Effect of a Pectinase-Surfactin Preparation on Extraction of Pigments and Total Polyphenol from Malbec Grape Skins

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Abstract: The effects of a surfactin from *Bacillus subtilis* C4 on the extraction of anthocyanins, other pigments, and total polyphenols by action of a pectinase from *Bacillu*s sp. SC-H during a short maceration of Malbec grape skins were studied. A prefermentative extraction from skins free of pulp and seeds with an extraction solution in absence of ethanol for 2 hours was carried out. Color was measured by tristimulus colorimetry and traditional indices (color index, shade, and total polyphenol content) and anthocyanins were determined by HPLC. The color index increased from 2.878 ± 0.281 for natural extraction, to 5.500 ± 0.107 for samples with pectinase plus 0.095% surfactin, and to 6.036 ± 1.013 for samples with pectinase plus 0.286% surfactin. Total polyphenol content increased from 555.77 ± 5.00 mg GAE/L for natural extraction to 769.71 ± 38.21 mg GAE/L and to 769.05 ± 8.40 mg GAE/L for the aforementioned samples. Anthocyanic compounds were readily released from grape skins, especially malvidin derivatives, which are the main pigments responsible for red wine color. Malvidin-3-glucoside was increased by 10% and 15% and malvidin-3-acetyl-glucoside by 21% and 29% with the enzymatic and enzyme-surfactin treatments, respectively. The coordinates and CIELAB color differences were improved with respect to natural extraction because of red pigments; thus, the macerates became darker and with a more vivid color, with the best values corresponding to the highest surfactin concentration studied. The simultaneous use of the enzyme and surfactin enhanced the indices and anthocyanin composition during a short extraction from grape skins. However, it is necessary to study the effects on entire berries and in real winemaking conditions. Further investigation is required in order to propose red wine maceration with the addition of pectinase-surfactin complex as a new tool in winemaking.

Key words: pectinase, surfactin, *Bacillus*, anthocyanin

Wine color is important because it is the first sensory factor perceived by the consumer and because of the positive correlation between color and quality (Jackson et al. 1978). Currently, the economic value of wine is determined not only by alcohol percentage and flavor but also by color intensity. Knowledge of wine color is necessary to ensure color evolution related to wine variety and quality. Polyphenols, particularly anthocyanins, are the compounds responsible for color in must and wines and contribute to wine astringency and bitterness. Phenolic compounds in red wines, especially flavonoids and stilbenes, have been identified as having several beneficial physiological effects, mostly because of their antioxidant properties (Frankel et al. 1993). Recent studies have highlighted the favorable effects of grape and wine polyphenols on human health (Rice-Evans et al. 1997, Lurton 2003).

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Grape skins represent ~5 to 10% of the total dry weight of the grape berry and act as a hydrophobic barrier to protect the grapes from physical and climatic injuries, dehydration, fungal infection, and UV light. Grape skins can be divided into three superimposed layers: (1) the outermost layer, the cuticle, composed of hydroxylated fatty acids and hydrophobic waxes; (2) the intermediate epidermis, which appears as a regular tilling of cells; and (3) the inner layer, the hypodermis, closest to the pulp, which contains most of the phenolics in grape skin (Lecas and Brillouet 1994). The cell wall of the grape berry forms a barrier to the diffusion of components, including aromas and phenols, and acts as a protection against external factors (Doco et al. 2003). The cell wall of grape skin is comprised of 30% neutral polysaccharides (cellulose, xyloglucan, arabinan, galactan, xylan, and mannan), 20% acidic pectin substances (of which 62% are methyl esterified), ~15% insoluble proanthocyanidins, and <5% structural proteins (Lecas and Brillouet 1994).

Pectinases belong to the group of carbohydrases that catalyze the breakdown of pectin substances. These enzymes are very specific in wine and lead to key benefits, such as a faster start to fermentation, higher must yield, easier pressing, more rapid and complete clarification of the obtained wine, and extraction of the phenolic and aromatic compounds in grape skins (Doco et al. 2007, Nicolini and Mattivi 1995). Pectinolytic preparations may also improve the stability, taste, and structure of red wines, as their enzymatic actions may release anthocyanins from the skins

and extract tannins that are bound to cell walls. As noted in the literature, the use of these preparations generally provides good results for red wine color (Bautista-Ortin et al. 2005, Revilla and González-San José 2003), although several authors have reported contradictory findings (Wightman et al. 1997, Nicolini et al. 1994). In our laboratory, a cold-active pectinase from *Bacillus* sp. SC-H was isolated and characterized (Cabeza et al. 2005). This pectinase was used in the research reported here.

Surfactin is a powerful biosurfactant with desirable properties, such as biodegradability, low toxicity, and beneficial biological activities (e.g., antitumor activity and antiviral action), and its unique structure provides new applications that classical surfactants may lack (Feignier et al. 1995, Pocalyko and Tallman 1998). In particular, our research group found that *Bacillus subtilis* C4 was able to synthesize surfactin (Sabaté et al. 2009). The present work studied the possibility that this surfactin could act as a coadjuvant agent of the cold-active pectinase. Surfactant components, due to their tensioactive nature, would help enzymes penetrate grape skin hydrophobic areas and therefore more easily reach their pectin substrate. Given that pectin cements the remaining components of cell walls when it is hydrolyzed, it would facilitate the releasing of other substances such as polyphenols and flavor compounds. Therefore, the use of biosurfactants could be an alternative to enhance or assist the release of molecules from grapes by enzymatic action.

