Functional Analyses of the Malolactic Wine Yeast ML01

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Abstract: Deacidification of grape musts and wines is important for the production of well-balanced wines. The bacterial malolactic fermentation (MLF) process is unreliable and stuck MLF often leads to spoilage of wines and the production of biogenic amines. The genetically engineered wine yeast, ML01, is a Prise de Mousse strain that contains the malate transport gene (*mae1*) from *Schizosaccharomyces pombe* and the malolactic gene (*mleA*) from *Oenococcus oeni*, stably integrated into the genome at the *URA3* locus. Both genes were isolated from wine-related microorganisms and are expressed under control of the *Saccharomyces cerevisiae PGK1* promoter and terminator sequences. ML01 is capable of decarboxylating up to 9.2 g/L of malate to equimolar amounts of lactate in Chardonnay grape must during the alcoholic fermentation. ML01 contains no antibiotic resistance marker genes or vector DNA sequences. 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 than wines produced with the parental yeast and a bacterial MLF. Analysis of the volatile compounds, sensory analyses, and industrial production of wine indicate that ML01 is suitable for the commercial production of quality wine.

Key words: wine, *Oenococcus oeni*, malolactic fermentation, L-malic acid

The bacterial 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. The MLF entails the biological deacidification of grape musts and is usually conducted by *Oenococcus oeni* and other lactic acid bacteria (LAB), preferably after the alcoholic fermentation by wine yeast strains of *Saccharomyces cerevisiae* (Lonvaud-Funel 1995). L-Malic and L-tartaric acids are the predominant organic acids found in grapes, accounting for >90% of total acidity (Beelman and Gallander 1979). *Oenococcus oeni* and other LAB deacidify wine by converting L-malic acid to L-lactic acid and carbon dioxide, 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 addition to deacidification of wine, MLF is also believed to modify the flavor profile of wine and enhance microbial stability (see Bartowsky and Henschke 2004, Kunkee 1991, Liu 2002).

During MLF, sulfur dioxide, temperature, pH, low nutrient content of wine, ethanol, fatty acids, and other microorganisms (yeast, bacteria, and bacteriophages) can inhibit the conventional bacterial MLF process (see Davis et al. 1985, Henick-Kling 1995, van Vuuren and Dicks 1993, Wibowo et al. 1985). Despite the use of commercially available bacterial malolactic starter cultures, stuck and sluggish MLFs are common, especially in Chardonnay wines. For the entire duration of the bacterial MLF, the wine is at risk from microbial spoilage and oxidation since the addition of SO_2 must be delayed and the temperature is often elevated to achieve a satisfactory MLF. Moreover, LAB, including *O. oeni*, can produce toxic substances such as biogenic amines and precursors of ethyl carbamate that are of serious concern to consumers (Liu 2002, Lonvaud-Funel 2001, Marcobal et al. 2006).

To avoid the negative aspects of bacterial MLF, winemakers can use blending, carbonate additions, precipitation of acids, dilution, and carbonic maceration to reduce the acidity of wine or grape must. Further alternative methods include the use of *Schizosaccharomyces pombe* (Gallander 1977, Silva et al. 2003) and immobilization of LAB or the malolactic enzyme on a variety of matrices (Maicas 2001). Unfortunately these methods often result

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in wine of inferior quality and are not applicable to production of quality wine on a commercial scale.

Saccharomyces cerevisiae does not contain a malate transport gene and cannot effectively metabolize extracellular malate (Grobler et al. 1995, Volschenk et al. 1997a,b). Furthermore, its NAD-dependent malic enzyme has a low substrate affinity (K_m = 50 mM) (Fuck et al. 1973), and this enzyme is also subject to catabolite repression (Redzepovic et al. 2003). We recently constructed an industrial *S. cerevisiae* wine yeast strain, ML01, capable of completing the malolactic fermentation within the first 9 days of the alcoholic fermentation depending on the fermentation temperature (Husnik et al. 2006). The malolactic cassette contains the malate transport (*mae1*) gene from the yeast *S. pombe* (Grobler et al. 1995) and the malolactic enzyme (*mleA*) from *O. oeni* (Husnik et al. 2006); both genes were constitutively expressed under control of the *S. cerevisiae PGK1* promoter and terminator sequences. The well-characterized DNA from these wine-associated organisms was stably integrated into the *URA3* locus of *S. cerevisiae* S92, yielding *S. cerevisiae* ML01 (Husnik et al. 2006). The parental strain, S92 (BioSpringer, Milwaukee, WI) belongs to a family of widely used yeast strains known as Prise de Mousse; S92 was isolated from the Champagne region of France. ML01 retained all of the qualities and characteristics of S92 as well as the ability to complete MLF during the initial stages of alcoholic fermentation. Rigorous scientific examination of the genotype, transcriptome, and proteome revealed that the ML01 yeast is substantially equivalent to the parental strain S92 (Husnik et al. 2006). The FDA has granted the ML01 yeast the same status, Generally Regarded As Safe (GRAS), as other wine yeast strains. The strain has been commercialized in Moldova and the United States by BioSpringer, a division of Lesaffre Yeast Corporation.

Here we report on the capability of ML01 to complete the MLF within the first 5 days of the alcoholic fermentation without negatively affecting ethanol production in high-acid Chardonnay must. Physicochemical, color properties, concentrations of volatile compounds, and sensory characteristics of the wines produced by ML01 are described. Specific characteristics of ML01 are also presented, such as growth kinetics, survival studies, and the residual populations of ML01 in must that are required to conduct a MLF.

