Malic Acid Distribution and Degradation in Grape Must During Skin Contact: The Influence of Recombinant Malo-Ethanolic Wine Yeast Strains

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Wine acidity plays an important role in determining wine quality and ensuring physiochemical and microbiological stability. In high-acid wines, the L-malic acid concentration is usually reduced through bacterial malolactic fermentation, while acidulation in low-acidity wines is usually done during final blending of the wine before bottling. This study showed that skin contact did not influence the relative concentration of L-malic acid in the pulp and juice fractions from Colombard, Ruby Cabernet and Cabernet Sauvignon grape musts, with 32%-44% of the L-malic acid present in the pulp fraction. Four recombinant malo-ethanolic (ME) *Saccharomyces* wine yeast strains containing the malic enzyme (*mae2*) and malate transporter (*mae1*) genes of *Schizosaccharomyces pombe*, effectively degraded the L-malic acid in both the juice and pulp fractions of all three cultivars, with a complete degradation of malic acid in the juice fraction within 2 days.

The acidity level of wine has a significant influence on the organoleptic and aesthetic character, as well as on the microbial stability of wine (Beelman & Gallander, 1979; Henick-Kling, 1993; Gao & Fleet, 1995). Grapes contain a variety of organic acids with tartaric acid and L-malic acid being the most abundant during harvesting, accounting for more than 70-90% of the total titritable acidity (Radler, 1993). Tartaric acid concentrations remain relatively stable during grape berry development, while L-malic acid, predominantly found in the central and peripheral zone of grape berries, accumulates early in berry development and declines during ripening due to dilution and respiration. Viticultural practices, the prevailing climate and grape cluster environments may directly affect respiration rates of L-malic acid. Residual L-malic acid affects the pH and titritable wine acidity and serves as a carbon source for contaminating lactic acid bacteria (LAB). Removal of excess L-malic acid, especially in wines from the cool climate viticultural regions, is therefore essential for the production of well-balanced wines and to improve the shelf-life of wines (Delcourt *et al*., 1995).

Commercial wine yeast strains of *Saccharomyces* have little or no effect on the final L-malic acid concentration in wines, as they are unable to degrade L-malic acid effectively (Radler, 1993; Volschenk *et al*., 2003). This has been ascribed to the low substrate specificity of the *S. cerevisiae* malic enzyme and its mitochondrial compartmentalisation, together with the absence of an active transport system for L-malic acid (Volschenk *et al*., 1997a, b). Biological deacidification of wines has therefore traditionally been obtained with bacterial malolactic fermentation (MLF), which reduces the total acidity of the wine and also contributes to the microbiological stability of wine (Davis *et al*., 1985; Nielsen & Richelieu, 1999). However, the inherently hostile environment of wine, i.e. nutrient scarcity, low pH, high ethanol and sulphur dioxide levels and low fermentation temperatures, often result in sluggish or stuck MLF with an increased risk of wine spoilage (Davis *et al*., 1985; Fleet, 1999; Maicas, 2001).

Co-expression of the *Schizosaccharomyces pombe* malate permease (*mae1*) gene together with the *Lactobacillus lactis* or the *Oenococcus oeni* malolactic enzyme genes resulted in recombinant *S. cerevisiae* laboratory strains that actively transported L-malic acid and simultaneously performed alcoholic and malolactic fermentation (Volschenk *et al*., 1997a, b). The *S. pombe* malate transporter gene (*mae1*) and the *O. oeni* malolactic enzyme gene (*mle*A) were subsequently integrated in the genomes of industrial wine yeast strains to develop commercially available wine yeast strains with the ability to degrade L-malic acid during alcoholic fermentation (Husnik, 2003).

Recombinant malo-ethanolic (ME) strains of *Saccharomyces* were also constructed by integrating the *S. pombe mae1* and *mae2* genes into the genomes of commercial wine yeast strains under regulation of the constitutive 3-phosphoglycerate kinase (*PGK1*) promoter and terminator elements of *S. cerevisiae* (Volschenk *et al*., 1997b, 2001, 2004). The recombinant strains were able to actively transport L-malic acid into the yeast cell and convert the L-malic acid to ethanol under fermentative conditions. However, the application of these recombinant ME strains under different winemaking conditions requires further investigation to ensure optimal results.

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In red wines, continual and deliberate contact between the grape skins and juice is used to extract flavour and colour from the grape skins. A combination of enzymatic activities, heat, ethanol and organic acids (including L-malic acid) results in the extraction of colour pigments and flavour precursors from the grape skins to produce the characteristic colour and flavour of red wines. Increases in the temperature and duration of skin contact can result in substantial variations in the character of the finished wine in terms of higher pH, potassium and total phenolic levels (Stephen *et al*., 1986; Ferreira *et al*., 1995; Darias-Martin *et al*., 2000).

