited to short distances over short periods of time and is largely favoured by the presence of water run-off (Valero et al., 2005). Despite the annually intensive dissemination of commercial yeast into the local environment, 94% of the commercial yeast strains were found between 10 to 200m from the winery. This underscores the limited range of possible environmental impact beyond the winery. Moreover, analysis of population variations from year to year indicated that commercial strains do not settle in the vineyard or predominate over the indigenous flora (Valero et al., 2005). It has also been shown that colonisation of damaged grapes, where the modified ecology favours fermenting yeast species, by a selected *S. cerevisiae* wine strain is no different from colonisation of undamaged grapes (Comitini and Ciani, 2006). In both cases the inoculated wine strain could not out-compete the indigenous microflora resident on the grapes (Comitini and Ciani, 2006).

Survival of GM yeast strains in a confined wine cellar and greenhouse vineyard has been studied (Bauer et al. 2003 – see Schuller and Casal, 2005). In this study, four GM yeast containing resistance markers (*KanMX* or *SMR1-140*) and expressing the transgenes (with strong yeast promoters) for a-amylase, endo-ß-1,4-glucanase, xylanase or pectate lyase were sprayed onto vines in a confined greenhouse vineyard. Results showed that despite high initial cell counts, few *S. cerevisiae* cells were isolated from grapes, leaves, stems and soil during weekly monitoring. Furthermore, no significant

difference between the occurrences of the modified strains compared to the parental strain was detected, including GM strains secreting glucanases and pectinases (modifications thought to provide a selective advantage). The total yeast population of treated vines was also very similar to the untreated control vines and spontaneous micro-vinifications resulted in no significant differences in the fermentations performances amongst the trials (Schuller and Casal, 2005). Survival of GM and "self-cloned" baker's yeast in a simulated natural environment (water and soil) also showed that GM and self-cloned yeast decreased at an equal or faster rate than the wild-type control (Ando et al., 2005). In this case, the GM and Self-Cloned yeast were both modified for freeze tolerance by disruption of the acid trehalase gene (*ATH1*).

The possibility of horizontal gene transfer to other organisms cannot be ignored. Horizontal transfer of DNA can occur via interspecies mating among yeast belonging to the *Saccharomyces* sensu stricto complex and across species barriers via transfer of plasmid DNA (Marinoni et al., 1999; Mentel et al., 2006; Nevoigt et al., 2000). As no antibiotic resistance markers are present in the ML01 strain, much of the concern associated with horizontal gene transfer is not applicable. The transfer of the entire malolactic cassette or part of it to another organism would also not constitute a threat to the environment. The transfer of both transgenes to another organism in the environment (most likely another *Saccharomyces* sensu stricto strain) may give that particular cell the ability to degrade malate, like ML01; an ability that is already present in numerous lactic acid bacteria and malate degrading yeast such as *S. pombe* present in the ecology in and around the winery. Individually the *mleA* and *mae1* genes are also predominant in the environment and it is presumable that if they conferred a selective advantage to an

organism this transfer of genetic material would have already occurred. The combination of *S. cerevisiae* promoter sequences with transgenes is also not a major concern since recognition of these regulatory sequences would only be effective in *S. cerevisiae* strains (and possibly other phylogenetically close species) and less so in distantly related organisms. It is also conceivable that due to the massive world-wide production and utilisation of baker's yeast, brewer's yeast and wine yeast (as well as other industries utilising *Saccharomyces* sp.) DNA fragments containing *PGK1* regulatory sequences have been dispersed into the environment over the centuries. The addition of the malolactic cassette to this mileu is of minimal concern. The well-characterised malolactic cassette is safe, does not confer any advantage to ML01 and should be considered as very low risk to the environment.

Specific social consequences of using the ML01 strain in the wine industry also exist. Wine is a traditional product that is perceived to be more than just an alcoholic beverage. Wine also has strong geographical ties to certain regions/countries and in many cases the wine industry in these regions is unreceptive to the use of GMOs. With this background in tradition it is very difficult to incorporate new technologies and if they are to be successful, they have to offer advantages to the consumer. In this regard the ML01 yeast can succeed if the consumer is educated about the toxic effects of naturally occurring bioamines and how improvements in winemaking (early sulphiting) and the use of ML01 can reduce or eliminate these compounds. In this respect the ML01 yeast is unique as a GMO since it provides a direct health advantage to consumers and a direct benefit to the producer (specifically a complete MLF during alcoholic fermentation).

Although there will always be wine makers that prefer inoculation of malolactic bacteria or even the use of the natural microflora of the wine cellar to complete the MLF, the malolactic yeast is an important additional tool for winemakers to produce wholesome, well-balanced high quality wines.

5 CONCLUSIONS

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 safety issues (e.g. mad cow disease and dioxin in chocolates) and are increasingly demanding safe food and beverages. Moderate consumption of wine protects consumers against cardiovascular disease, dietary cancers, ischaemic stroke, peripheral vascular disease, diabetes, hypertension, peptic ulcers, kidney stones and macular degeneration (Bisson et al., 2002). These potential health benefits may have contributed to the increased 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. œni*, 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 and diamine oxidase 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. 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 EC, a well-known carcinogen in wines (Coulon et al., 2006). Moreover, powerful genomic, transcriptomic,

proteomic and metabolomic techniques are now available to demonstrate that recombinant yeasts are substantially equivalent to the parental strains.

The genetically stable commercial wine yeast strain, ML01, was constructed 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*. The malolactic cassette integrated into the *URA3* locus of ML01 contains no vector sequences or antibiotic resistance marker genes. 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. DNA microarray and iTRAQ analyses of the transcriptome and proteome of the yeast indicated that the introduction of the malolactic cassette had little effect on global gene expression patterns and protein levels. Phenotypic results show that the novel malolactic yeast ML01 is capable of efficiently decarboxylating malate to lactate within the first five days of the alcoholic fermentation at 20°C; at lower temperatures (13°C) MLF by ML01 can take up to nine days. Wines produced by the ML01 yeast had lower volatile acidity than wine produced with the parental strain S92 and a bacterial MLF. ML01 also produced Chardonnay wines lighter in color than wine produced by the parental strain and Cabernet Sauvignon wines darker in color than wines produced with S92 and a bacterial MLF. GC/MS analysis of volatile compounds and sensory analyses of wine produced by ML01, the parental yeast S92 and S92 plus *O. œni* indicated that ML01 is ideal for the

production of wine on a commercial scale as ML01-produced wines were judged to be superior in overall quality by trained tasters.