Materials and Methods

Strains and media. *Saccharomyces cerevisiae* ML01 (Husnik et al. 2006) and S92 (BioSpringer, Milwaukee, WI) were cultured in YPD broth (Difco, Becton, Dickinson and Co., Sparks, MD) according to standard methods (Ausubel et al. 1995). Active dry yeasts (ADY) were used either directly or after rehydration in sterile distilled water for 15 min at 37°C and were mixed intermittently during rehydration and before inoculation. Synthetic must was prepared by adding 1 mL/L of Tween 80 to the synthetic medium described by Denayrolles et al. (1995); pH was adjusted to 3.5 with KOH. Pasteurized, commercially available Chardonnay grape juice (Vine Fresh; Wine Kitz, Vancouver, Canada) (22.65 Brix, pH 2.9, titratable acidity [TA] 4.39 g/L, 0.92 g/L malate, YAN 348.1 mg/L) was used to study growth kinetics and viability of ML01 postfermentation. Malate concentration was adjusted to 4.5 g/L and the juice 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), 20 g/L peptone (Difco), 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).

Winemaking. Wines were made from Chardonnay and Cabernet Sauvignon grapes. Chardonnay must from fruit harvested in 2000 (22.5 Brix, pH 3.18, TA 13.45 g/L, 9.2 g/L malate, YAN 285.7 mg/L, 60 mg/L total SO_2) was obtained from Quails' Gate Estate Winery, Okanagan Valley, BC. Two carboys (11.7 L capacity) and two flasks (3 L capacity) of Chardonnay 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 alcoholic and MLF fermentations were completed at 20°C by the malolactic yeast ML01, the wines were racked, topped up, sulfited (40 mg/L total SO_2), and aged at 7°C for 9 months. After alcoholic fermentation by the parental strain S92, the two carboys were racked, topped up, sulfited (40 mg/L total SO_2), and aged at 7°C for 9 months. Alcoholic fermentations were considered complete once the wines reached a specific gravity of 0.990 to 0.996. Wines in the remaining two carboys fermented by S92 were racked, topped up, and inoculated with a freeze-dried preparation of *O. oeni* per manufacturer's recommendations (Vinoflora Oenos, Chr. Hansen, Hoersholm, Denmark) and placed at 20°C. The two carboys inoculated with *O. oeni* were reinoculated one week later with *O. oeni* (recommended concentrations) and again two weeks later with a double inoculum of *O. oeni* and placed at 25°C. After 6 months from the initial inoculation with *O. oeni*, the malolactic fermentation (MLF) was stuck at 0.25 g malate/L in one carboy and at 2.98 g/L in the second carboy. Wine was racked, sulfited (40 mg/L total SO_2), and aged at 7°C for 3 months. After aging, all wines were racked and bottled (total SO_2 adjusted to 40 mg/L). Sensory analyses were performed after 4 months and again after 4 years of bottle aging at 14°C. Physical, chemical, and sensory analyses were performed on three-bottle replicates from one carboy. Analyses of wines inoculated with S92 and *O. oeni* were conducted on the carboy with a residual of 0.25 g malate/L.

Since sufficient biological replicates were required to quantify volatile compounds in wine by gas chromatography-mass spectrometry (GC-MS), we repeated the fermentations with ML01, S92, and S92 plus *O. oeni* 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, YAN 401.2 mg/L, 25 mg/L total SO_2) by Calona Vineyards, Okanagan Valley, BC. 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 hr, then 13°C for 1 week, and finally 19°C until completion of the fermentation. After the alcoholic and MLF fermentations were completed by ML01, the wines were racked, topped up, sulfite 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 of the wines were racked, topped up, sulfite levels adjusted to 0.8 mg/L molecular SO_2, and aged at 4°C for 11 months. Remaining wines were racked, topped up, and inoculated with *O. oeni* per manufacturer's recommendations (Vinoflora Oenos) and placed at 20°C. The S92 wine inoculated with *O. oeni* was reinoculated 2 weeks later with a double inoculum of *O. oeni*. 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. oeni* and 50 mg/L of Leucofood (Gusmer Enterprises, Mountainside, NJ) was added. Nine months from the initial inoculation with *O. oeni*, the bacterial MLF was completed. The wine was racked, sulfite adjusted to 0.8 mg/L molecular SO_2, and aged at 4°C for 2 months. After aging, all wines were racked and bottled.

Cabernet Sauvignon juice (22.9 Brix, pH 3.72, TA 7.41 g/L, malate 6.2 g/L, YAN 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. oeni* (Vinoflora Oenos) after the completion of the alcoholic fermentation were reinoculated two weeks later with *O. oeni* (recommended concentrations). After completion of MLF, the wine was racked, sulfited (80 mg/L total SO_2), and stored at 7°C for 8 months. After aging, all wines were racked and molecular SO_2 adjusted to 0.4 mg/L before bottling.

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). Plates were incubated for 2 and 7 days at 25°C to enumerate LAB and yeast, respectively. Plating was done in duplicate. Soluble solids (Brix) in wines were determined by specific gravity and with an ABBE Mark II Refractometer (Reichert Analytical Instruments, Depew, NY). Titratable acidity was determined according to AOAC method number 926.12 and by using a 686 titroprocessor (Metrohm, Herisau, Switzerland) (tartaric acid as reference). The pH was determined using a 455 pH/ion analyzer (Corning, Corning, NY) and a 686 titroprocessor (Metrohm). The viscosity of the wine was determined with a viscometer (model DV-II, Brookfield Engineering Labs, Stoughton, MA) equipped with a LV spindle. The viscometer was set at 60 rpm at 25°C. Color according to CIELAB tristimulus scales was measured in Chardonnay 2000 wines and Cabernet Sauvignon after 4 months of bottle aging at 14°C in the dark. The wines were analyzed with a DU640B scanning spectrophotometer (Beckman Coulter, Fullerton, CA) and $A_{420nm+520nm}$ with a Ultrospec 3000 (GE Healthcare, Chalfont St. Giles, UK). All color analyses were completed without dilutions. Malate, lactate, acetate, and ethanol were determined by enzymatic analyses (Megazyme, Wicklow, Ireland).