Approximately 40% of the total L-malic acid remains in the pulp fraction after skin contact and pressing of grapes (K Hunter, ARC-Nietvoorbij, 2002, personal communication). The ability of the ME strains to degrade L-malic acid in both the pulp and juice fractions are important to winemakers for the management of acidity levels in wine. In this study, the effect of skin contact on the relative concentrations of L-malic acid in the juice and pulp fractions was investigated at different vinification stages in one white and two red grape varieties. Furthermore, the efficacy of recombinant *Saccharomyces* ME strains in reducing the L-malic acid content in both the pulp and juice fractions were determined.

MATERIALS AND METHODS

Yeast strains and media

Four commercial wine yeast strains of *Saccharomyces* (Table 1) with divergent genetic backgrounds were obtained from Lesaffre International, France, and used for the transformation and integration of the *PGK1*$_p$-*mae1*-*PGK1*$_t$-*PGK1*$_p$-*mae2*-*PGK1*$_t$ expression cassettes as previously described by Volschenk *et al*. (2001). Transformants were screened on optimised GMIA media for their ability to degrade L-malic acid (Volschenk *et al*., 2001). Precultures of stable transformants and host yeast strains were grown to high cell density in 20 ml YEPD broth at 28°C, harvested by centrifugation at 8000 rpm and washed twice in sterile grape juice prior to inoculation.

Large-scale wine fermentations

Colombard, Ruby Cabernet and Cabernet Sauvignon grape must were evaluated in commercial-scale fermentations at ARC-Nietvoorbij (Stellenbosch, South Africa) to determine the L-malic acid distribution between the pulp and juice. The Colombard grapes (sugar index of 23.7°B [degree Brix, representing g sugar/100 gram juice], total acids of 6.27 g/L, pH of 3.53) were destemmed, crushed, pressed and left on the skins for less than 48 h before inoculation and allowed to ferment at 15°C. The Ruby Cabernet (21.8°B, 7.16 g/L total acids, pH 3.77) and Cabernet Sauvignon (22.3°B, 7.32 g/L total acids, pH 3.34) musts were subjected to skin contact for four days at 23°C with daily submerging. After inoculation, all further treatments and fermentations were done according to standard winemaking practices. Must samples were taken twice daily for L-malic acid analyses.

Must preparation and small-scale fermentations

To determine the efficacy of the recombinant strains, grapes of the same three cultivars were used to monitor L-malic acid concentrations during skin contact and the early stages of fermentation. The grapes were crushed by hand and treated with sulphur dioxide (SO_2) at concentrations of 50 ppm SO_2 for red must and 30 ppm SO_2 for white must. The crushed berries and juice were divided into eight batches of 250 mL Ruby Cabernet and Cabernet Sauvignon, or eight batches of 100 mL Colombard must. The mixtures were inoculated with ca. 2 x 10^6 cells/mL of precultured host or recombinant *Saccharomyces* ME strains (Table 1). The L-malic acid concentration was measured prior to inoculation and twice daily after inoculation (during skin contact), as well as towards the end of fermentation after the crushed berries were hand-pressed through a mesh cloth. Alcoholic fermentation was considered complete when the weight of the bottles remained stable for three days.

Sample preparation and malic acid assays

Monitoring of L-malic acid concentration was done in the grapes prior to crushing, after crushing, during skin contact, after press-

TABLE 1

Description of *Saccharomyces* strains used in this study.

Strain	Description
L1001	Neutral strain of *Saccharomyces cerevisiae*, starts rapidly with the alcoholic fermentation, but passing with difficulty above 13% (v/v) alcohol, rapidly autolysing itself at the end of the fermentation, type new Beaujolais.
L1003	*S. cerevisiae* killer strain for white wines, aromatic, able to reach 15% (v/v) alcohol.
L1005	*Saccharomyces bayanus* strain, neutral towards killer toxin, with high potential to produce alcohol superior to 15% (v/v) alcohol.
L1006	*S. cerevisiae* strain isolated in Spain, often stops fermentation at 10.5% (v/v) alcohol.
ME L1001	L1001 containing integrated *PGK1*$_p$-*mae1*-*PGK1*$_t$-*PGK1*$_p$-*mae2*-*PGK1*$_t$ cassette.
ME L1003	L1003 containing integrated *PGK1*$_p$-*mae1*-*PGK1*$_t$-*PGK1*$_p$-*mae2*-*PGK1*$_t$ cassette.
ME L1005	L1005 containing integrated *PGK1*$_p$-*mae1*-*PGK1*$_t$-*PGK1*$_p$-*mae2*-*PGK1*$_t$ cassette.
ME L1006	L1006 containing integrated *PGK1*$_p$-*mae1*-*PGK1*$_t$-*PGK1*$_p$-*mae2*-*PGK1*$_t$ cassette.