The bacterial MLF is unpredictable and often results in stuck MLF, and the production of off-flavors and biogenic amines. *S. cerevisiae* ML01 conducts an efficient malolactic fermentation that will solve the important issue of cellar capacity and prevent oxidation and microbial spoilage of wines that result in financial losses to wineries. Early sulfiting of wine produced with ML01 will prevent the growth of undesirable lactic acid bacteria that produce biogenic amines. It is therefore conceivable that 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.

This thesis represents an integrated approach, conducting analyses at the phenotypic, DNA, RNA, and protein levels to determine if recombinant yeast ML01 is substantially equivalent to the parental strain S92. Based on these results it was concluded that the ML01 strain is substantially equivalent to the parental strain S92. ML01 has been approved for use in Canada (Appendix Q) and has GRAS status with the US FDA (Appendix R). It is the first metabolically engineered yeast to be commercialised by the wine industry and is currently available in Canada, the USA and Moldova. Notifications are currently being submitted to all of the major wine producing countries in the world.

5.1 Future Directions

Several lines of future investigations with the novel ML01 yeast and its impact on the wine industry should be pursued. First, analysis of the bioamine content of wines produced by ML01 in several different commercial wineries should be conducted. It is expected that wines produced by the ML01 yeast would contain significantly lower levels of bioamines and these data could be used to educate wine consumers on how yeast biotechnology can reduce the toxic properties of naturally occurring biogenic amines in wine. Secondly, the ageing of commercially prepared wines using ML01 and appropriate control wines should be studied. This experiment would provide important data to the wine industry which places high regard on wines that age well. Thirdly, the construction of other strains containing the malolactic cassette would be of particular importance to winemakers. Although the strain S92 can be used to produce red and white wines, it is generally preferred for white wine production. Creating other malolactic strains well suited to red wine production could increase the options available to winemakers seeking to complete the MLF during the alcoholic fermentation of their red wines.

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APPENDIX A

Schematic Representation of pUT332 and Probes Used to Confirm the Absence of Antibiotic Markers in ML01

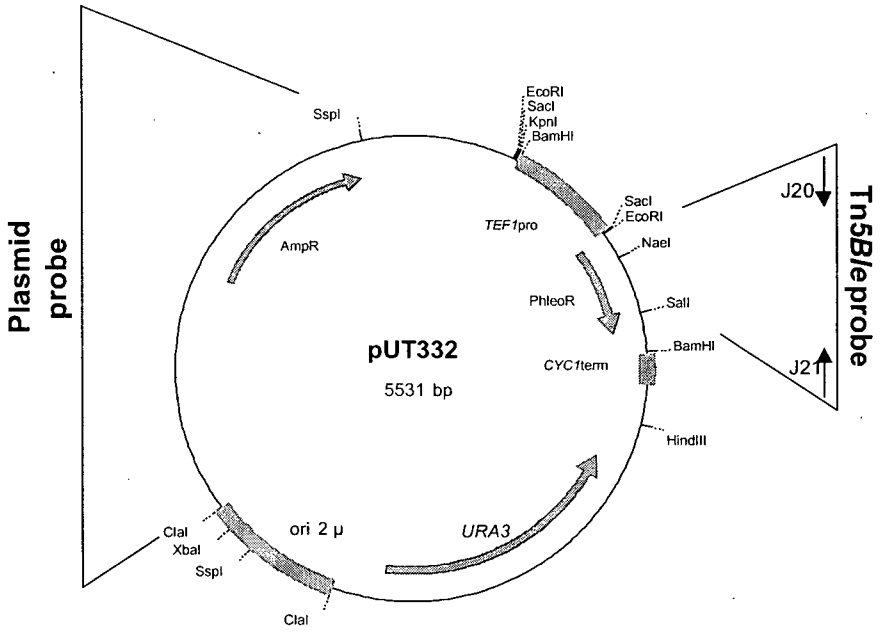

Figure 21. Plasmid map of pUT332 and schematic representation of the probes used in Southern blot experiments to confirm the absence of antibiotic markers in *S. cerevisiae* ML01. The *bla* gene (ampicillin resistance) probe was retrieved as a *Cla*I-*Ssp*I fragment after restriction digest of the pUT332 plasmid. This probe includes an additional 970 bp of plasmid pUT332 which trace back directly to plasmid pUC19 (Gatignol *et al.*, 1990). The 1758 bp plasmid probe comprises 67.1% of all the pUC19 sequences distributed throughout the sequence of pUT332. The Tn*5ble* probe was retrieved by PCR amplification of a region of the pUT332 plasmid using primers J20 and J21.