The definition and objective assessment of wine color is complicated. A reference method has been proposed by the OIV, although it may not be precise for highly colored wines (Heredia and Guzmán-Chozas 1991). The application of colorimetric systems, such as the standard method of the Commission Internationale de L'Eclairage (CIELAB), is of great value in the quantification and characterization of anthocyanin chromatic properties (Heredia et al. 1998). In addition, chromaticity value has been related to pigment concentration (Ihl et al. 1994) and to physicochemical parameters (Heredia and Guzmán-Chozas 1991).

The aim of the present work was to explore the effects of surfactin on the extraction of pigments and total polyphenols from grape skins by action of a pectinase in order to assay at laboratory scale a new strategy that could be applied to the maceration stage in red wine vinification.

Materials and Methods

Pectinase production. *Bacillus* sp. SC-H (Cabeza et al. 2005) was cultivated in the following medium (g/L): pectin, 2.0; yeast extract, 1.0; meat peptone, 10.0; soy peptone, 10.0; agar, 10.0; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $(NH_4)_2SO_4$, 3; $MnSO_4.6H_2O$, 0.05; $FeSO_4.7H_20$, 0.015; $MgSO_4$, 0.8 (pH 5.0). First, *Bacillus* sp. SC-H was activated by three successive subcultures, followed by cultivation in a 2-L flask containing 500 mL of the medium. The culture was incubated for 6 days at 35°C, and centrifuged (10,000 x *g*, 15 min, 4°C). The supernatant was filtered through 0.22-µm membrane and concentrated 5.3 times using a rotary evaporator under vacuum at 40°C. Specific enzymatic activity of *Bacillus* SC-H enzymatic concentrate was 6.302 U/mg protein, where one unit of enzymatic activity (U) was defined as the amount of enzyme required to release 1 µmol reducing sugar per minute at 30°C.

Surfactin sample preparation. *Bacillus subtilis* C4, isolated in previous works (Sabaté et al. 2009), was grown on brain-heart infusion broth (Britania, Buenos Aires, Argentina) for 12 hr at 37°C, without shaking. Bacterial cells were removed by centrifugation (10,000 × *g* for 15 min at 4°C) and the cell-free supernatant (CFS) was filtered (0.45 µm) and maintained at 4°C until use. Lipopeptides were precipitated by adding concentrated HCl to the CFS to pH 2.0 (Desai and Banat 1997). The resulting precipitate was recovered by centrifugation (14,000 × *g*, 25 min, 4°C) and surfactin was later extracted with methanol (Youssef et al. 2004). Finally, the methanolic extract was evaporated and dissolved in sterile distilled water. Surfactin synthesis was confirmed by HPLC isocratic analysis. Samples (25 µL) were injected onto a Spherisorb C18 column (5 µm, 250 x 4.6 mm) (Waters Corp., Milford, MA) at 0.5 mL/min flow rate using acetonitrile-water 90:10 (4% TFA) mobile phase at room temperature, and detected at 220 nm. Commercial surfactin used as control was purchased from Sigma Chemical Co. (St. Louis, MO). The surfactin concentration in the final sample was 10 mg/mL.

Extraction of pigments and polyphenols from skins. The tests were performed on Malbec grapes (*Vitis vinifera*) grown in a vineyard located in the Rama Caída subregion, from the San Rafael Designation of Origin, in southern Mendoza (Argentina). The berries were obtained in the 2008 vintage, at optimum ripeness (24.7% w/v reducing sugar, 4.65 g/L total acidity, pH 3.44) and in good sanitary conditions. Only the skins were used for the analysis.

The grape berries were manually destemmed and crushed, and the skins were separated from the pulp and seeds. The peels were split and unfolded to expose the internal part where color material is located and thus to increase the contact area for the extraction solution. The fragmented peels (1.65 g) were introduced in test tubes, and 2.5 mL extraction solution was added, which was comprised of a constant volume (150 µL) of *Bacillus* SC-H enzymatic concentrate, an increasing amount of surfactin (0.048, 0.095, 0.143, 0.190, 0.238, and 0.286%, w/v), and 0.15 M acetic-sodium acetate, pH 5 to bring the mixture to a constant final volume. Three reaction blanks were conducted: enzyme, surfactin, and total blank (natural extraction). Skins were contacted with the extraction solution and were incubated at 30°C with shaking (130 rpm) for 2 hr.

Macerates were centrifuged (5,000 x *g,* 10 min) and supernatants were analyzed by spectrophotometry at 420, 520, 620, and 750 nm wavelengths, to calculate color index (CI) (Glories 1984), shade (Sudraud 1958), total polyphenol content (TPC), which was quantified using the Folin-Ciocalteu method with gallic acid as standard (Singleton and Rossi 1965), and at 450, 520, 570. and 630 nm to determine CIELAB coordinates, using MSCV software (Ayala et al.

