

Reduction of Malic Acid in Wine Using Natural and Genetically Enhanced Microorganisms

Gary L. Main,^{1*} Renee T. Threlfall,¹ and Justin R. Morris²

Abstract: Naturally selected yeast ICV-GRE and 71B, malolactic bacteria Lalvin 31, and genetically enhanced yeast ML01 were compared for biodeacidification of malic acid in production of Vignoles wines. ICV-GRE yeast consumed 18% of malic acid with no lactic acid production. Lalvin 31 added to the wine fermented with ICV-GRE converted the remainder of the malic acid to lactic acid and consumed some citric acid. The ICV-GRE + Lalvin 31 treatment produced less lactic acid compared with the ML01 treatment due to malic acid consumption by the ICV-GRE yeast and had the lowest titratable acidity. ML01 was effective at converting 5.7 g/L (100%) malic to lactic acid during the first 60 hr of fermentation. The 71B yeast consumed 1.9 g/L (33%) of the malic acid with no lactic acid production. Wine produced with ML01 had higher levels of total sulfur dioxide (SO₂) than the other treatments. A secondary experiment found that the ML01 yeast produced 34.6 mg/L SO₂, which was three times as much as ICV-GRE and six times as much as 71B. The amount of lactic acid and SO₂ produced by ML01 yeast could be of concern to enologists depending on style of wine desired.

Key words: malic, Vignoles, genetically modified, ML01

The white grape Vignoles (interspecific hybrid Seibel 6905 X Pinot Noir) can have high L-malic acid levels (4 to 8 g/L) that result in tart wines. Biological deacidification by bacteria is the most common approach to acid reduction. Winemakers often use malolactic fermentation (MLF) to reduce wine acidity and enhance microbial stability. *Oenococcus oeni* is the species of lactic acid bacterium commonly used in wine for MLF. This bacterium uses the malolactic enzyme *mleA* to decarboxylate L-malate to L-lactate and carbon dioxide, which results in an increase in wine pH and a reduction in titratable acidity (Bartowsky 2005).

The standard wine yeast *Saccharomyces cerevisiae* is capable of consuming some malic acid, but the amounts are considered negligible or weak as compared with *O. oeni* (Husnik et al. 2006, Redzepovic et al. 2003, Volschenk et al. 2003). This yeast species does not have an active malate transport system. Malic acid passes through the yeast cellular membrane by simple diffusion (Delcourt et al. 1995, Pretorius 2000, Volschenk et al. 2003) where it is metabolized into succinate by the citric acid cycle, or more predominately ethanol, through a malo-ethanolic pathway (Pretorius 2000, Ramon-Portugal et al. 1999, Redzepovic et al. 2003, Volschenk et al. 2003). Some glucose must be present for malic consumption to occur (Delcourt et al. 1995), and an increase in malic enzyme transcription

occurs near the end of fermentation (Redzepovic et al. 2003). Higher concentrations of malic acid favor consumption (Delcourt et al. 1995, Ramon-Portugal et al. 1999), and there is an increase in malic enzyme transcription at higher malic acid concentrations (Redzepovic et al. 2003). Low pH also favors malic acid consumption, as only the undissociated form of the acid is able to pass through the cellular membrane (Ramon-Portugal et al. 1999). The undissociated form of malic acid in water ranges from 64% at pH 3.2 to 41% at pH 3.6. Malic acid consumption by yeast in the changing medium during fermentation is difficult to predict but should increase at lower pH values and with higher malic acid concentrations.

The ability of commercial strains of *S. cerevisiae* to consume malic acid varies but can range from zero to 40%, with most strains at the lower end of the range. In Chardonnay, *S. cerevisiae* strain 71B consumed about 2 g (30%) of malic acid as compared with 0.7 g (10%) for *S. bayanus* strain EC1118 (Pilone and Ryan 1996). Other studies have shown variable amounts consumed by strain 71B: 18% (Redzepovic et al. 2003) and 30 to 33% (Henick-Kling and Park 1994). The manufacturer of 71B (Lallemend, Montreal, Canada) reports that the yeast consumes up to 40% of the malic acid present.