APPENDIX B

Strategy for Sequencing the Malolactic Cassette from the Genome of ML01

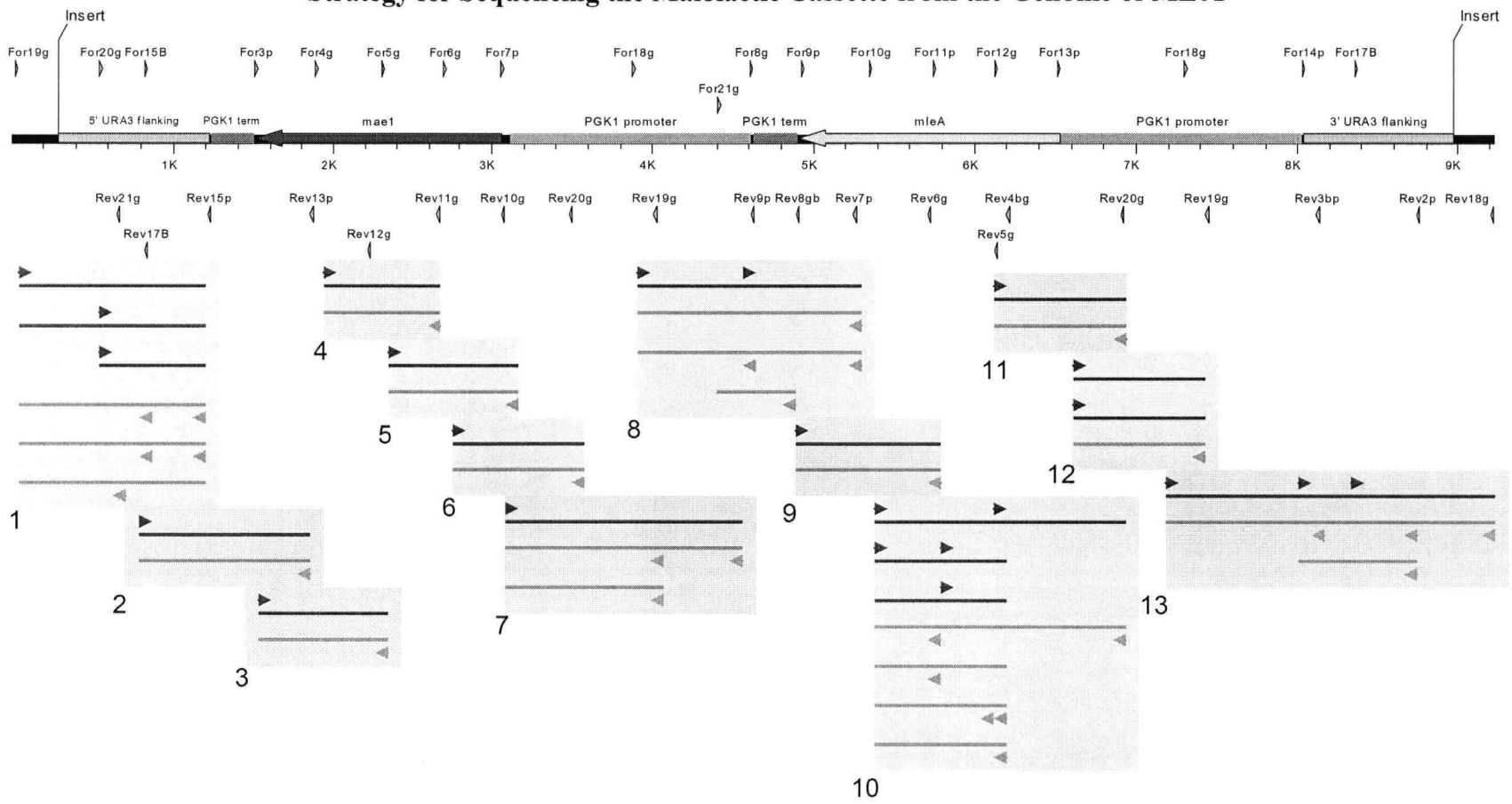

Figure 22. Strategy used to sequence the malolactic cassette from the genome of *S. cerevisiae* ML01. A minimum of two sets of 13 unique PCR templates (lines), spanning the entire malolactic cassette, were used in the sequencing. Primers for sequencing of each strand are indicated as arrows.

APPENDIX D

Participant Consent Form

Title of Study: Sensory evaluation of wine produced by the malolactic yeast MLOl

Principal Investigator: Professor Hennie J.J. van Vuuren (Ph.D.)
Wine Research Centre
UBC
(604) 822-0418
Sponsors: South African Wine Industry 1994-1996; Bio-Springer 1997-2002

1. INTRODUCTION
You are being invited to take part in this research study because of your expertise and extensive experience in the sensory evaluation of wine. You will not receive any remuneration to participate in this tasting.

2. YOUR PARTICIPATION IS VOLUNTARY
Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. If you wish to participate, you will be asked to sign this form. If you do decide to take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision. Please take time to read the following information carefully.

3. WHO IS CONDUCTING THE STUDY?
This study was initiated in the laboratory of Dr. Hennie van Vuuren at the University of Stellenbosch in August 1994. Financial support was received from the South African Wine Industry and Bio Springer. John Husnik, Ph.D. student under supervision of Dr. Hennie J.J. van Vuuren, continued with the research in the Wine Research Centre at UBC. Bio Springer sponsored the research at UBC from January I, 2001 - June 2002.

4. BACKGROUND
Most red wines and some white wines undergo the bacterial malolactic fermentation that catalyses the bio-conversion of L-malate to L-lactate. Some naturally occurring lactic acid bacterial strains in wine produce undesirable compounds, such as biogenic amines, from amino acids present in grape musts. The presence of bio-amines can be of great concern for consumers since these molecules, particularly histamine, have been shown to be the causative agent of head aches and other allergenic symptoms such as, diarrhea,

palpitations, rashes and vomiting. Moreover, strong scientific evidence suggests that other biogenic amines such as cadaverine, putrescine, spermine, and tyramine can potentiate the toxic effect of histamine. During the last two decades new technologies such as metabolic engineering, protein engineering, and novel enzyme and fermentation technologies have been developed that have greatly enhanced our capabilities to produce safer wines of a higher quality. The malolactic yeast ML01 is such an example. Over the last 10 years we have succeeded to genetically enhance an industrial wine yeast strain to perform the alcoholic and malolactic fermentations simultaneously. We have conducted rigorous scientific examination of the MLOl malolactic yeast and this yeast has now received Generally Regarded as Safe (GRAS) status from the FDA. All food grade microorganisms are required to have this status before they can be applied in the food or wine industry. The application of the ML01 malolactic yeast will minimize or prevent growth of lactic acid bacteria capable of producing allergens and at least ensure a reduction, or elimination, of these allergens from wine. Consumers sensitive to bioamines are unlikely to get headaches when drinking wines produced with the ML01 yeast.

5. WHAT IS THE PURPOSE OF THE STUDY?
The purpose of this study is to evaluate and compare the colour, aroma, flavour and overall quality of the wine produced by the malolactic wine yeast, wine produced with the parental yeast, and wine produced with the parental yeast and malolactic bacteria. The yeast is being used for the production of commercial wines in Moldavia and large-scale fermentation trials have been conducted in South Africa and are currently being conducted in the USA. To the best of our knowledge, wine produced with the malolactic yeast MLOl is safe to consume.

6. WHO CAN PARTICIPATE IN THE STUDY?
Any experienced wine taster.

7. WHAT DOES THE STUDY INVOLVE?
Sensory evaluation of the wines at PARC in Summerland. Wines will be tasted blind and each panel member will be given a questionnaire to complete. The time required for tasting is approximately 2 hours.

8. AFTER THE STUDY IS FINISHED
Mr. Husnik will use the data as part of his Ph.D. thesis and in publications resulting from this study. Every member of the panel will receive a copy of the paper.

9. WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?
Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure.

10. WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY DURING MY PARTICIPATION?

If you have any questions or desire further information about this study before or during participation, you can contact Dr. Hennie J.J. van Vuuren at (604) 822-0418. If you have any concerns about your rights as a research subject and/or your experiences while participating in this study, contact the 'Research Subject Information Line in the University of British Columbia Office of Research Services' at (604) 822-8598.

Thanks for your willingness to participate in this study.

Sincerely yours

Hennie J.J. van Vuuren (Ph.D.)
Professor and Eagles Chair
Director

September 22, 2003

CONSENT

I understand that my participation in this study is entirely voluntary and that I may refuse to participate or withdraw from this study at any time. **To the best of our knowledge, wine produced with the malolactic yeast MLOl is safe to consume and there are no foreseeable risks.**
There are no direct benefits to subjects from participating in this tasting.

I have received a copy of this consent form for my own records.

I consent to participate in this study.