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

Volatile compounds were analyzed and quantified using a 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) interfaced to a 5973N mass selective detector (MSD). A 60 m x 0.25 mm i.d., 0.25-µm thickness DBWAX fused silica open tubular column (J&W Scientific, Folsom, CA) 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 MSD was operated in scan mode (35 to 400). The analysis was completed in triplicate, data were analyzed using Enhanced Chemstation software (MSD Chemstation Build 75, Agilent Technologies), and compounds found were matched with the Wiley275 library (Wiley and Sons, Hoboken, NJ).

Sensory analysis. Chardonnay wines produced in 2000 were evaluated in duplicate after 4 months and again after 4 years of bottle aging at 14°C for color (relative degree of intensity from light to dark yellow), aroma, flavor-by-mouth, and overall quality (composite response of visual, aroma, flavor, and aftertaste). Prior to the evaluation, five experienced wine judges selected the following attributes to characterize the wines: fruity aroma, buttery aroma (diacetyl and/or lactic qualities), fruity flavor, buttery flavor (diacetyl and/or lactic qualities), sweetness, acidity, and body (mouthcoat and/or ethanol).

Thirteen experienced judges (six females and seven males) evaluated the wines in duplicate using a completely randomized design. Judges participated in a training session to practice the tasting/rinsing protocol, which required judges to swirl and sniff the glass for the aroma assessment, then sip the wine for the flavor and quality assessments. Judges rinsed with water. All aroma, flavor,

and quality assessments were conducted in individual tasting booths, with 30-mL wine samples presented at room temperature in 250-mL IANO-ISO glasses, labeled with three-digit random numbers, and covered with plastic petri dishes. Color assessment was conducted on 20-mL samples in 25-mL plastic Petri dishes, against a white background and under natural light. Different random codes were used for the color, aroma, flavor, and quality 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 color), respectively.

Effect of residual ML01 populations. The effect of different concentrations of ML01 on the decarboxylation of malic acid to lactic acid was examined in 500-mL Kimax 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, 55, 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 optical density, individual samples were centrifuged (18,000 x *g*, 10 min) and the supernatants were frozen. 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 Excel 2000 (Microsoft Corp., Redmond, WA). Fermentations were conducted in duplicate.

Genetic stability of ML01. The ML01 strain was cultivated in YPD at 30°C for 100 generations. Number of generations was calculated using OD_{600nm} measurements taken at the start and end of each subculture. After 100 generations, ML01 yeast cells were plated on YPD medium and incubated at 30°C for 4 days. One hundred randomly selected ML01 colonies were analyzed by colony PCR for the presence of the malolactic cassette (S92 colonies were used as a negative control). Each colony was picked with a sterile toothpick and deposited into100 U/mL Zymolyase solution (Seikagaku Corp, Tokyo, Japan) for 40 min at 37°C in a total volume of 30 µL. Two microliters of zymolyase-treated cells were used as the template for each PCR reaction. Taq DNA polymerase (Fermentas, Burlington, Canada) and primers 5'-GTTGTAATGTGACCA ATGAG-3' (inside the cassette, *PGK1* promoter) and 5'-CTCTTTATATTTACATGCTAAAAATGG-3' (outside the cassette, 3'-end *URA3* flanking region) were used according to manufacturer recommendations. The 1095 bp PCR product was visualized by 0.8% agarose gel electrophoresis and ethidium bromide staining (Ausubel et al. 1995).

Growth kinetics. A Bioscreen Automated Microbiology Growth Curve Analysis System (Thermo Electron, Waltham, MA) was used to examine the growth kinetics of the ML01 or S92 yeasts in commercially available Chardonnay must. A single colony from culture plates was inoculated into 5 mL of YPD broth that was incubated at 30°C until the culture reached stationary phase. One microliter of the culture was then inoculated into one well of a microtiter plate (Thermo Electron) containing 99 µL of Chardonnay must. The plate was incubated at 30°C for 64 hr with continuous shaking at high intensity. Optical densities for all wells were measured every 10 min using the wide-band measurement filter (OD_{600nm}). A total of nine replicates and one blank control were performed. 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.

Postfermentation viability of ML01. Fermentation flasks (250-mL capacity) containing 200 mL Chardonnay grape juice were inoculated with 100 mg/L of rehydrated ML01 or S92 yeast. Each fermentation flask was fitted with an ethanol-disinfected vapor lock and 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.

Utilization of malate as sole carbon source. Single colonies from culture plates of the parental strain S92 or the ML01 strain were transferred to 5 mL YPD broth and incubated at 30°C until the cultures reached the stationary phase. Four 1-L Erlenmeyer flasks were filled with 150 mL of modified YPD containing 2% L-malic acid and sealed 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 periodically removed and optical density measured spectrophotometrically at 600 nm. The samples were then centrifuged in a microcentrifuge at 18,000 x *g* for 10 min (Eppendorf, Hamburg, Germany), and supernatants were frozen at -30°C for later analysis. L-Malic and L-lactic acid concentrations were determined for samples at 194 and 350 hr by enzymatic analysis (Roche, Basel, Switzerland). The OD_{600nm} readings from the two ML01 flasks and the two S92 flasks were averaged and growth curves were generated using Excel 2000.

Statistical analysis. 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) to track panel consistency, judge reproducibility, and sample-to-sample variation, respectively. Mean scores and least significant differences (Fisher LSD $p < 0.05$) were calculated. Mean scores with significant differences among wines were plotted

using a cobweb diagram. A correlation principal component analysis (PCA) was performed on the mean sensory scores (n = 13) from each of the replications (n = 2), allowing the location of both replicates to be on the diagram. ANOVAs were used to preselect the most relevant attributes before PCA analysis. The term "overall quality" was not included in the PCA in order to delineate clearly the objective flavor profile analysis (attribute intensities) from the more subjective quality assessment.