Concurrent alcoholic and malolactic fermentations are possible through genetically enhanced yeast. The yeast strain ML01 is a genetically stable industrial strain of *S. cerevisiae* constructed by integrating a malate permease gene (*mae1*) from *Schizosaccharomyces pombe* and a malolactic gene (*mleA*) from *O. oeni* (Husnik et al. 2006, 2007). In effect, the yeast has been engineered to have an active transport system to enable malic acid to enter the yeast cell and an efficient enzyme system within the cell to convert malic acid to lactic acid. The use of this yeast

¹Postdoctoral associate, ²Distinguished professor, University of Arkansas, Institute of Food Science and Engineering, 2650 North Young Avenue, Fayetteville, AR 72704.

*Corresponding author (email: gary.main@uark.edu; fax: 479 575-2165)

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eliminates the need for additional MLF using malolactic bacteria and speeds the winemaking process by eliminating the time required for bacterial conversion of malate to lactate. ML01 does not produce the aroma and flavor compounds produced by malolactic bacteria. ML01 has received Generally Recognized as Safe status by the U.S. Food and Drug Administration (GRAS notice no. 000120). However, the use of genetically modified yeasts has received opposition in the European Union, Australia, and South Africa. BioSpringer, a division of Lesaffre Yeast Corporation, has commercialized ML01 in the United States and the Republic of Moldova (Husnik et al. 2007).

In this experiment, naturally selected yeast and malolactic bacteria were compared to the genetically enhanced yeast ML01 for biodeacidification of malic acid in production of Vignoles wines. The use of ML01 as compared to commercial wine yeast has not been documented.

Materials and Methods

Experimental design and analysis. Four fermentation treatments using the yeasts 71B, ICV-GRE (control), ICV-GRE + Lalvin 31 (ML bacteria), and ML01 were established in a completely randomized design with three replications. Data were analyzed using analysis of variance with JMP statistical software (version 6.0.2; SAS Institute, Cary, NC). Separation of mean values was determined using Standard Least Squares to fit the model with ismeans separation using Tukey's HSD test at the $p \leq 0.05$ level of significance.

Juice processing. Vignoles grapes were hand-harvested at the University of Arkansas Agricultural Research and Extension Center, Fayetteville. Juice was produced using conventional methods with 30 mg/kg sulfur dioxide (SO_2) from potassium metabisulfite and 33 mL/1000 kg of the pectinase Scottzyme Cinn-Free (Scott Laboratories, Petaluma, CA) added during crush. Must was held with skin contact in closed containers for 48 hr at 2°C before pressing. The must was pressed, and juice was cold-settled overnight at 2°C to reduce suspended solids. The juice was racked, warmed to 18°C, mixed, and ~18.2 L of juice was pumped into 18.9-L glass carboys. Air-locks containing water were placed on each carboy after yeast addition.

Microorganisms and rehydration process. Yeasts 71B and ICV-GRE, Lalvin 31 (a MBR strain of *O. oeni* for direct inoculation), and yeast nutrients Go-Ferm and Fermaid K were supplied by Lallemant (Montreal, Canada). The genetically modified yeast ML01 was produced by Springer Oenologie (BioSpringer, Maisons-Alfort, France) and purchased from American Tartaric Products (Larchmont, NY). Yeast inoculums followed manufacturer's recommendations: 0.26 g/L for 71B and ICV-GRE and 0.075 g/L for ML01. All yeasts were rehydrated in 50 mL of water at 40°C containing 6 g of Go-Ferm. Fermentation temperature was 18°C. The yeast nutrient Fermaid K at a rate of 0.26 g/L was added to all treatments 36 hr after yeast inocula-

tion. Lalvin 31 was added to the ICV-GRE + Lalvin 31 treatment 14 days after yeast addition at a rate of 0.01 g/L.