2001) and CIELAB color differences ($\Delta E_{r,s}^* = [(L^* - L^*_A)^2 + (a^* - a^*_A)^2 + (b^* - b^*_A)^2]^{1/2}$, with L^*, a^* and b^* the CIELAB coordinates and subscript $_A$ corresponding to natural extraction). All assays were performed in triplicate and averaged.

HPLC analysis of anthocyanins. The chromatograph was a Shimadzu (Kyoto, Japan) LC10 HPLC, with UV/VIS Photodiode Array Detector SPD-M10Avp, fitted with Shimadzu software. Samples were analyzed, previously filtered through a 0.45-µm pore size membrane, on a LiChrospher RP-18 reverse-phase column (4.6 mm x 250 mm, 5-µm particle size) (Merck, Darmstadt, Germany) equipped with a precolumn (RP-18; 2 mm x 20 mm, 30 to 40-µm particle size) at a constant temperature of 25°C. Solvents used were water/formic acid/acetonitrile (87:10:3) (A) and water/formic acid/acetonitrile (40:10:50) (B). Anthocyanic compounds were eluted under the following conditions: 0.8 mL/min flow rate, elution with linear gradients from 6 to 30% B in 15 min, from 30 to 50% B in 15 min, from 50 to 60% B in 5 min, from 60 to 6% B in 6 min, followed by washing and reconditioning of the column. The UV-visible spectra (scanning from 200 nm to 600 nm) were recorded for all peaks. Identification of anthocyanins was obtained using authentic standards and by comparing the retention times and spectra with those found in the literature (Mazza et al. 1999, Revilla et al. 1998). Quantification of anthocyanins was based on peak areas at 518 nm. Delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, peonidin-3-acetyl-glucoside, malvidin-3-acetyl-glucoside, peonidin-3-*p*-coumaroyl-glucoside, and malvidin-3-*p*-coumaroyl-glucoside were used as standards (Sigma).

Three samples were analyzed by HPLC: the first sample had no treatment, with no enzymatic extract or surfactin added (natural extraction); the second sample only had enzymatic concentrate (enzyme); and the third sample contained both enzymatic concentrate and surfactin at the highest surfactin concentration (0.286%) (enzyme-surfactin).

Surfactin and pectinolytic activity. Pectinolytic activity was assayed by quantification of reducing sugars released from pectin solution (0.25% pectin in 0.15 M acetic-sodium acetate buffer, pH 5) using 3,5-dinitrosalicylic acid reagent (DNS) (Miller 1959). Galacturonic acid was used as standard (Sigma). Samples contained 0.45 mL substrate and 0.05 mL enzymatic solution, the latter comprised of a constant volume of *Bacillus* SC-H enzymatic concentrate, a variable amount of surfactin (0, 0.048, 0.095, 0.143, 0.190, 0.238, and 0.286%, w/v), and a corresponding volume of 0.15 M acetic-sodium acetate buffer, pH 5, to bring the mixture to a constant final volume. These solutions were incubated at 30°C for 30 min. The reaction was stopped by adding 0.5 mL DNS reagent, followed by immersion in a boiling water bath for 15 min. After cooling, 1.5 mL distilled water was added and the absorbance measured at 530 nm. One unit of pectinase activity (U) was defined as the amount of enzyme required to release 1 µmol of reducing sugar per min at 30°C.

Accession number. 16S rRNA partial sequence data of *Bacillus* sp. SC-H and *Bacillus subtilis* C4 reported in this article have been submitted to GenBank nucleotide sequence databases under the accession numbers FJ626869 and EU195328, respectively.

Statistical analysis. ANOVA and multiple range tests were applied to analyze all the results, using StatGraphics Plus 5.1 (Manugistics, Rockville, MD). Differences between means were considered statistically significant when the F test was $p < 0.05$ (95.0% confidence level).

Results and Discussion

Color index, shade, and TPC. Natural extraction produced 2.878 ± 0.281 of color index (CI), 1.153 ± 0.05 of shade, and 555.77 ± 5.00 mg GAE/L of total polyphenol content (TPC) (Figure 1, Figure 2). Enzyme, surfactin,

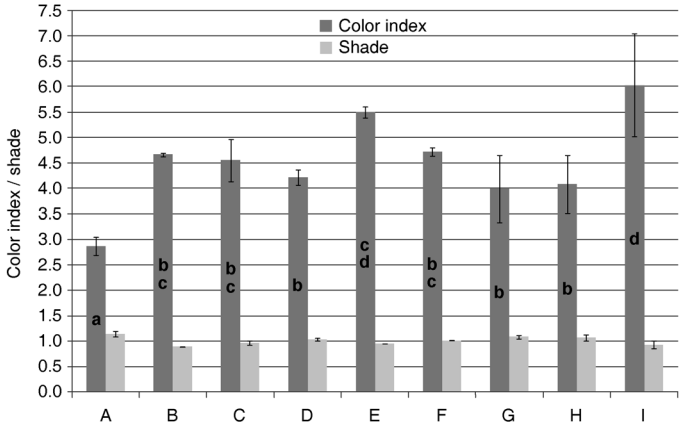

Figure 1 Surfactin effect over color index and shade (mean ± SD, n = 3). A, natural extraction (without enzyme or surfactin); B, 0.286% surfactin (w/v); C, 150 µL SC-H enzymatic concentrate; and D, E, F, G, H, and I, 150 µL SC-H enzymatic concentrate and 0.048%, 0.095%, 0.143%, 0.190%, 0.238%, and 0.286% surfactin (w/v), respectively. Different lowercase letters indicate significant differences among samples ($p < 0.05$).