One-way ANOVAs were used to evaluate the variation in physicochemical 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 were evaluated using two-tailed independent samples *t*-tests ($p < 0.05$). All statistical calculations were performed using Minitab 14 (Minitab Inc., State College, PA), SPSS (SPSS Inc, Chicago, IL), and Excel 2002.

Results

MLF in musts by *S. cerevisiae* ML01. The sulfited and cold-stabilized Chardonnay must (2000) contained 105 cfu/mL of yeast and 40 cfu/mL of LAB prior to inoculation with ML01 or S92 ADY. The ML01 and S92 strain both attained a final specific gravity of 0.996 in high-acid Chardonnay at the end of fermentation. The ML01 strain completed the alcoholic fermentation in 22 days and the S92 strain in 32 days (Figure 1). The ML01 strain consumed 9.04 g/L of malate (n = 2) within the first five days of the alcoholic fermentation (Figure 2A) and produced an approximately equimolar amount of 6 g/L of lactate in the must by day seven (Figure 2B). In contrast, the S92 strain consumed only 0.93 g/L of malate (n = 4) and no lactate was produced in the Chardonnay wine at the end of the alcoholic fermentation. *Oenococcus oeni* required 171 days after alcoholic fermentation to consume 5.29 and 8.02 g/L of malate and produce 3.96 and 5.42 g/L of lactate, respectively, in wine fermented with S92 (Figure 2). No further decarboxylation of malic acid was observed. Analysis of TA, acetate, pH, viscosity, and color properties of the Chardonnay wines produced by ML01, S92, and S92 plus *O. oeni* are shown in Table 1.

The sulfited Cabernet Sauvignon must (without skin contact) contained 7.2 x 10^3 cfu/mL of yeast and no LAB 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 fermentation. ML01 consumed 6.13 g/L of malate (n = 2) within the first four days of the alcoholic fermentation. In contrast, S92 consumed 1.87 g/L of malate (n = 2) by the end of the alcoholic fermentation. *Oenococcus oeni* required 42 days after alcoholic fermentation to consume 4.33 g/L of malate (n = 2) in wine fermented with S92. Analysis of TA, acetate, pH, and color properties of the

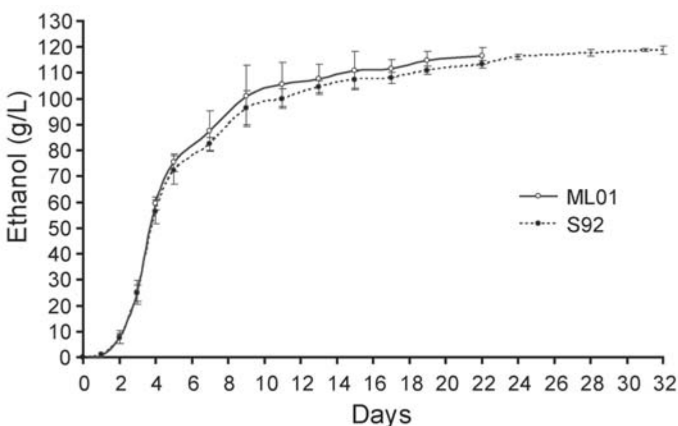

Figure 1 Ethanol production by ML01 and S92 in high-acid Chardonnay grape must fermented at 20°C. ML01 completed the alcoholic fermentation in 22 days and the parental strain in 32 days. Final specific gravity of both wines was 0.996. Duplicate biological replications were analyzed in triplicate.

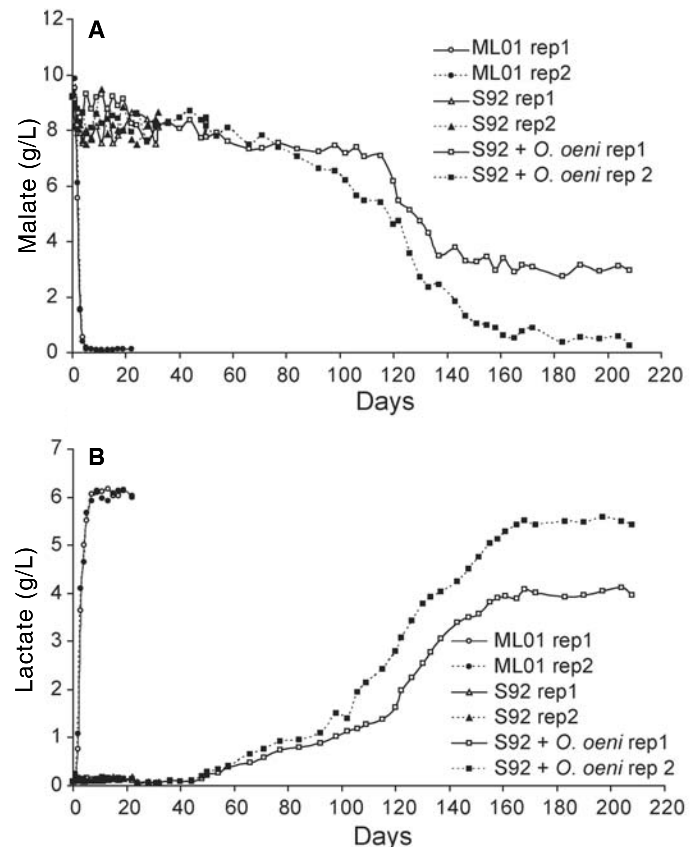

Figure 2 MLF by ML01 was completed in the first 5 days of the alcoholic fermentation in high-acid Chardonnay grape must (9.2 g/L). (**A**) Malate degradation and (**B**) lactate production by ML01 and S92 and S92 + *O. oeni*. The ML01 strain fully and efficiently degraded 9.08 g/L of malate and produced equimolar amounts of lactate (6.07 g/L of lactate). Wine inoculated with *O. oeni* required 171 days post-alcoholic fermentation to complete the MLF in carboy #2 (0.25 g/L residual malate). A stuck MLF was observed for S92 + *O. oeni* in carboy #1; 2.98 g/L of malate remained despite four inoculums of *O. oeni*. The parental strain on its own consumed 10.1% of the malate during the alcoholic fermentation.