ing and clarification, and finally at completion of fermentation just before bottling. Initial L-malic acid concentrations were determined by diluting 50 g grapes with 100 mL water and homogenisation for 1 min, followed by heating at 60°C for 30 min to extract any solutes still bound to the skin. The paste was centrifuged at 3000 rpm for 5 min and the supernatant filter-sterilised through a 0.22 µm Cameo Nylon Syringe Filter (GE Osmonics, USA) and kept on ice. Must samples taken after crushing of the grapes, during skin contact and after pressing, were sieved (2 mm) to separate free-flow juice from the pulp and skin fractions. Free-flow juice was filter-sterilised and kept on ice, whereas samples of 50 g pulp were treated as described for the grape berries to determine the residual L-malic acid concentration. The clarified juice was filter-sterilised as described above and kept on ice. L-Malic acid assays were done with the L-malic acid test kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. All treatments were done in duplicate.

RESULTS AND DISCUSSION

Distribution of L-malic acid between pulp and juice in large-scale fermentations

There was little degradation of L-malic acid in the Cabernet Sauvignon, Ruby Cabernet and Colombard must during the skin contact period (Figs. 1A, 2A & 3A). Furthermore, the relative ratio of L-malic acid concentration in the juice and pulp fractions remained relatively stable throughout the skin contact period, with an average of 34%, 38% and 44% of the total L-malic acid present in the pulp fractions of the Cabernet Sauvignon, Ruby Cabernet and Colombard fermentations musts, respectively. Even after pressing, the skin fractions still contained 31%-44% of the total L-malic acid concentration.

Effect of skin contact on efficacy of recombinant ME strains

Four commercial *Saccharomyces* strains and the corresponding recombinant ME strains were evaluated in small-scale fermentations for their ability to degrade L-malic acid in all three grape cultivars. In Cabernet Sauvignon must, all the recombinant ME strains effectively degraded the L-malic acid within 28 h after crushing (Fig. 1B). The host strain L1005 degraded 25% of the L-malic acid after 118 h, while the other host strains showed no degradation. Similarly, all the recombinant ME strains effectively removed the L-malic acid in the Ruby Cabernet juice within 33 h (Fig. 2B), with host strain L1005 degrading 15% of the L-malic acid at 115 h. In the Colombard must, ca. 83% of the L-malic acid in the juice was degraded by the recombinant ME strains at the time of pressing (32 h), with complete degradation within 47 h after crushing of the grapes (Fig. 3B). At the end of fermentation (115 h), the host strain L1001 showed little degradation of L-malic acid, while strains L1003, L1005 and L1006 degraded 32%, 47% and 22% of the L-malic acid, respectively.

Effect of recombinant ME strains on relative concentration of malic acid in juice and pulp fractions

After skin contact and fermentation for 5 days, the Cabernet Sauvignon and Ruby Cabernet must was pressed and the L-malic acid concentration in the juice and the pulp determined (Figs. 1C & 2C). The host strains had little effect on the ratio of L-malic acid in the pulp and juice fractions, with 34%-41% of the L-malic acid remaining in the pulp fraction. For the recombinant strains, a significant reduction in the L-malic acid content was noticed in both the juice and pulp fractions with more than 90% of the L-malic acid removed in both fractions (relative to the host strains).