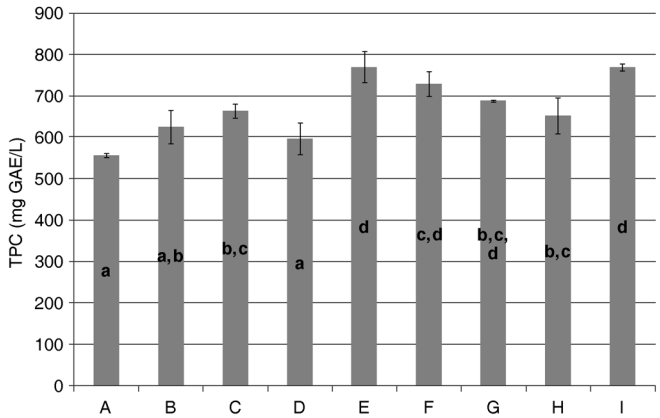

Figure 2 Surfactin effect over total polyphenol content (in GAE/L, gallic acid equivalent per liter) (mean ± SD, n = 3). A, natural extraction (without enzyme or surfactin); B, 0.286% surfactin (w/v); C, 150 µL SC-H enzymatic concentrate; and D, E, F, G, H, and I, 150 µL SC-H enzymatic concentrate and 0.048%, 0.095%, 0.143%, 0.190%, 0.238%, and 0.286% surfactin (w/v), respectively. Different lowercase letters indicate significant differences among samples ($p < 0.05$).

and surfactin concentrations acting simultaneously with the enzyme increased both CI and TPC; shade, however, remained practically invariable as significant differences were not observed (Figure 1, Figure 2). Thus, the releasing of yellow pigments did not increase significantly with any treatment in respect to the natural extraction, conferring a good characteristic to red wine quality.

For color index, the most important augments were produced by 0.095% (sample E) and 0.286% (sample I) surfactin concentrations (Figure 1). It was surprising that the relatively low surfactin concentration of sample E produced higher CI. Total polyphenol content (Figure 2) behaved similarly to color index. It would appear that there was no surfactin dose effect on these two parameters; however, both CI and TPC were increased in almost all samples with respect to natural extraction independently of the surfactin concentration.

Surprisingly, for CI the 0.048% surfactin level (sample D) produced a similar effect as the enzyme (sample C) and surfactin (sample B) treatments. However, a significant increase in TPC index in respect to natural extraction was not observed, probably because of the partial inhibitory effect of surfactin on the enzyme (discussed below), which would be compensated by increasing surfactin concentrations (due to its tensioactive effect).

The effects could be due to the surfactin action on grape cell wall, more specifically on the layer that contains the hydroxylated fatty acids and hydrophobic waxes; therefore, surfactin helps the enzyme to access the substrate. These results confirm the hypothesis proposed in our work, which was to evaluate the possible coadjuvant effect of the surfactin on the enzyme in the penetration into hydrophobic sections and the enzyme action over pectin, hence, the releasing of substances such as polyphenols (either pigments or not).

CIELAB coordinates and color differences. The CIELAB coordinates and differences on supernatants were determined and colorimetric changes among the different treatments can be seen (Table 1). Lightness (L^*) and hue angle (H^*) of almost all macerates decreased significantly in respect to natural extraction, presenting the maximum diminution sample I (17%) for the L^* parameter and E (89%) for the H^* parameter. While chroma or saturation (C^*) increased significantly in each tested sample in respect to the control macerate, sample I had the greatest effect (140%). Thus, whereas the enzyme alone, the surfactin alone, and the enzyme plus an increasing amount of surfactin were present in the samples, the macerates became darker with more bluish hues and a more vivid color. This effect was observed most markedly in samples E and I. In all cases, a^* coordinate (red color intensity measure) increased when the enzyme-surfactin preparations were added, with sample I having the greatest effect. The b^* coordinate (yellow color intensity measure) decreased significantly only in samples B and E, in respect to natural extraction.

Hence, these values might suggest that there was no direct correlation among lightness (L^*), saturation (C^*, contribution of a^* and b^* in the total color), and red color intensity measure (a^*) and the surfactin concentration. However, C^* and a^* values were significantly increased and L^* was reduced when the enzyme alone, the surfactin alone, and both enzyme and surfactin at any concentration were present in the extraction solution (in respect to natural extraction). Hue angles (H^*) lower than 20° were achieved when enzyme alone, surfactin alone, and enzyme-surfactin were added (in respect to natural extraction). Purple and bluish colors predominated in these macerates. The resultant supernatant color gave it a desirable characteristic (more red color intensity than yellow), since during the aging process and wine oxidation, yellow and brown color intensities increase and red intensity decreases, conferring an aged aspect and a russet color to red wine. Thus, it is important to achieve a better color in wines before the beginning of aging process.