Subject signature	Printed name	Date
Signature of a witness	Printed name	Date
Signature of PI	Printed name	Date

Version 2 November 18, 2003

APPENDIX E

PCR Confirmation of the Screening Method

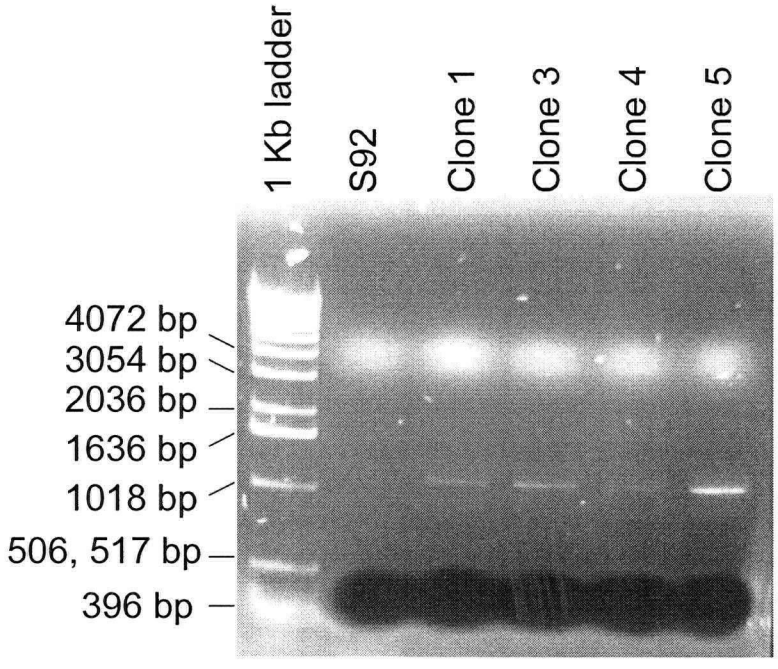

Figure 23. PCR confirmation of the screening method used to detect integration of the malolactic cassette into the *URA3* locus of S92. The 1095 bp PCR product was visualized by 0.8% agarose gel electrophoresis and ethidium bromide staining. PCR primers specific to *PGK1*p and outside the malolactic cassette (3'-end) were used.

APPENDIX F

Degradation of Malate and Consumption of Glucose and Fructose by Malolactic Wine Yeast Clones

Table 15. Degradation of malate (g/L) by individual colonies of malolactic clones inoculated into synthetic must containing 4.5 g/L of malate.

Day	Malolactic clone 1			Malolactic clone 3			Malolactic clone 4			Malolactic clone 5			S92	S92 + YCplac33-KanMX	S92 + pJH13
	1	2	3	1	2	3	1	2	3	1	2	3			
0	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38
1	2.38	2.27	3.26	3.08	3.29	3.30	3.94	4.08	4.05	4.02	3.67	4.34	4.29	4.35	3.94
2	0.28	0.25	0.30	0.91	0.36	0.33	0.88	1.20	1.11	1.88	2.50	4.08	4.31	4.19	1.39
3	0.07	0.07	0.07	0.11	0.07	0.07	0.08	0.08	0.17	0.16	0.50	nd	3.84	4.04	0.15
4	0.07	0.07	0.07	0.06	0.06	0.06	0.06	0.05	0.08	0.06	0.07	nd	3.89	3.88	0.05
5	0.06	0.06	0.06	0.06	0.05	0.05	0.04	0.04	0.05	0.05	0.06	nd	3.90	3.95	0.04
6	0.05	0.04	0.05	0.05	0.04	0.04	0.03	0.02	0.04	0.04	0.05	nd	3.91	3.61	0.03
7	0.04	0.04	0.04	0.04	0.05	0.04	0.02	0.02	0.03	0.04	0.04	nd	3.31	3.33	0.03
14	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.03	3.48	2.98	3.21	0.01

Table 16. Consumption of glucose (g/L) by individual colonies of malolactic clones inoculated into synthetic must containing 100 g/L of glucose (and 100 g/L of fructose).

Day	Malolactic clone 1			Malolactic clone 3			Malolactic clone 4			Malolactic clone 5			S92	S92 + YCplac33-KanMX	S92 + pJH13
	1	2	3	1	2	3	1	2	3	1	2	3			
0	107	107	107	107	107	107	107	107	107	107	107	107	107	107	107
7	10.0	5.18	9.18	20.5	11.2	8.04	1.19	.704	1.27	5.46	10.2	19.6	3.22	6.31	1.46
14	0.13	0.08	0.10	0.21	0.10	0.10	0.05	0.06	0.06	0.07	0.17	0.46	0.05	0.05	0.04

Table 17. Consumption of fructose (g/L) by individual colonies of malolactic clones inoculated into synthetic must containing 100 g/L of fructose (and 100 g/L of glucose).

Day	Malolactic clone 1			Malolactic clone 3			Malolactic clone 4			Malolactic clone 5			S92	S92 + YCplac33-KanMX	S92 + pJH13
	1	2	3	1	2	3	1	2	3	1	2	3			
0	105	105	105	105	105	105	105	105	105	105	105	105	105	105	105
7	23.9	30.6	29.5	27.5	21.8	20.5	12.7	10.3	12.1	20.2	33.0	43.4	22.9	26.8	12.3
14	3.07	1.52	2.60	4.34	2.22	2.10	0.57	1.24	0.93	1.82	3.41	16.5	1.58	1.76	0.71

APPENDIX G

Confirmation of the Identity of the Parental Strain by PCR Amplification of the d Elements of the Ty1 Retrotransposon

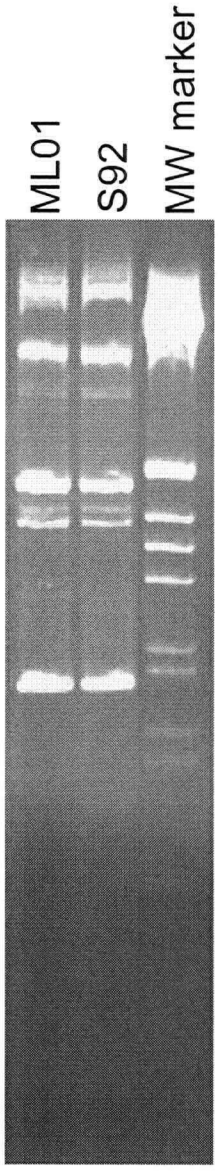

Figure 24. Genetic patterns of the ML01 and the S92 yeast strains based on amplification of genomic DNA regions in between d elements of the Ty1 retrotransposon.
A detailed method of the PCR amplification procedure is given by Ness et al., 1993.