Cabernet Sauvignon wines produced by ML01, S92, and S92 plus *O. oeni* are shown in Table 1.

GC-MS analysis of wines. GC-MS headspace analysis (Table 2) revealed that wine produced by ML01 did not contain any compounds that were not detected in wine produced with the parental strain S92. One compound, ethyl 2-methylbutanoate, was detected in wine produced with S92 plus *O. oeni* 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. oeni*.

When the three wines were compared, no significant differences were found for 19 out of 30 compounds (Table 2). 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. oeni* was 295.88 mg/L. Wines produced with S92 plus *O. oeni* also contained significantly higher concentrations of dimethylsulfide, ethyl acetate, ethyl isovalerate, isobutyl alcohol, ethyl octanoate, acetic acid, ethyl decanoate, and diethyl succinate than the other two wines. Acetoin was also significantly higher in wines produced with S92 plus *O. oeni* than in wines produced with S92 without MLF.

Sensory analysis. The ANOVA results of the sensory attribute ratings for wines produced by ML01, S92, and S92 with MLF are summarized in Table 3. Significant differences among wines were observed for seven of the nine sensory attributes. Judge variation was significant for all attributes except acidity, which was expected due to individual physiological and scoring differences. Panel inconsistencies as indicated by significant judge*wine interactions (Table 3) were present for two of the seven significantly different attributes observed for wines (fruity aroma and overall quality). Therefore, F-values were recalculated for these two attributes using a more conservative random effects model ($MS_{treatment}/MS_{j*w}$) (Goniak and

Table 1 Physicochemical and color measurements of Chardonnay and Cabernet Sauvignon wines produced by ML01, S92, and S92 plus *O. oeni.* Mean values for bottle replicates are given for all quantities (n = 3).

	ML01	S92	S92 + *O. oeni*	*p*[a]
Chardonnay				
TA (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	*
Color 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
Cabernet Sauvignon				
TA (g/L)	4.39 a	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	***
Color 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	***

[a]*, **, ***, and ns: significant at $p < 0.05$, 0.01, 0.001, and not significant, respectively.
[b]Means separated at $p < 0.05$ by Duncan's post-hoc test.

Table 2 Concentration of volatile compounds in Chardonnay wines produced with ML01, S92, and S92 plus *O. oeni*. Wines were analyzed by GC-MS headspace assay. Mean values for biological replicates are given for all compounds (n = 3).

Compound	ML01 (mg/L)	S92 (mg/L)	*O. oeni* S92 + (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

[a]*, **, ***, and ns: significant at $p < 0.05$, 0.01, 0.001, and not significant, respectively.
[b]Means separated at $p < 0.05$ by Duncan's post-hoc test.

Noble 1987). While the new F-value for fruity aroma (2.29) was nonsignificant, the new F-value for overall quality (7.99) was statistically significant, indicating that panel inconsistencies were relatively minor compared with the magnitude of the overall quality effects.

The significantly different mean sensory attributes were plotted on a cobweb diagram (Figure 3) and a PCA plot (Figure 4). Wine produced by ML01 was significantly highest in overall quality, body, and perception of sweetness and lowest in acidity when compared with wines produced by S92 with and without MLF. Wine produced by ML01 was also significantly highest for fruity taste when compared with wines produced by S92 with a bacterial MLF. The main characteristics of wine produced by S92 without MLF were darker color and high acidity. Wines produced by S92 with a bacterial MLF were judged more acidic, less sweet, have less body and less fruity taste, and lower in quality than wines produced with ML01.

Table 3 F-values from analysis of variance of Chardonnay wines for sensory attributes (three wines, 14 judges, two replications).

Descriptor	Wine	Judge	Rep	Judge x Wine	Judge x Rep	Wine x Rep
Yellow color	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
Sweetness	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]*, **, and ***: significant at $p < 0.05$, 0.01, and 0.001, respectively.

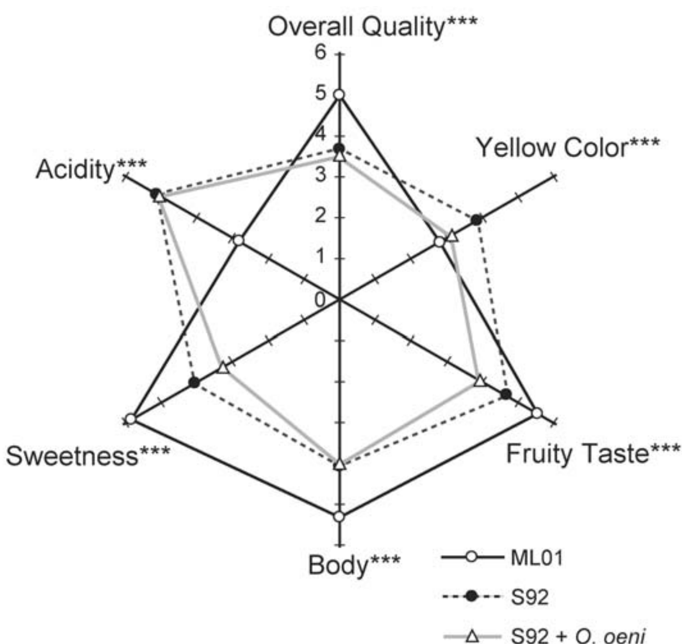

Figure 3 Significantly different mean sensory attributes of Chardonnay wines produced by ML01, S92, and S92 plus *O. oeni* (n = 26) (*** indicates significance at $p < 0.001$).