CIELAB color differences ($\Delta E_{r,s}$) were calculated between the different samples and natural extraction (sample A) (Table 1). A study with trained panelists comparing the color of red wines in wineglasses found that 2.7 CIELAB color difference units is the minimum color difference

Table 1 Enzyme–surfactin complex effect over CIELAB color.

Treatment[a]	L^*	C^*	H^*	a^*	b^*	$\Delta E_{r,s}$ (CIELAB units)[b]
A (natural extraction)	82.8 a[c]	9.7 a	23.0 a	8.9 a	3.30 a,b	0
B (0.266% surfactin)	74.1 c,d	19.1 c,d	357.4 d	19.0 c,d	-0.86 d	14.1
C (150 µL SC-H)	75.3 b,c,d	17.9 b,c,d	5.2 b,c,d	17.9 b,c,d	1.49 b,c	11.9
D (0.048%)	77.4 b,c	16.3 b,c	7.7 b,c,d	16.1 b,c	2.19 a,b,c	9.1
E (0.095%)	70.9 d,e	21.3 d,e	2.4 c,d	21.3 d,e	0.91 c,d	17.4
F (0.143%)	74.9 b,c,d	17.7 b,c,d	7.5 b,c,d	17.6 b,c,d	2.31 a,b,c	11.8
G (0.190%)	78.9 b	15.3 b	14.8 a,b	14.8 b	3.90 a	7.0
H (0.238%)	78.4 b,c	15.6 b	13.8 a,b	15.2 b	3.73 a	7.6
I (0.286%)	68.9 f	23.2 e	10.5 b,c	22.8 e	4.22 a	20.2

[a]A, natural extraction (without enzyme or surfactin); B, 0.286% surfactin (w/v); C, 150 µL SC-H enzymatic concentrate; D, E, F, G, H, and I, 150 µL SC-H enzymatic concentrate plus 0.048%, 0.095%, 0.143%, 0.190%, 0.238%, and 0.286% surfactin (w/v), respectively.
[b]$\Delta E_{r,s} = ((L^* - L^*_A)^2 + (a^* - a^*_A)^2 + (b^* - b^*_A)^2)^{1/2}$.
[c]Different letters indicate significant differences among samples ($p < 0.05$).

discernable between two wine samples (Martínez et al. 2001). Therefore it was confirmed that an observer would perceive disparities in all tested samples, presenting the highest difference in sample I (20.2 CIELAB units).

Anthocyanin composition. Anthocyanin composition was compared with natural extraction to determine the influence of the enzymatic treatment and the effect of enzyme-surfactin complex on the extraction stage in red wine vinification. Chromatograms were recorded at 518 nm of grape skin macerates with the natural extraction, the enzymatic concentrate (enzyme), and the enzymatic concentrate and surfactin complex at the highest surfactin concentration (0.286%) (enzyme-surfactin) (Figure 3). Nine different peaks were identified and were assigned to 3-glucosides of delphinidin (1), cyanidin (2), petunidin (3), peonidin (4), malvidin (5), the 3-acetyl-glucosides of peonidin (6), malvidin (7), and 3-*p*-coumaroyl-glucosides of peonidin (8) and malvidin (9), on the basis of their retention times and UV-visible spectra, compared with authentic standards.

Anthocyanin profiles of the three macerates were similar and the different treatments did not produce a selective effect on any anthocyanin, as previously reported (Kelebek et al. 2007). Glycosylated anthocyanins were predominant in the three macerates (~82%), followed by acetylated (~13%), and coumarylated (~5%). In the three aforementioned fractions of all samples, malvidin derivatives were the most abundant anthocyanin compounds; malvidin-3-glucoside presented the highest amount of all anthocyanin compounds and malvidin-3-acetyl-glucoside was the second most abundant pigment. Similar results were also found in red wines treated with pectolytic enzymes, where malvidin-3-glucoside was the most dominant anthocyanin (Kelebek et al. 2007). Glycosides of peonidin and petunidin, the third most representative compounds, decreased in enzyme and enzyme-surfactin treatments. Thus, enzyme and enzyme-surfactin treatments only produced an augment of the malvidin derivatives with respect to natural extraction.

Analysis of the concentrations of these anthocyanins in both enzyme and enzyme-surfactin treatments with respect to natural extraction (Table 2) revealed an increase of 10% and 15% of malvidin-3-glucoside, 21% and 29% of malvidin-3-acetyl-glucoside, respectively, and an increase of 75% of malvidin-3-coumaryl-glucoside in enzyme sample, since this component decreased by 15% in the enzyme-surfactin sample.