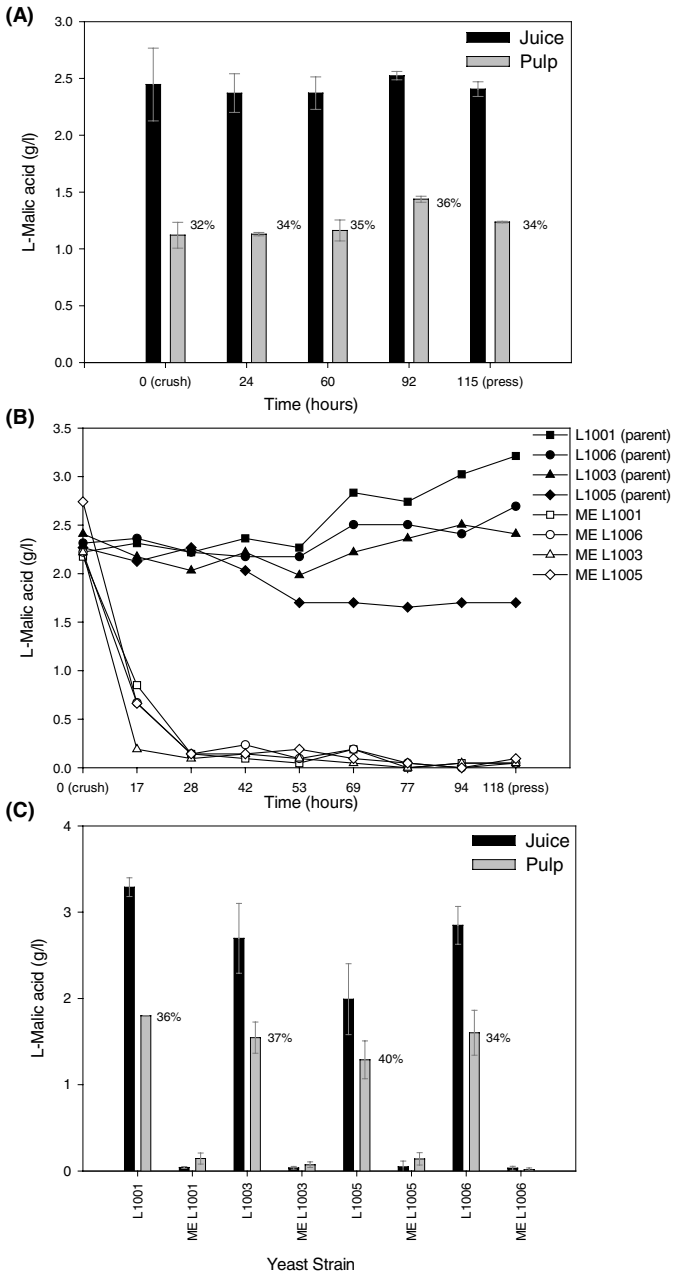

FIGURE 1

Cabernet Sauvignon: (A) Distribution of L-malic acid between the juice and pulp fractions during skin contact in commercial-scale fermentation. (B) Degradation of L-malic acid by ME strains of *Saccharomyces* during skin contact in a small-scale fermentation. (C) Relative concentrations of L-malic acid in juice and pulp fractions after pressing in small-scale fermentation. Percentages indicate the relative L-malic acid concentration in the pulp fraction.

CONCLUSIONS

We have shown that the duration of skin contact does not influence the relative concentration of L-malic acid in the pulp and juice fractions in the red or white grape varieties investigated. However, the ME *Saccharomyces* strains effectively degraded the L-malic acid in the juice fraction, as well as the L-malic acid normally discarded with the pulp fraction after pressing. This rapid