The first two factors of the PCA mean sensory scores accounted for 97.7% of the variance among the wines (Figure 4). The attributes fruity taste, body, sweetness, and acidity were most heavily loaded on PC1, accounting for 82.4% of the variability. Color 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° angles between the vectors. Body was positively correlated with sweetness and fruity taste, as indicated by the narrow angles. In contrast, yellow color and fruity taste were uncorrelated, as shown by their 90° orientation. The wines from ML01, located on the right, were characterized by full body, sweet taste, and fruity flavor. In contrast, S92 and S92 plus *O. oeni* 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 a darker yellow color. These traits are consistent with characteristics identified from the cobweb diagram (Figure 3).

Effect of increasing populations of ML01. In order 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% or less of the total yeast cell population at the beginning of the alcoholic fermentation (Figure 5A, B). Fermentations containing a 10% ML01 inoculum resulted in a partial (33.3%) MLF (Figure 5C, D). If 50% of the yeast population comprised ML01, then an almost complete (95.3%) MLF was observed (data not shown).

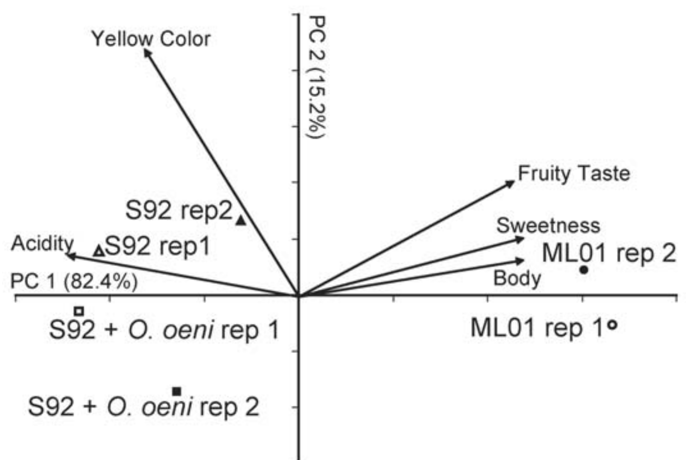

Figure 4 Principal component analysis of significantly different mean sensory data for Chardonnay wines produced by ML01, S92, and S92 plus *O. oeni*. PCA factors 1 and 2 explain 82.4% and 15.2% of the variability, respectively (n = 13).

Genetic stability and phenotypic characteristics of ML01. All 100 randomly chosen colonies tested positive for the malolactic cassette after 100 generations on a nonselective growth medium. Integration of the malolactic cassette in the *URA3* locus of S92 strain is therefore stable for ADY production and winemaking processes. In Chardonnay must, no statistical difference was observed between maximum specific growth rate (μ_{max}) for ML01 (0.37 ± 0.03 h^{-1}) and the parental strain S92 (0.37 ± 0.02 h^{-1}); corresponding generation times were 1.88 ± 0.13 hr and 1.86 ± 0.08 hr for ML01 and S92, respectively. Growth during the commercial production of ADY ML01 was also not affected by introduction of the malolactic cassette into the *URA3* locus of S92 (Lesaffre Development, personal communication). Viability of yeast cells postfermentation declined at similar rates for ML01 and S92 (Figure 6).

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 7), and no malate was consumed by ML01 or S92. Malate concentration after 350 hr in media inoculated with ML01 and with S92 was 20.4 ± 0.99 g/L and 20.2 ± 1.03 g/L, respectively.

Discussion

Malolactic fermentation has long been a difficult step in the winemaking process, especially in high-acid Chardonnay musts. We have recently reported the construction of the genetically stable malolactic wine yeast ML01 (Husnik et al. 2006), which is capable of efficiently performing MLF during the alcoholic fermentation in a variety of grape musts, including high-acid Chardonnay must. The ML01 strain and the parental strain, S92 (Bio-Springer), have the same maximum specific growth rate in Chardonnay must, die at a similar rate (Figure 6), and both strains are unable to consume malate as a sole carbon source (Figure 7).

The parental strain S92 required 10 more days to complete the alcoholic fermentation than the ML01 strain. 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