Therefore, enzyme and enzyme-surfactin preparations greatly increased the level of malvidin derivatives compared with natural extraction, although other anthocyanic monoglucoside derivatives decreased in respect to the control macerate. Nevertheless, given the short maceration time assayed in the present work, these results indicate that anthocyanic compounds were readily released from grape skin cell-wall matrices to the must, using both enzyme and enzyme-surfactin preparations.

The increase of malvidin derivatives due to enzyme and enzyme-surfactin treatments, mainly malvidin-3-

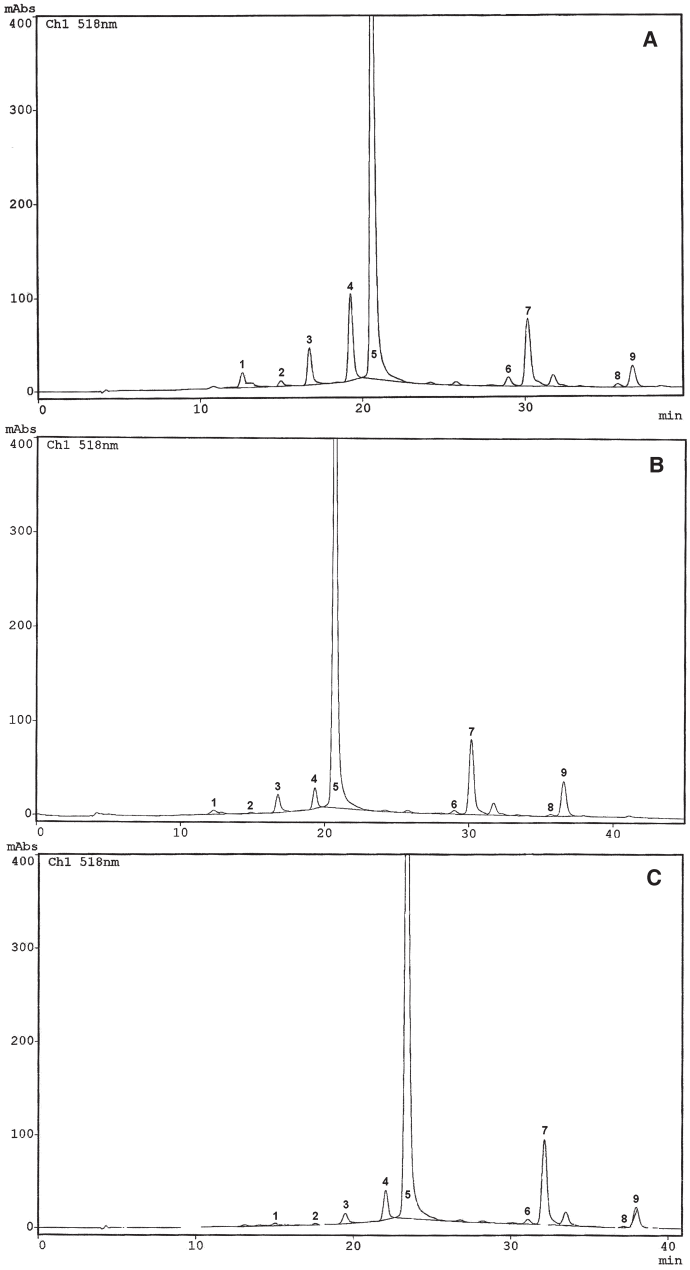

Figure 3 HPLC chromatograms of anthocyanins in grape skin macerates (at 518 nm). (**A**) natural extraction (without enzyme or surfactin), (**B**) enzymatic concentrate (enzyme) only, and (**C**) treatment with both enzymatic concentrate and surfactin at the highest surfactin concentration (0.286%) (enzyme-surfactin). Peak identification: 1, delphinidin-3-glucoside; 2, cyanidin-3-glucoside; 3, petunidin-3-glucoside; 4, peonidin-3-glucoside; 5, malvidin-3-glucoside; 6, peonidin-3-glucoside-acetate; 7, malvidin-3-glucoside-acetate; 8, peonidin-3-glucoside-*p*-coumarate; and 9, malvidin-3-glucoside-*p*-coumarate.

Table 2 Principal anthocyanin concentrations (percent) in three treatments: A, natural extraction (sample without enzyme or surfactin); C, 150 µL SC-H enzymatic concentrate; and I, 150 µL SC-H enzymatic concentrate plus 0.286% surfactin (w/v).

Peak no	Anthocyanin	A	C	I
5	Malvidin-3-glucoside	66.90%	74.06%	76.76%
7	Malvidin-3-acetyl-glucoside	10.48%	12.72%	13.48%
9	Malvidin-3-*p*-coumaroyl-glucoside	3.35%	5.87%	2.86%

acetyl-glucoside, could be the responsible for the augment of the CI and TPC parameters in respect to natural extraction (Figures 1 and 2, samples C and I, respectively). Moreover, similar conclusions could be drawn about CIELAB coordinates (Table 1), particularly C^* and a^*, which are associated with the rapid release of free anthocyanins. Recent data (Gómez-Míguez et al. 2007) has shown that the most abundant anthocyanic compound in wines, malvidin-3-monoglucoside, was the greatest contributor to the prediction of color parameters. Acetylated derivatives of malvidin were highly correlated with H^* and b^*; thus, they are very important in predicting the tonality of wine color.