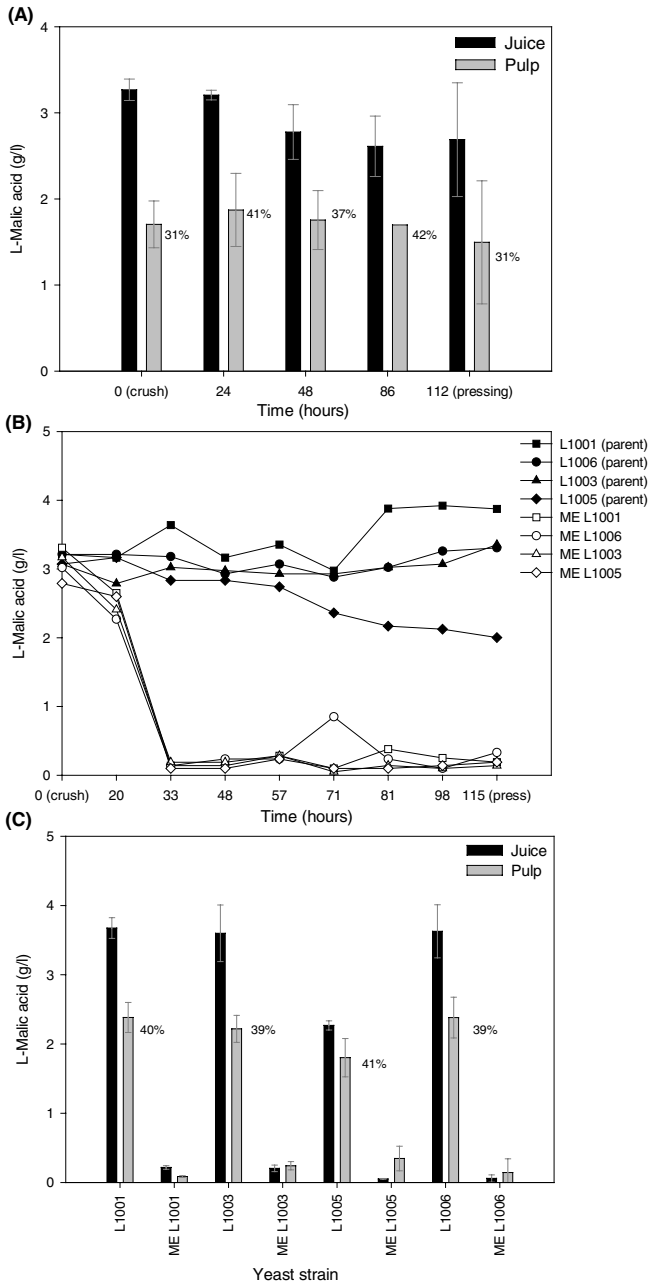

FIGURE 2

Ruby Cabernet: (A) Distribution of L-malic acid between the juice and pulp fractions during skin contact in commercial-scale fermentation. (B) Degradation of L-malic acid by ME strains of *Saccharomyces* during skin contact in a small-scale fermentation. (C) Relative concentrations of L-malic acid in juice and pulp fractions after pressing in small-scale fermentation. Percentages indicate the relative L-malic acid concentration in the pulp fraction.

biological deacidification can be especially useful to replace the unreliable bacterial malolactic fermentation, especially in the cool viticulture regions, where the wine acidity tends to be higher. Furthermore, the complete degradation of L-malic acid from both the juice and pulp fractions and its subsequent conversion to ethanol could have a potential benefit in the production of rebate wine for distilled beverages that require high alcohol content. However, precaution must be taken with the application of the ME *Saccharomyces* strains during skin contact, since the rapid removal of L-malic acid from the juice may affect the extraction

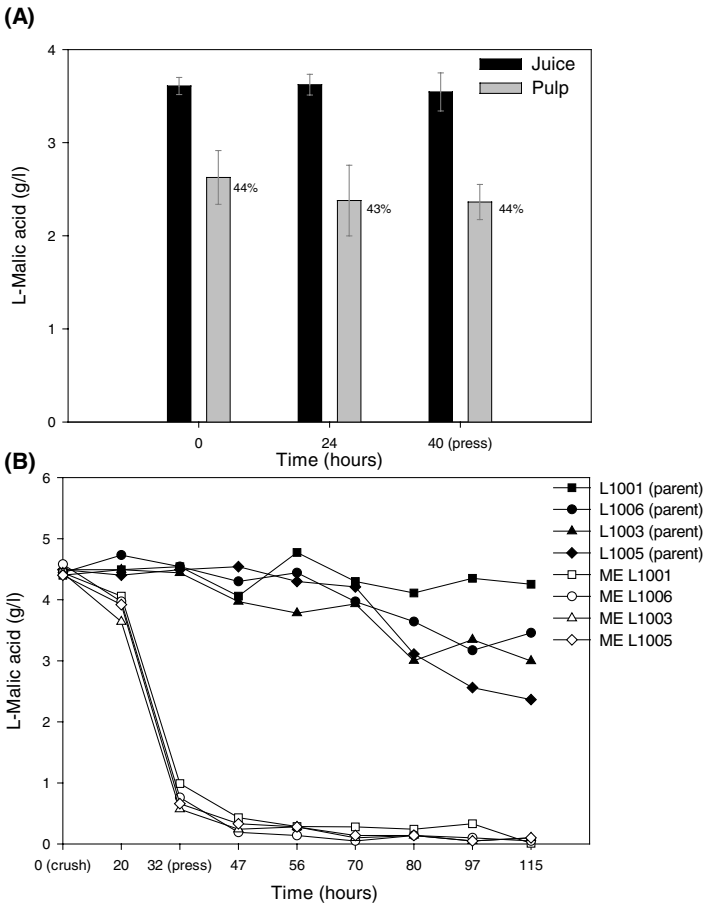

FIGURE 3

Colombard: (A) Distribution of L-malic acid between the juice and pulp fractions during skin contact in commercial-scale fermentation. Percentages indicate the relative L-malic acid concentration in the pulp fraction. (B) Degradation of L-malic acid by ME strains of *Saccharomyces* during skin contact in a small-scale fermentation.

efficiency of colour and flavour compounds. It is therefore advisable to apply the recombinant ME yeast strains after skin contact to eliminate poor colour and flavour extraction as well as elevated ethanol levels that could influence survival of the yeast cells.

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