APPENDIX H

Southern Blot Confirming Integration of the Malolactic Cassette into the *URA3* Locus of S92 (*PGK1* promoter probe)

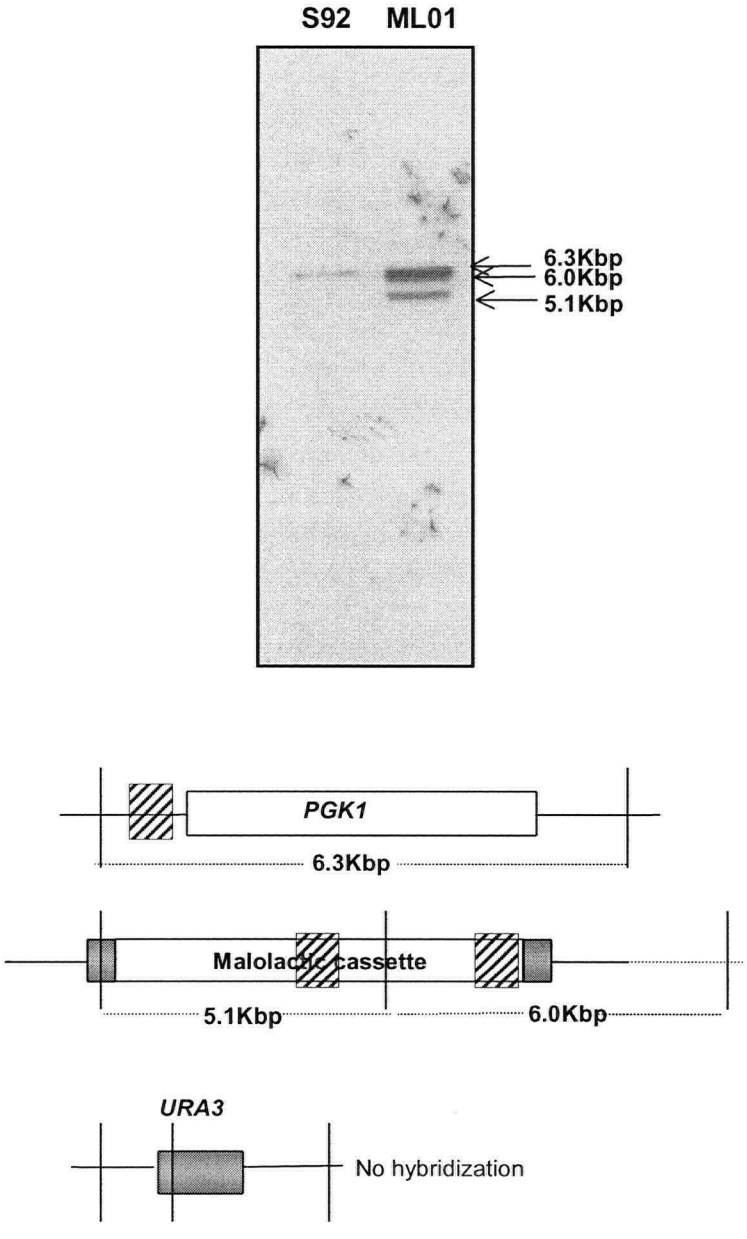

Figure 25. Integration of the malolactic cassette into the *URA3* locus of S92 was confirmed by Southern blot analysis using a *PGK1* promoter probe. The schematic representation illustrates the *Eco*RV restriction sites with vertical lines and the *PGK1* promoter probe hybridization site is depicted as hatched boxes in the lower panel.

APPENDIX I

Ascospore formation by the ML01 and S92 strains

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.

Table 18. Comparison of the ML01 and S92 strains capability to sporulate. Following spore formation on SAA medium, the percentage of ascospores (containing 3 or 4 spores) was determined by microscopic evaluation.

	ML01	S92
Total counted	782	612
Percentage of triads and tetrads formed per total number of cells	11%	8%

Analysis of the ML01 spores revealed that cells from two spores were auxotrophic for uracil but positive for MLF and cells from the remaining two ascospores were prototrophic for uracil and negative for MLF.

APPENDIX J

Detailed Description of the DNA Sequences that Comprise the Malolactic Cassette

Table 19. The source and description of the malolactic cassette DNA sequences.

Nucleotide position	Designation	Reference for cloning details	Source
1-4	***SrfI* half cloning site**	**This study**	***Saccharomyces cerevisiae* GC210**
1-928	***URA3* sequence**	**This study**	***Saccharomyces cerevisiae* GC210**
1-508	5' non coding sequence		
509-928	Part of open reading frame		
929-934	***Kpn*I cloning site**	**This study**	**Synthetic**
935-1198	***PGK1* terminator**	**Crous *et al.*, 1995**	***Saccharomyces cerevisiae* AB972**
935-940	*Hind*III site		
941-1194	Rest of *PGK1* terminator		
1195-1198	Remainder of *Cla*I site		
1199-1218	**Part of linker used in cloning strategy**	**Crous *et al.*, 1995**	**Synthetic**
1199	C residue from linker		
1200-1204	Remainder of *Bgl*II cloning site		
1205	C residue		
1206-1211	*Xho*I cloning site		
1212	G residue		
1213-1218	*Bgl*II cloning site		
1219-2818	***mae1* gene**	**Grobler *et al.*, 1995 Volschenk *et al.*, 1997a and 1997b**	***Schizosaccharomyces pombe* 972 h⁻ (leu1-32)**
1219-1224	*Nde*I site		
1225-1456	3' non coding region		
1457-1459	STOP codon		
1460-2770	Coding region		
2771-2773	START codon		

Nucleotide position	Designation	Reference for cloning details	Source
2774-2812	5' non coding region		
2813-2818	*Bal*I site		
2819-2829	**Part of linker used in cloning strategy**	**Crous *et al.*, 1995**	**Synthetic**
2819-2824	*Eco*RI cloning site		
2825	C residue		
2826-2829	remainder of *Bgl*II cloning site		
2830-4316	***PGK1* promoter**	**Crous *et al.*, 1995**	***Saccharomyces cerevisiae* AB972**
2830-4310	Part of *PGK1*p		
4311-4316	*Hind*III cloning site		
4317-4322	***Kpn*I cloning site**	**This study**	**Synthetic**
4323-4330	***NotI* cloning site**	**This study**	**Synthetic**
4331-4594	***PGK1* terminator**	**Crous *et al.*, 1995**	***Saccharomyces cerevisiae* AB972**
4331-4336	*Hind*III site		
4337-4590	Rest of PGK1t		
4591-4594	Remainder of *Cla*I site		
4595-4614	**Part of linker used in cloning strategy**	**Crous *et al.*, 1995**	**Synthetic**
4595	C residue		
4596-4600	Remainder of *Bgl*II cloning site		
4601	C residue		
4602-4607	*Xho*I cloning site		
4608	G residue		
4609-4614	*Bgl*II cloning site		
4615-4616	**CA residues left from oligonucleotide used for *mleA* amplification**	**Volschenk, unpublished**	**Synthetic**
4617-6242	***mleA* gene**	**Volschenk, unpublished**	***Œnococcus œni* Lo 8413**
4617-4619	STOP codon		