Pectinolytic activity. The effect of surfactin on the extraction carried out by the enzyme required a check of the direct effect of the surfactant on the enzymatic activity. The determination was performed on 0.25% pectin dispersions in 0.15 M acetic-sodium acetate buffer, pH 5. The sample without surfactin was significantly different from the other samples and resulted in the highest value of pectinolytic activity (Figure 4). In general, a major decrease of enzymatic activity was produced. Enzymatic activity was reduced to approximately 40% of the control value when surfactin was added at any concentration.

It could be concluded that there is a partial inhibiting effect of the surfactin on enzymatic activity, which was observed with all tested surfactin concentrations. Particularly, the significant decrease of enzymatic activity could be due to a partial unfolding effect of the surfactin on the enzyme or to the ion pairing effect. It was recently demonstrated that the preparation of ionic paired enzymes causes unexpected changes in the specificity of the substrate when it is compared with the native suspension (Altreuter et al. 2002). One proposal is that interactions between the surfactant and specific sites of enzymes occur through the formation of an active conformational complex with the enzyme (Yahya et al. 1998). A study on the adverse effect of sodium dodecyl sulfate on the activity and stability of cutinase suggested that SDS causes local conformation changes in the active site that result in inhibition, partial reversible unfolding, and subsequent inactivation (Pocalyko and Tallman 1998). The same mechanism could explain the partial inhibition of surfactin on pectinase observed in this work.

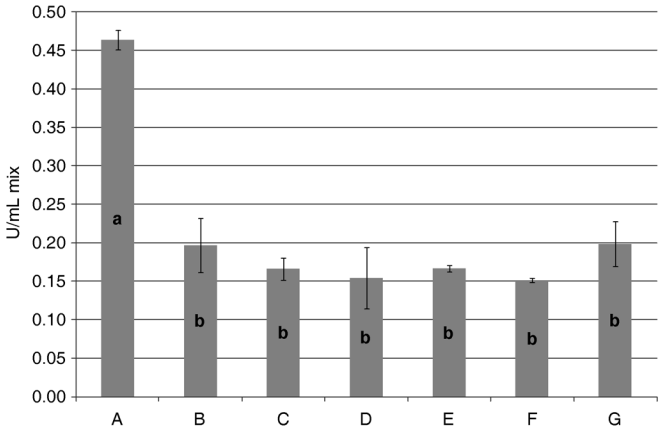

Figure 4 Surfactin effect over pectinolytic activity (mean ± SD, n = 3). Different mixes are formed with SC-H enzymatic concentrate and a variable amount of surfactin (w/v): A, 0%, B, 0.048%, C, 0.095%, D, 0.143%, E, 0.190%, F, 0.238%, and G, 0.286%. Different lowercase letters indicate significant differences among samples ($p < 0.05$). One unit of pectinase activity (U) was defined as the amount of enzyme required to release 1 µmol of reducing sugar per min at 30°C.

Nevertheless, the observed partial inhibiting effect could be due to the environment under which the pectinolytic activity assay was conducted, an aqueous medium in which the enzyme was in contact only with surfactin. No grape skins were present, so these assay conditions were different from the color extraction conditions. Surfactin in the pectinolytic activity assay could act directly on the enzyme, decreasing its activity. Surfactin could behave differently in the extraction solution with the grape skins as substrate. This is supported by the results of the color extraction test (according to CI, TPC, and CIELAB coordinates) for enzyme-surfactin samples E and I, where these parameters were superior with respect to samples with enzyme only or surfactin only. Surfactin by itself did not show pectinolytic activity (data not shown).

The present study is a preliminary work, aimed at assaying the effect of the surfactin on the enzymatic extraction, with the purpose of using the enzyme-surfactin complex in the maceration stage of red wine vinification. Therefore, this is an approach to the real vinification process, at laboratory scale and under optimum conditions for the enzyme (30°C and pH 5) and only a 2-hour maceration. Further investigations are needed to study the contribution of this new tool in the maceration stage of red wine vinification under real conditions of wine production.

In recent years, low maceration temperatures (5 to 15°C) prior to fermentation have also been tested in order to produce red wines. This method—known as cold-maceration or cold soak—is a French technique that was designed to improve the extraction of pigments, tannins, and aromas from the grape skins to the wine (Goumy et al. 1996). Hence, the extraction of these compounds occurs in the absence of ethanol. Red wines produced after low-temperature maceration (15°C) resulted in more colored and less brown wines (Gómez-Míguez et al. 2007). In addition, our investigative group has a cold-active pectinase from *Bacillus* sp. SC-H, which was used in this work. Cold maceration and the new enzyme-surfactin complex could possibly work together to produce better effects in the extraction of different compounds from grape skins.

Future investigations could focus on a strain of *Bacillus* that produces both pectinase and surfactin in order to obtain these metabolites in the same extract, reducing industrial production costs. Furthermore, the role of surfactin in penetration of pectinases could be used in other processes that involve the extraction of vegetal substances, such as olive oil. This practice would be greatly facilitated given the high lipid content contained in this vegetal material, which would be more adequate for surfactin action.