Equipment and analysis. Fermenting musts were mixed every 12 hr until completion of alcoholic fermentation by rapidly swirling the top of the carboy 20 times. This caused visible circulation and lifting sediment from the bottom of the carboy. After mixing, a 16-mL sample was taken using a disposable pipette. The samples were placed in centrifuge tubes and sonicated for 10 min (Branson 2510 Ultrasonic Cleaner, Danbury, CT) to degas. The samples were then centrifuged for 10 min at 4,192 *g* using an Eppendorf 5804 centrifuge with swing bucket rotor, type A-4-44 (Brinkmann Instruments, Westbury, NY). Every 12 hr samples were prepared using a nylon mini-UniPrep 0.45- μm syringeless filter (Whatman, Florham Park, NJ) and injected into the HPLC. Glucose, fructose, citric, tartaric, malic, succinic and lactic acids, glycerol, and ethanol were measured using HPLC procedures described elsewhere (Walker et al. 2003).

Titratable acidity and pH were measured at 24-hr intervals. The pH was measured with a Beckman pH meter (model 250; Beckman Coulter, Fullerton, CA) with a probe using a three-point calibration (1.68, 4.0, and 7.0). Titratable acidity (tartaric acid in g/L) was measured by placing 5 mL of juice or degassed wine into 125 mL of degassed deionized water and titrating with 0.1 N NaOH to an end point of pH 8.2. In an effort to keep the wine pH below 3.7 during fermentation for quality and stability control, tartaric acid was used to adjust pH to 3.5 on day three for the 71B and ML01 treatments, and all wines were adjusted on day seven. The ICV-GRE + Lalvin 31 treatment was further adjusted to pH 3.5 after completion of malolactic fermentation. Adjustment to pH 3.5 was based on previous experience with Vignoles grown in our vineyards and helps to limit growth of bacterial spoilage organisms (Bauer and Dicks 2004).

At the end of fermentation, wines were racked and 75 mg/L SO_2 was added. After cold stabilization for three months at 2°C, wines were filtered through a 1- μm pad filter and free SO_2 was adjusted to 60 mg/L before bottling in 750-mL bottles. Free SO_2 was measured in juice and wine using "sulfite in wine Titrets" (CHEMetrics, Calverton, VA), and total SO_2 in the finished wines was measured using the aeration-oxidation procedure (Zoecklein et al. 1995).

Determination of SO_2 production by yeast. At the end of the first experiment there were differences in total SO_2 even though all Vignoles wines had been produced with similar amounts of added SO_2 . An additional experiment was established to determine if SO_2 production varied among the yeasts ICV-GRE, 71B, and ML01. Vignoles juice was no longer available, so juice was produced from hand-destemmed Thompson Seedless grapes using a Squeeze strainer (LEMRA Products, Danielson, CT). The juice was cold-settled overnight at 2°C to reduce insoluble solids. Juice pH was adjusted to 3.5 with KOH to be similar in pH to the Vignoles juice. Juice chemistry prior to fermentation

was soluble solids 20.9, pH 3.53, titratable acidity 7.3 g/L (as tartaric), malic acid 3.8 g/L, and with total SO₂ below detection limits. Fermaid K (0.26 g/L) was added to the juice. The juice was heated to 82°C in a 3.8-L glass container to pasteurize, then sealed and cooled overnight. Juice was poured into sterile 500-mL Erlenmeyer flasks with airlocks to ferment. Rehydration and yeast inoculum were as previously described with three replications of each yeast and duplicate SO₂ analysis. The malolactic bacteria Lalvin 31 was not included in the experiment as it did not increase SO₂ in Vignoles. Other fermentation conditions were the same as the Vignoles experiment.