Nucleotide position	Designation	Reference for cloning details	Source
4620-6239	*mleA* open reading frame		
6240-6242	START codon		
6243-6253	**Part of linker used in cloning strategy**	**Crous *et al.*, 1995**	**Synthetic**
6243-6248	*EcoR*I cloning site		
6249	C residue		
6250-6253	Remainder of *Bgl*II cloning site		
6254-7740	***PGK1* promoter**	**Crous *et al.*, 1995**	***Saccharomyces cerevisiae* AB972**
6254-7734	Part of *PGK1*p		
7735-7740	*Hind*III cloning site		
7741-7748	***NotI* cloning site**	**This study**	**Synthetic**
7749-8683	***URA3* sequence**	**This study**	***Saccharomyces cerevisiae* GC210**
7749-8132	Part of open reading frame		
8133-8683	3' non coding region		
8680-8683	***Srf*I half cloning site**	**This study**	***Saccharomyces cerevisiae* GC210**

APPENDIX K

Discrepancies between the Integrated Malolactic Cassette and Published Sequences

Table 20. Discrepancies found when comparing the sequence of the integrated malolactic cassette and previously published sequences.

Nucleotide position	Description
821	Difference in the 5' region of the *URA3* open reading frame
929-934	Additional sequence resulting from cloning strategy
1199-1218	Additional sequence resulting from cloning strategy
2819-2829	Additional sequence resulting from cloning strategy
2896	Difference in the *PGK1*p sequence
4298	Difference in the *PGK1*p sequence
4317-4330	Additional sequence resulting from cloning strategy
4595-4614	Additional sequence resulting from cloning strategy
4629	Difference in the *mleA* open reading frame. This difference corresponds to a change of amino acids from aspartic acid (in the published sequence) to glutamic acid (in the
5247	Difference in the *mleA* open reading frame. This difference corresponds to **no change** of the amino acid sequence
6243-6253	Additional sequence resulting from cloning strategy
7741-7748	Additional sequence resulting from cloning strategy
7751	Difference in the 3' region of the *URA3* open reading frame
8234	Difference in the 3' region of the *URA3* non coding sequence
8543	Difference in the 3' region of the *URA3* non coding sequence

APPENDIX L

Confirmation of DNA Microarray Data by Real-Time Reverse Transcription PCR

Table 21. Comparison of fold change data for ten genes as determined by DNA microarray and by real-time reverse transcription PCR.

Gene Symbol	Fold change by semi-quantitative RT--PCR	Fold change by DNA microarray
	48 h	**48 h**
DIP5	4.92	2.81
PHO84	3.60	2.18
SUE1	-3.15	-5.13
PRR2	-1.11	-3.44
CTT1	-2.99	-3.29
PUT4	-6.33	-3.13
YPC1	-1.74	-2.18
	144 h	**144 h**
ENA2	Below threshold	5.27
AQR1	-2.74	-3.23
YML089C	-3.14	-3.14

APPENDIX M

Transcripts of *mae1*, *mleA* and *URA3* in the ML01 yeast

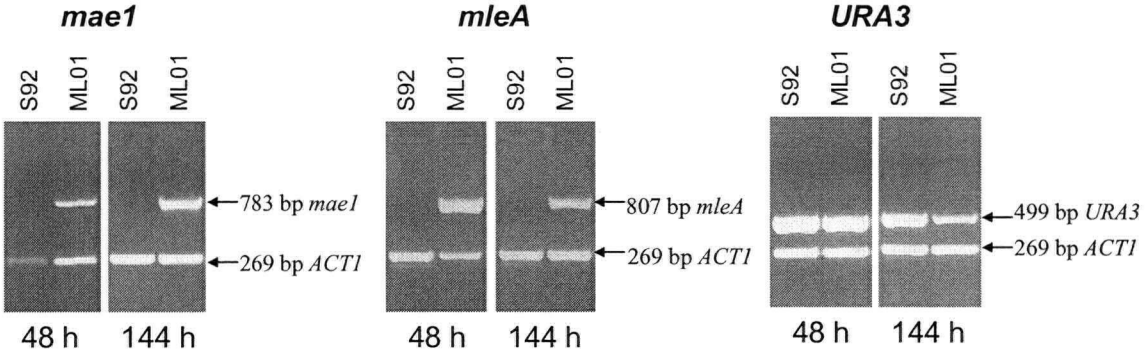

Figure 26. The presence of the *mae1*, *mleA* and *URA3* transcripts in the ML01 yeast during fermentation. Reverse transcriptase PCR was conducted on total RNA extracted from cells harvested from fermentations at 48 and 144 hours. The *mae1* and *mleA* transcripts are absent in S92 at both time points.

APPENDIX N

Vinification Trials with ML01 and S92 in Chardonnay Must (2004 harvest)