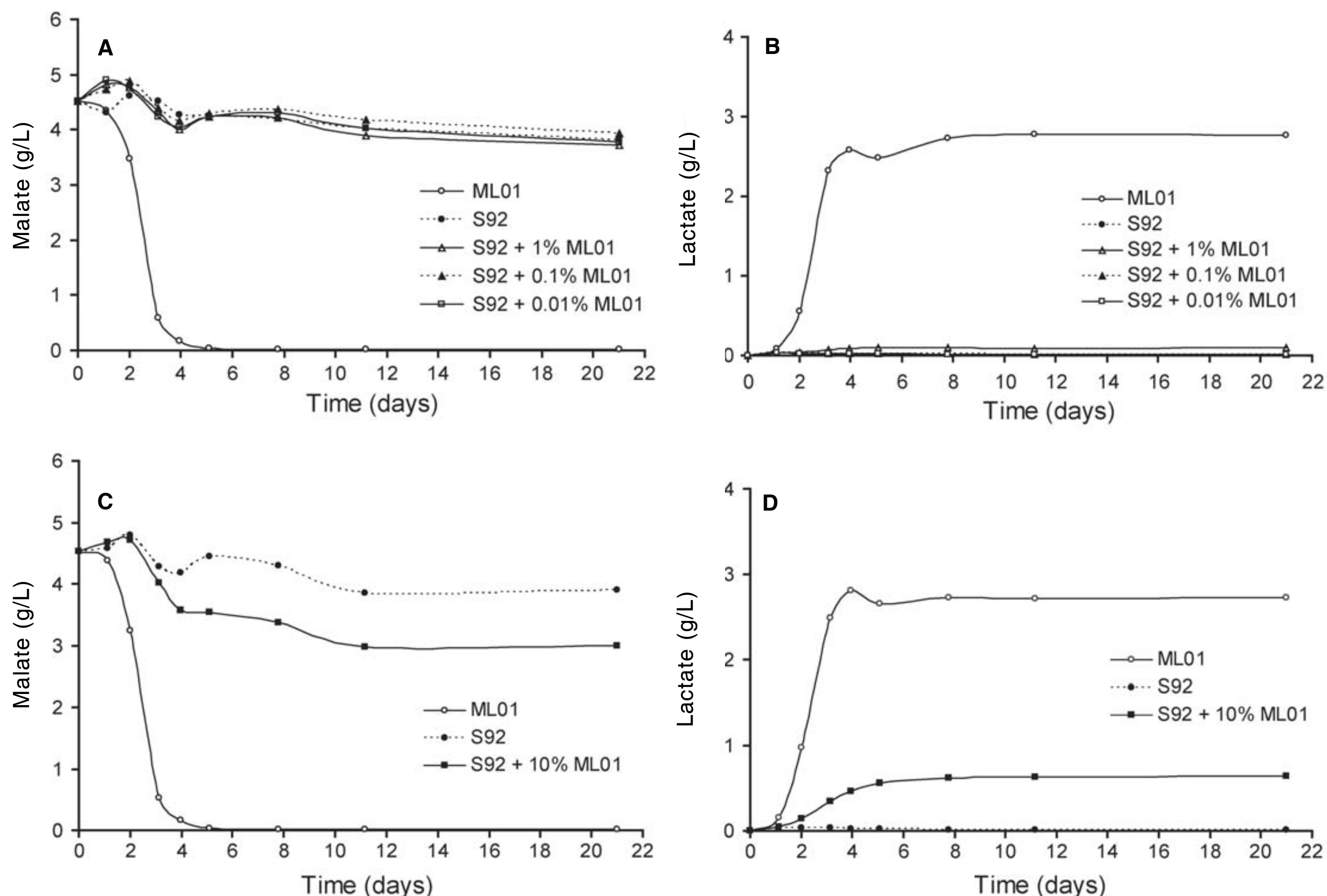

Figure 5 MLF was not detected in wines containing an inoculum less than 1% of ML01 yeast. (**A**) Malate degradation and (**B**) lactate production by ML01 and S92 and by S92 + 1%, 0.1%, and 0.01% ML01 co-cultures in synthetic must containing 4.5 g/L of malate. (**C**) Malate degradation and (**D**) 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).

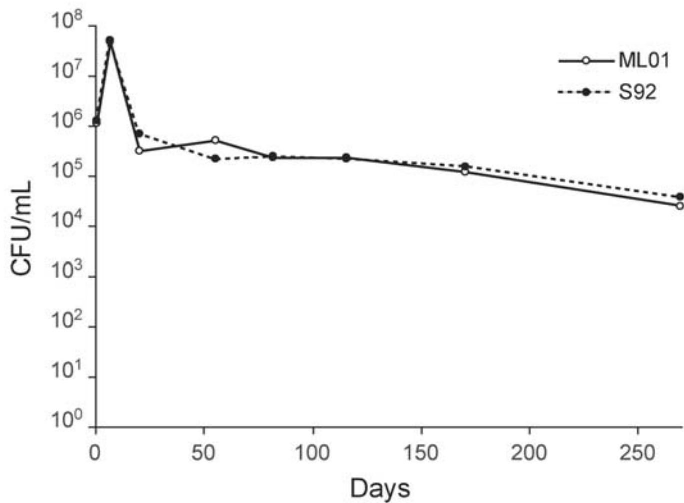

Figure 6 Postfermentation viability of ML01 and S92 in Chardonnay wine. Both strains were inoculated (100 mg/L) in duplicate into filter-sterilized Chardonnay must and viability of cells was determined by plate counts on YPD for 269 days (n = 2).

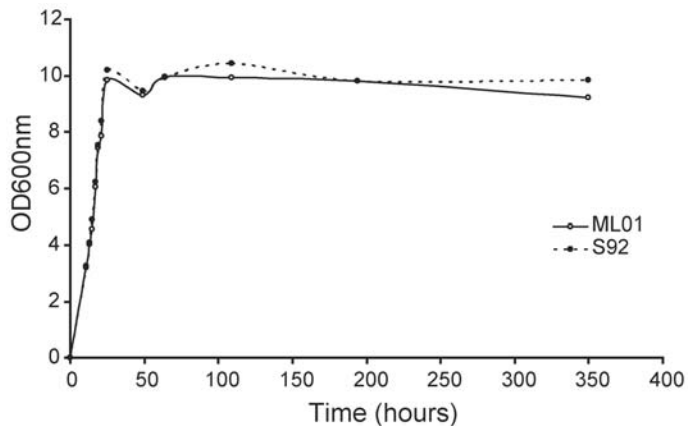

Figure 7 ML01 and S92 cannot consume L-malate as a sole carbon source. Strains were inoculated (0.01 OD_{600nm} final concentration) into modified YPD medium containing 20 g/L malate and 5 g/L glucose and grown aerobically for 350 hr. Subsequent analysis showed no reduction in malate levels in the medium; 20.4 ± 0.99 g/L and 20.2 ± 1.03 g/L of malate remained in media inoculated by ML01 and S92, respectively (n = 2).

more easily because of the slight pH increase due to malate degradation over the first 5 days. In higher pH wines such as Cabernet Sauvignon, the alcoholic fermentations by the two yeast strains were completed at the same time (data not shown). The data indicate that the presence of the malolactic cassette in ML01 does not affect ethanol production when compared to the parental strain S92.