Results and Discussion

The Vignoles juice was 21.2% soluble solids, 3.53 pH, titratable acidity 7.8 g/L as tartaric acid, 5.7 g/L L-malic acid, and 11 mg/L free SO₂ prior to inoculation. The ML01 yeast completed fermentation about 12 hr after the other yeasts (Figure 1A), perhaps because of the reduced inoculation rate recommended by the manufacturer as compared with the other yeasts used. A signed memorandum of understanding was required for purchase of ML01. That memorandum implied that acid conversion occurred during the initial biomass buildup and that a low inoculation rate (5–10 g/hL) was required for acid conversion to complete. Glucose was consumed at a faster rate than fructose in all yeasts (data not shown). All yeasts reduced fermentable sugars (glucose and fructose) to 2 g/L or less after 108 hr of fermentation. Although all wines were considered dry (residual sugar <2 g/L), wine produced using ML01 had more residual sugars than the other treatments (Table 1).

Malic acid was completely converted to lactic acid by ML01 in 60 hr (Figure 1B, C). Malolactic fermentation was considered complete when less than 0.1 g/L malic acid remained in the wine (Henick-Kling and Park 1994). The malolactic fermentation by Lalvin 31 was complete within 21 days of inoculation.

During fermentation, the pH rose on the ICV-GRE, 71B, and ML01 treatments to 3.57, 3.64, and 3.72, respectively, after 72 hr. A pH increase during yeast fermentation occurs due to either the loss of malic acid in a malo-ethanolic pathway (ICV-GRE and 71B) or the decarboxylation of malic acid to the weaker lactic acid (ML01). A pH change may also be associated with precipitation of tartaric acid as potassium bitartrate (Zoecklein et al. 1995). Tartaric acid was added to reduce the pH to 3.5 on day three and again on day seven when pH values were 3.73, 3.78, and 3.80 for ICV-GRE, 71B, and ML01, respectively. Wine pH was further adjusted to 3.5 on all treatments, if necessary, before cold stabilization. This pH adjustment was made to increase microbial stability (Bauer and Dicks 2004) and to produce wines with lower pH after cold stabilization (Zoecklein et al. 1995). After cold stabilization and bottling, the pH averaged 3.38 ± 0.02 on all treatments. Titratable acidity in the finished wine differed with each treatment (Table 1). The ICV-GRE + Lalvin 31 had the greatest de-