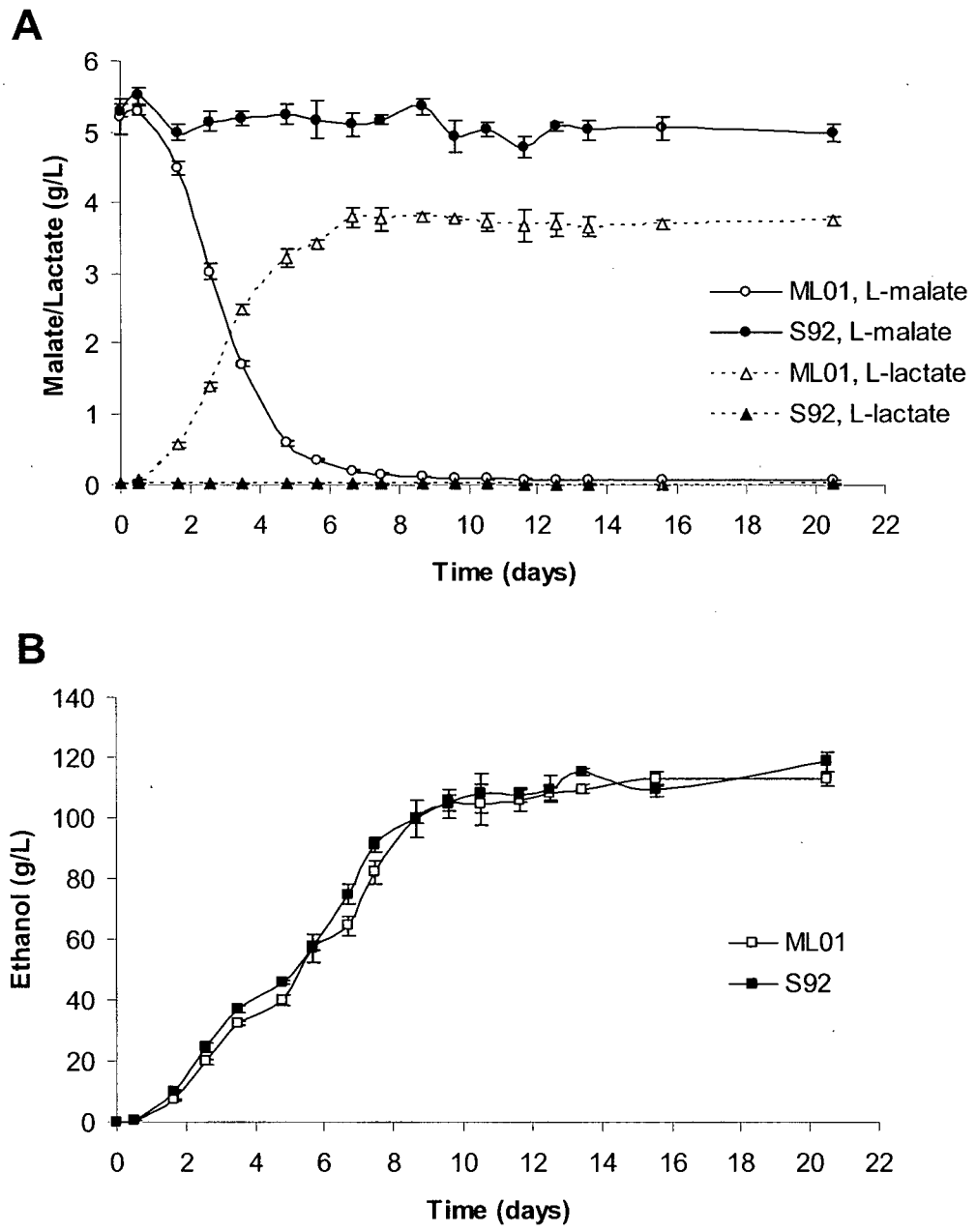

Figure 27. Malate degradation and lactate and ethanol production by ML01 and S92 in Chardonnay must (from fruit harvested in 2004). (A) Efficient conversion of L-malate (?) to L-lactate (△) during alcoholic fermentation by ML01; no significant degradation of L-malate (?) or production of L-lactate (?) was observed for S92. (B) Production of ethanol by ML01 (?) and S92 (¦); introduction of the malolactic cassette did not affect ethanol formation.

APPENDIX O

Physicochemical Characteristics of Chardonnay Wines (2004 Harvest) Produced by ML01, S92 and S92 plus *O. œni*

Table 22. Physicochemical and colour measurements[a] of Chardonnay wines (2004 harvest) produced by ML01, S92 and S92 plus *O. œni*.

	ML01	**S92**	**S92 + *O. œni***	***p*[b]**
Titratable acidity (g/L)	6.1 a[c]	7.8 b	6.3a	**
Acetate (g/L)	0.25 a	0.328 b	0.424 c	***
pH	3.68 a	3.52 b	3.78 c	***
Colour measurements				
L (degree of lightness)	97.86 a	97.96 b	96.55 a	***
a (greenness)	-1.85 a	-1.73 b	-2.39 c	***
b (yellowness)	9.76 a	9.47 b	13.95 c	***
$A_{420nm} + A_{520nm}$	0.198 a	0.179 a	0.255 b	**

[a]The mean values for biological replicates are given for all quantities (n=3)
[b]*, **, ***, ns: significant at $p < 0.05$, 0.01, 0.001, or not significant
[c]Means separated at $p < 0.05$ by Duncan's post-hoc test

APPENDIX P

Correlation Matrix

Table 23. Correlation matrix obtained from the sensory analysis of Chardonnay wines.

	Overall Quality	*Yellow Colour*	*Fruity Taste*	*Body*	*Sweetness*	*Acidity*
Overall Quality	1.00000					
Yellow Colour	-0.69810	1.00000				
Fruity Taste	0.93026	-0.38672	1.00000			
Body	0.99958	-0.71850	0.91927	1.00000		
Sweetness	0.97510	-0.52192	0.98847	0.96828	1.00000	
Acidity	-0.99459	0.76872	-0.88710	-0.99718	-0.94677	1.00000

APPENDIX Q

Health Canada Approval to use ML01 for the Commercial Production of Wine in Canada

Health Canada / Santé Canada
Health Products and Food Branch / Direction générale des produits de santé et des aliments

Tunney's Pasture
Postal Locator 0701A5
OTTAWA, Ontario
K1A 0L2

06-115381-853

JUL 12 2006

Dr. Hennie J.J. van Vuuren
Faculty of Agricultural Sciences
University of British Columbia
Suite 230, 2205 East Mall
VANCOUVER, British Columbia
V6T 1Z4

Dear Dr. van Vuuren:

This letter refers to the Novel Food Submission concerning Wine Yeast ML-01 and its use in winemaking in Canada. Officers of the Health Products and Food Branch have reviewed the information you provided for assessment of the acceptability of the Wine Yeast ML-01 and of the wines derived from it for human food use in Canada.

According to the submitted information, this yeast strain carries two novel genes which permits the yeast to conduct malolactic fermentation.

Based on our evaluation of the submitted data, we have no objection to the food use of the Wine Yeast ML-01 in Canada.

It should be noted that this opinion is solely with respect to the suitability for sale as food of products derived from Wine Yeast ML-01.

../2

Canada

APPENDIX R

US Food and Drug Administration GRAS Notice

CFSAN/Office of Food Additive Safety
June 30, 2003

Agency Response Letter
GRAS Notice No. GRN 000120

Robert Biwersi
Lesaffre Yeast Corporation
433 East Michigan Street
Milwaukee, WI 53202

Re: GRAS Notice No. GRN 000120

Dear Mr. Biwersi:

The Food and Drug Administration (FDA) is responding to the notice, dated January 2, 2003, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on January 7, 2003, filed it on January 10, 2003, and designated it as GRAS Notice No. GRN 000120.

The subject of the notice is *Saccharomyces cerevisiae* strain ML01 (*S. cerevisiae* strain ML01) carrying a gene encoding malolactic enzyme from *Oenococcus œni* and a gene encoding malate permease from *Schizosaccharomyces pombe*. The notice informs FDA of the view of Lesaffre Yeast Corporation (Lesaffre) that *S. cerevisiae* strain ML01 is GRAS, through scientific procedures, for use in winemaking as a yeast starter culture for grape must fermentation. Lesaffre recommends using between 0.1 to 0.2 grams of active dry yeast per liter of wine.