The MLF conducted by ML01 in Chardonnay must proceeded rapidly, consuming 98.3% (n = 2) of the malate in the first five days of the alcoholic fermentation; an equimolar amount of lactate was produced by day seven (Figure 2). The slight delay in detection of equimolar amounts of lactate is probably due to the slow diffusion of lactic acid out of the cell, compared with malate that is actively transported into the yeast. The parental strain S92 consumed only 10.1% of the malate (n = 4) in the media and no lactate was produced. The two carboys inoculated with *O. oeni* 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 2). Wines had to be kept at a relatively high temperature that is conducive for growth of *O. oeni* 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 would allow for early stabilization of wine in the cellar.

In Chardonnay wines, titratable acidity was considerably reduced in wines that had undergone a MLF, also reflected in the increase in pH (Table 1). The wine with the lowest pH was produced by S92 without a MLF. The consistently higher pH of wines fermented with S92 and *O. oeni* can probably be ascribed to the fact that S92 consumes 10.1% of the malate prior to inoculation with malolactic bacteria. *Oenococcus oeni* therefore produced less lactate (5.42 g/L) than ML01 (6.07 g/L) from malate (Figure 2B), which influenced the pH. Chardonnay wine produced with ML01 had significantly lower levels of acetate (0.452 g/L), the main component of volatile acids in wine, 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). Acetate concentration in Cabernet Sauvignon wines was 0.324 g/L for ML01, 0.355 g/L for S92 with a bacterial MLF, and 0.237 g/L for S92 alone. Titratable acidity and pH of Cabernet Sauvignon wines produced similar results to Chardonnay wines (Table 1).

Although the ML01 yeast efficiently decarboxylated malate to lactate in a variety of musts, it could not decarboxylate malic acid to lactic acid when present at levels below 1% of the total inoculum (Figure 5A, B). Even at inoculum levels of 10% ML01, the decarboxylation of malate to lactate was limited (33.3 %) and did not continue after the first few days (Figure 5C, D). Hence, cross-contamination of ML01 yeast in must not destined for deacidification in wineries does not appear to be a concern under commercial conditions.

CIELAB color measurements indicated that the Chardonnay wines produced by ML01 and S92 with a bacterial MLF had a similar degree of lightness; wine fermented with S92 without a MLF had the lowest degree of lightness. These data also correlated well with visual scores determined by the panelists, who chose S92 without a MLF to be darker in color during sensory evaluations (Figures 3 and 4). Chardonnay wine produced with ML01 also had the lowest amount of greenness and yellowness and S92 with a MLF had the highest amount of greenness and yellowness (Table 1). Importantly, Cabernet Sauvignon wines produced by ML01 and S92 without a MLF had a darker color than wine produced with S92 with a bacterial MLF. Wines produced with S92 had the highest

value for redness and the lowest value for yellowness. Wine produced with S92 and a bacterial MLF was significantly lighter with the least redness. The wine with the lowest pH was produced by S92 without a MLF (pH 3.80) and the pH of the wines produced by ML01 and S92 with a MLF were 3.98 and 4.05, respectively. These data indicate that the bacterial MLF had a negative effect on red wine color (Table 1); loss of color was previously attributed to the increase in pH. However, it is now clear that the metabolic activity of *O. oeni* impacts negatively on anthocyanins in red wine.

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). Wine produced with ML01 did not contain any compound that was not present in wine produced with the parental strain without a MLF. However, concentrations of certain compounds such as ethyl lactate, an aroma compound (buttery) that also gives wine a broader, fuller taste (Henick-Kling 2002), do vary greatly. Ethyl lactate concentrations were high in wines produced by ML01 and S92 with a bacterial MLF but were low in wine produced by the parental strain without a MLF (Table 2).

Sensory analysis of Chardonnay wines aged for four years showed that wine produced by ML01 was judged highest for overall quality (Figure 3). Overall quality was also strongly correlated with body (data not shown). Chardonnay wine produced by S92 without a MLF was judged to be significantly darker than wines produced by ML01 or S92 with MLF, correlating well to the degree of lightness measured by CIELAB (Table 1). 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 quality, fruity taste, sweetness (perceived because of a lack of acidity when compared to other wines) and body, whereas dark color and high acidity are attributes of wine produced with S92 without a MLF (Figures 3 and 4). Mean sensory attributes of these Chardonnay wines tasted after 4 months of aging were similar to those obtained after 4 years of aging (data not shown).

The presence of biogenic amines in wines has been well documented and is of great concern to some consumers (Husnik et al. 2006). More than 20 amines, notably histamine, cadaverine, phenylethylamine, putrescine, and tyramine, have been found in wine (Lehtonen 1996). During MLF these toxic compounds are produced by LAB from corresponding amino acids in wine (Lonvaud-Funel 2001, Marcobal et al. 2006). We did not analyze wines produced in this study for biogenic amines since fermentations were conducted under clean conditions in the laboratory. In contrast to the bacterial MLF, the use of ML01 in commercial wineries allows for early sulfiting of must and wine that will limit or prevent the growth of LAB that produce biogenic amines. It is therefore conceivable that the use of ML01 could limit or prevent the production of biogenic amines in commercial wines.

Conclusions

Results show that the 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 9 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. oeni* indicated that ML01 appears to be suitable for the production of wine on a commercial scale, as ML01-produced wines that were judged by trained panelists to be superior in overall quality relative to wines made using the parental yeasts. The bacterial MLF is unpredictable and often results in stuck MLF and the production of off-flavors and biogenic amines. Early sulfiting of wine produced with ML01 will prevent the growth of undesirable LAB that produce biogenic amines. It is therefore conceivable that the use of ML01 could limit or prevent the production of biogenic amines by LAB in commercial wines.

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