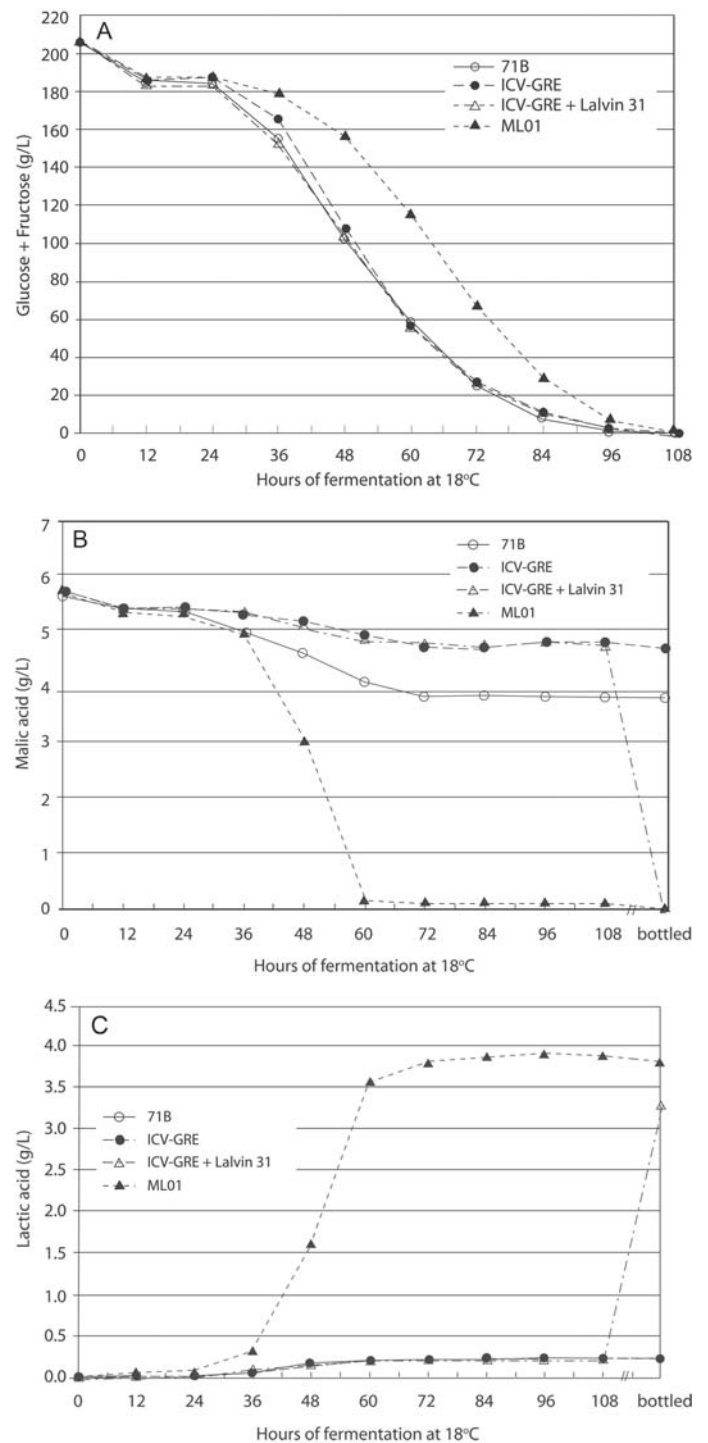


Figure 1 Sugar utilization (A), malic acid reduction (B), and lactic acid formation (C) during fermentation of Vignoles using natural (71B and ICV-GRE) and genetically enhanced (ML01) yeast and natural Lalvin 31 malolactic bacteria (ICV-GRE + Lalvin 31).

acidification effect in terms of titratable acidity reduction, followed by 71B and ML01. Although differing amounts of tartaric acid were added to each treatment to achieve similar pH, the final tartaric acid content varied only 0.28 g/L among treatments in the finished wine.

The yeasts 71B and ICV-GRE consumed 1.89 g/L (33%) and 1.03 g/L (18%) of the malic acid during fermentation,

Table 1 Effect of natural yeast (71B and ICV-GRE), genetically enhanced yeast (ML01), and natural malolactic bacteria (Lalvin 31) on Vignoles wine.

| Treatment | Glucose + fructose (g/L) | Titrateable acidity ^a (g/L) | Tartaric acid (g/L) | Citric acid (g/L) | L-malic acid (g/L) | L-lactic acid (g/L) | Succinic acid (g/L) | Glycerol (g/L) | Ethanol (% v/v) | Total SO ₂ (mg/L) |
|----------------------------|--------------------------|--|---------------------|-------------------|--------------------|---------------------|---------------------|----------------|-----------------|------------------------------|
| 71B | 0.88 b ^b | 8.1 b | 1.53 b | 0.38 c | 3.81 b | 0.03 c | 0.50 a | 6.06 a | 12.3 a | 82 b |
| ICV-GRE | 0.65 c | 8.6 a | 1.56 ab | 0.41 b | 4.67 a | 0.03 c | 0.43 b | 5.37 d | 12.2 b | 92 ab |
| ICV-GRE + L31 ^c | 0.57 c | 6.7 d | 1.74 ab | 0.18 d | 0.06 c | 3.11 b | 0.44 b | 5.44 c | 12.3 a | 73 b |
| ML01 | 1.48 a | 7.1 c | 1.81 a | 0.44 a | 0.05 c | 3.64 a | 0.35 c | 5.95 b | 12.0 c | 121 a |

^aAs tartaric acid.

^bMeans within column with the same letter(s) are not significantly different at the $p \leq 0.05$ level of significance.