Lesaffre describes generally available information about traditional manufacturing processes for the production of wine from grapes. These processes include the harvesting, de-stemming and crushing of grapes (resulting in must), the separation of the juice from the skins and seeds, one or more distinct types of microbial fermentation, clarification, stabilization, and bottling. Winemakers may vary the sequence of operational steps or modify procedures, depending upon the desired characteristics and nature of the wine.

Lesaffre describes generally available information about two distinct fermentation processes (i.e., alcoholic fermentation and malolactic fermentation) that occur either through the action of microorganisms that already are present on the grapes or through the action of microorganisms that are specifically added by the winemaker. Alcoholic fermentation (i.e., a process whereby the sugars glucose and fructose are converted to ethanol) is mediated by metabolic pathways associated with yeast (usually *S. cerevisiae* or closely related species). Malolactic fermentation (i.e., a process whereby the dicarboxylic acid malic acid is decarboxylated to the monocarboxylic acid lactic acid) is mediated by lactic acid bacteria through the combined action of a protein (called malate permease) that transports malic acid from the wine into the bacteria and an enzyme (called malolactic enzyme) that converts the malic acid to lactic acid. Because malolactic fermentation reduces the number of carboxylic acid groups on organic acids present in the wine, it reduces the acidity of the must. Although alcoholic fermentation is an inherent process associated with all winemaking, malolactic fermentation is a secondary process that may or may not be induced by the winemaker, depending on the desired characteristics and nature of the wine.

Lesaffre describes generally available information about clarification of wine, which can occur either at the end of the alcoholic fermentation or after the wine has been kept on the lees (the sediment formed by spent yeast cells and grape particulate matter). Wine clarification encompasses the removal of solid particles in the wine via gravity or centrifugation and subsequent elimination of the sediment or pellet. When clarification occurs at the end of fermentation, the clarification process removes most yeast cells. When the wine is kept on the lees before clarification, the yeast cells undergo autolysis, which releases cellular material that ultimately is degraded through the action of enzymes such as proteases.

Lesaffre describes generally available information about stabilization processes, which differ depending on whether the wine is a white wine or a red wine. For white wine, stabilization involves removing proteins via filtration with bentonite. For red wine, stabilization involves adding gelatin or egg white albumin to precipitate colloidal structures that include tannin-protein complexes. Prior to bottling, most wines undergo filtration (e.g., with diatomaceous earth, cellulose filters, or membrane filters) that eliminates any remaining yeast cells.

Lesaffre describes published articles about bioengineered strains of *S. cerevisiae*, including strains of *S. cerevisiae* that have been modified to conduct malolactic fermentation. Lesaffre notes that the use of bioengineered strains that can conduct both

alcoholic and malolactic fermentation eliminates the need for separate additions of two distinct microorganisms (i.e., yeast and lactic acid bacteria).

Lesaffre describes the development of its own bioengineered strain of *S. cerevisiae*. The host strain, *S. cerevisiae* strain S92, was isolated from the Champagne region in France and is closely related or identical to commercial strains commonly used in winemaking. The microbial source of malate permease (i.e., *Schizosaccharomyces pombe*), is a yeast[(1)] that was first isolated from African beer and has frequently been found in sugar-containing products in tropical and sub-tropical regions and in grape must and cider in moderate climates. The microbial source of malolactic enzyme (i.e., *Oenococcus œni*) is a lactic acid bacterium that has been isolated from wines and related habitats such as wineries and vineyards. It is the preferred, and most commonly used, lactic acid bacterium for malolactic fermentation of wines. The malate permease is a 49 kDa protein with a hydrophobicity profile typical of membrane transport proteins. It contains a peptide sequence (composed of proline, glutamic acid, serine and threonine) that characterizes proteins with a rapid turnover. The malolactic enzyme is a dimer with a total molecular weight of approximately 130 kDa.

Lesaffre describes the construction of an integration cassette that contains genes encoding malate permease from *S. pombe* and the malolactic enzyme from *O. œni*, regulatory sequences associated with the expression of these genes, and sequences used for integration into an appropriate chromosomal site in *S. cerevisiae* strain S92. Lesaffre also describes the transformation strategy that it used to reduce the numbers of potentially transformed yeasts that needed to be screened for the successful integration of the integration cassette. This strategy involved co-transformation of *S. cerevisiae* strain S92 with a plasmid (pUT322) that carries a selectable marker conferring resistance to the antibiotic phleomycin and was based on the hypothesis that cells transformed with plasmid pUT322 are more likely to also have been transformed with the integration cassette. Using this strategy, Lesaffre first screened transformed yeast for resistance to phleomycin and then screened the selected phleomycin-resistant yeast for the ability to produce lactic acid. Lesaffre obtained a phleomycin-sensitive isolate and confirmed that it is free of plasmid pUT332 sequences. Lesaffre designated this strain as ML01.

Based on DNA analysis, Lesaffre concluded that the chromosomal patterns of *S. cerevisiae* strains S92 and ML01 are the same except for the presence of the integration cassette. Lesaffre found that the integration cassette remained stably incorporated after 100 generations. Lesaffre also found that *S. cerevisiae* strain ML01 functions as intended in that it efficiently degrades malic acid. Based on studies that evaluated yeast physiology under different culture conditions, Lesaffre concluded that *S. cerevisiae* strain ML01 has the same growth kinetics, fermentation rate, and ethanol yield as *S. cerevisiae* strain S92 under winemaking conditions and that uptake and utilization of malic acid did not confer a growth advantage to *S. cerevisiae* strain ML01.

Lesaffre describes the method for routine production of *S. cerevisiae* strain ML01 and notes that this method is based on well-established procedures for the production of active dry yeast. The yeast is grown primarily under aerobic conditions to promote yeast

propagation rather than alcohol production. The yeast is harvested via centrifugation and is subsequently dewatered with a rotary vacuum filter, processed through an extruder, and dried, resulting in active dry yeast. The yeast is packaged in vacuum foil pouches prior to shipping.

Lesaffre discusses potential dietary intake of *S. cerevisiae* strain ML01 and of the proteins that Lesaffre has introduced into that strain. Lesaffre considers that exposure to the yeast itself or to the newly introduced proteins would be negligible because the processing procedures used in winemaking remove intact yeast cells, debris associated with autolyzed yeast cells, and proteins released during autolysis of yeast cells.

Based on the information provided by Lesaffre, as well as other information available to FDA, the agency has no questions at this time regarding Lesaffre's conclusion that *Saccharomyces cerevisiae* strain ML01 is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of *S. cerevisiae* strain ML01. As always, it is the continuing responsibility of Lesaffre to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
/s/
Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

[1]Although malolactic fermentation is usually mediated by lactic acid bacteria, Lesaffre chose a yeast (rather than a lactic acid bacterium) as a source of the permease, because the permease must function in the membrane of the yeast *S. cerevisiae*.