^cICV-GRE + Lalvin 31.

whereas ML01 converted all the malic acid to lactic acid. The amount of malic acid consumed by ICV-GRE was more than expected since the manufacturer had not reported it as a consumer of malic acid. The Institut Coopératif du Vin experimental winery (Montpellier, Languedoc, France), has measured 0.8 g/L (25%) malic acid consumption by ICV-GRE in several wine cultivars (G. Specht, Lallemant, personal communication, 2006). The bacteria Lalvin 31 converted the remainder of the malate to lactate in the ICV-GRE + Lalvin 31 treatment. The ICV-GRE + Lalvin 31 treatment also consumed citric acid. Lalvin 31 is a known consumer of citric acid, as are most *O. oeni* bacteria (Rosi et al. 2003).

The ICV-GRE yeast consumed some of the malate, presumably via a malo-ethanolic pathway (Volschenk et al. 2003), since no L-lactic acid was produced at the end of alcoholic fermentation. The ICV-GRE + Lalvin 31 treatment produced less lactate (3.11 g/L) than ML01 (3.64 g/L). Husnik et al. (2007) reported less lactate in wine made from the nongenetically enhanced parent yeast strain of ML01, S92 + *O. oeni*, than in ML01 due to consumption of 10% of the malic acid by S92. Inoculation with malolactic bacteria can be during or after fermentation, but the success of MLF depends on a host of factors including specific yeast strain/bacteria strain interactions (Alexandre et al. 2004). Addition of the bacteria at the end of alcoholic fermentation should decrease the amount of lactate produced by the bacteria as compared to inoculation at the beginning of fermentation because of malate consumption by the yeast.

During fermentation, yeast cells adjust physiologically to the changing juice/wine medium. Succinic acid and glycerol are produced by yeast; the amount produced can affect acidity and mouthfeel and depends on yeast species and strain, fermentation temperature, and nutrients, among other factors (Rainieri et al. 1998). The yeast 71B produced the most succinic acid, and ML01 produced the least, but the amount produced was within a range of 0.35 to 0.50 g/L. The yeasts produced differing amounts of glycerol, with 71B producing the most and ICV-GRE producing the least within a range of 5.37 to 6.06 g/L. The narrow range for succinic acid and glycerol produced by the yeasts should not have imparted a quality difference among the wines. Varying fermentation parameters to in-

crease stress on the yeast may change succinate and glycerol production. Ethanol was slightly lower in ML01 than the other treatments, but all treatments ranged from 12 to 12.3% by volume.

The total amount of SO₂ added from crush to bottling varied with treatment by about 20 mg/L because wines were adjusted to the same free SO₂ level at bottling. Added SO₂ averaged among the three yeast fermentation replicates was 131 g/L ± 4 (71B), 132 g/L ± 7 (ICV-GRE), 125 g/L ± 7 (ICV-GRE + Lalvin 31), and 137 g/L ± 7 (ML01). These values did not correspond with the total SO₂ in the finished wine (Table 1). Some of the reduced amount of total SO₂ in the wine could have been due to SO₂ bound to solids that were removed during racking and filtering. Since all fermentations were treated similarly, the loss in SO₂ among treatments should have been similar. However, the ML01 treatment had 30 to 50 mg/L more SO₂ than the other treatments. This difference could not be explained unless the yeasts were producing different amounts of SO₂.

To account for the variation in the total SO₂ levels in the wines, a second experiment was initiated using Thompson Seedless grape juice to determine if SO₂ production by the yeast could explain the observed SO₂ differences. The SO₂ produced by the yeasts were 4.6 mg/L ± 0.75 (71B), 10.1 mg/L ± 0.55 (ICV-GRE), and 34.6 mg/L ± 1.03 (ML01). The ML01 yeast produced three times as much SO₂ as ICV-GRE and six times as much as 71B. The yeast strain 71B has been previously reported to produce low amounts of SO₂ during fermentation (Henick-Kling and Park 1994). Prise de Mousse Lalvin EC1118 yeast is in the same family as ML01 (Husnik et al. 2007). The manufacturer of Lalvin EC1118 reports this yeast as a high SO₂ producer at 30 mg/L. The relative differences between SO₂ produced by the yeasts in the Thompson Seedless juice and the Vignoles juice should be similar. Assuming that SO₂ production was similar among yeasts between the two juices, the differences in total SO₂ can be explained by the additional SO₂ produced by ML01.

Conclusions

Reduction and/or elimination of malic acid in wines were accomplished with both natural and genetically en-

hanced organisms. The naturally selected wine yeast ICV-GRE and 71B reduced the malic acid content in wine by 18 and 33%, respectively, while ML01 converted all the malic acid to lactic acid. The wine fermented with ICV-GRE + Lalvin 31 had lower lactic acid and titratable acidity than ML01 because of malic consumption by the yeast before MLF. ML01 produced more SO₂ than the other yeasts.

Literature Cited

- Alexandre, H., P.J. Costello, F. Remize, J. Guzzo, and M. Guilloux-Benatier. 2004. *Saccharomyces cerevisiae*–*Oenococcus oeni* interactions in wine: Current knowledge and perspectives. *Int. J. Food Microbiol.* 93:141-154.
- Bartowsky, E.J. 2005. *Oenococcus oeni* and malolactic fermentation—Moving into the molecular arena. *Aust. J. Grape Wine Res.* 11:174-187.
- Bauer, R., and L.M.T. Dicks. 2004. Control of malolactic fermentation in wine. A review. *S. Afr. J. Enol. Vitic.* 25:74-88.
- Delcourt, F., P. Taillandier, F. Vidal, and P. Strehaniano. 1995. Influence of pH, malic acid and glucose concentrations on malic acid consumption by *Saccharomyces cerevisiae*. *App. Microbiol. Biotechnol.* 43:321-324.
- Henick-Kling, T., and Y.H. Park. 1994. Considerations for the use of yeast and bacterial starter cultures: SO₂ and timing of inoculation. *Am. J. Enol. Vitic.* 45:464-469.
- Husnik, J., H. Volschenk, J. Bauer, D. Colavizza, Z. Luo, and H.J.J. van Vuuren. 2006. Metabolic engineering of malolactic wine yeast. *Metabolic Eng.* 8:315-323.
- Husnik, J.I., P.J. Delaquis, M.A. Cliff, and H.J.J. van Vuuren. 2007. Functional analyses of the malolactic wine yeast ML01. *Am. J. Enol. Vitic.* 58:42-52.
- Pretorius, I.S. 2000. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking (review). *Yeast* 16:675-729.
- Pilone, G.J., and F.A. Ryan. 1996. A New Zealand experience in yeast inoculation for acid reduction. *Wine Indust. J.* 11(4):83-86.
- Rainieri, S., C. Zambonelli, V. Tini, L. Castellari, and P. Giudici. 1998. The enological traits of thermotolerant *Saccharomyces* strains. *Am. J. Enol. Vitic.* 49:319-324.
- Ramon-Portugal, F., I. Seiller, P. Taillandier, J.L. Favarel, F. Nepveu, and P. Strehaniano. 1999. Kinetics of production and consumption of organic acids during alcoholic fermentation by *Saccharomyces cerevisiae*. *Food Technol. Biotechnol.* 37(4):235-240.
- Redzepovic, S., S. Orlic, A. Majdak, B. Kozina, H. Volschenk, and M. Viljoen-Bloom. 2003. Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. *Intl. J. Food Microbiol.* 83:49-61.
- Rosi, I., F. Giovanna, and V. Canuti. 2003. Influence of different pH values and inoculation time on the growth and malolactic activity of a strain of *Oenococcus oeni*. *Aust. J. Grape Wine Res.* 9:194-199.
- Volschenk, H., H.J.J. van Vuuren, and M. Viljoen-Bloom. 2003. Malo-ethanolic fermentation in *Saccharomyces* and *Schizosaccharomyces*. *Curr Gent.* 43:379-391.
- Walker, T., J. Morris, R. Threlfall, and G. Main. 2003. Analysis of wine composition in Cynthiana and Syrah wines. *J. Agric. Food Chem.* 51:1543-1547.
- Zoecklein, B.W., K.C. Fugelsang, B.H. Gump, and F.S. Nury. 1995. *Wine Analysis and Production*. Chapman & Hall, New York.