Conclusion

Results indicate that enzyme-surfactin complexes applied in the maceration of Malbec grape skins are useful in the extraction of readily extractable anthocyanin and other phenolic compounds. A better extraction of red pigments than yellow pigments was achieved, therefore darker and less brown macerates, which is a very desirable and valued feature in red wine composition. The results suggest the possibility of adding the pectinolytic preparation supplemented with surfactin during maceration of red grapes, allowing for a potential application in winemaking. Therefore, it is necessary to conduct further experiments using entire berries and in actual vinification conditions to propose the addition of pectinase-surfactin complex in red wine maceration as a new tool in winemaking.

Literature Cited

Altreuter, D.H., J.S. Dordick, and D.S. Clark. 2002. Optimization of ion-paired lipase for non-aqueous media: Acylation of doxorubicin based on surface models of fatty acid esterification. Enzyme Microb. Technol. 31:10-19.

Ayala, F., J.F. Echávarri, and A.I. Negueruela. 2001. Método Simplificado para el Color del vino. Universidad de La Rioja y de Zaragoza, España (www.unizar.es/negueruela/descargas/MSCVes.zip).

Bautista-Ortin, A.B., A. Martinez-Cutillas, J.M. Ros-Garcia, J.M. López-Roca, and E. Gómez-Plaza. 2005. Improving colour extraction and stability in red wines: The use of maceration enzymes and enological tannins. Int. J. Food Sci. Tech. 40:867-878.

Cabeza, M.S., F.S. Baca de Giménez, E. Muñoz Puntes, and V.I. Morata de Ambrosini. 2005. Microorganismos productores de pectinasas activas a bajas temperaturas para vinificación. *In* CIBIA V, Libro de Art. Extenso, Tomo III, Art. 6. Puerto Vallarta, México.

Desai, J.D., and I.M. Banat. 1997. Microbial production of surfactant and their commercial potential. Microbiol. Mol. Biol. R. 61:47-64.

Doco, T., P. Williams, and V. Cheynier. 2007. Effect of flash release and pectinolytic treatments on wine polysaccharide composition. J. Agric. Food Chem. 55:6643-6649.

Doco, T., P. Williams, M. Pauly, M.A. O'Neill, and P. Pellerin. 2003. Polysaccharides from grape berry cell walls. Part II. Structural characterization of the xyloglucan polysaccharides. Carbohyd. Polym. 53:253-261.

Feignier, C., F. Besson, and G. Michel. 1995. Studies on lipopeptide biosynthesis by *Bacillus subtilis*: Isolation and characterization of iturin$^-$, surfactin$^+$ mutants. FEMS Microbiol. Lett. 127:11-15.

Frankel, E., J. Kanner, J.B. German, E. Parks, and J.E. Kinsella. 1993. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341:454-457.

Glories, Y. 1984. La couleur des vins rouges. Première partie: Les equilibres des anthocyanes et des tanins. Conn. Vigne Vin 18:195-217.

Gómez-Míguez, M., M.L. González-Miret, and F.J. Heredia. 2007. Evolution of colour and anthocyanin composition of Syrah wines elaborated with pre-fermentative cold maceration. J. Food Eng. 79:271-278.

Goumy, D., M. Couasnon, and O. Seze. 1996. Caroxyque Française, France. French Patent Application, FR 2 731 228 A1.

Heredia, F.J., and M. Guzmán-Chozas. 1991. Reliability of the Spanish official method for colour of red wines in comparison with CIE 1931 (x,y) method. Food Chem. 39:167-174.

Heredia, F.J., E. Francia-Aricha, J.C. Rivas-Gonzalo, I.M. Vicario, and C. Santos-Buelga. 1998. Chromatic characterization of anthocyanins from red grapes. I. pH effect. Food Chem. 63:491-498.

Ihl, M., C. Shene, E. Scheuermann, and V. Bifani. 1994. Correlation for pigment content through colour determination using tristimulus values in a green leafy vegetable, Swiss chard. J. Sci. Food Agric. 66:527-531.

Jackson, M., C. Timberlake, P. Briddle, and L. Vallis. 1978. Red wine quality: Correlations between colour, aroma and flavor and pigment and other parameters of young Beaujolais wines. J. Sci. Food Agric. 29:715-727.

Kelebek, H., A. Canbas, T. Cabarouglu, and S. Selli. 2007. Improvement of anthocyanin content in the cv. Öküzgözü wines by using pectolytic enzymes. Food Chem. 105:334-339.

Lecas, M., and J.M. Brillouet. 1994. Cell wall composition of grape berry skins. Phytochemistry 35:1241-1243.

Lurton, L. 2003. Grape polyphenols: A new powerful health ingredient. Innov. Food Tech. 18:28-30.

Martínez, J.A., M. Melgosa, M.M. Pérez, E. Hita, and A.I. Negueruela. 2001. Visual and instrumental color evaluation in red wines. Food Sci. Technol. Int. 7:439-444.

Mazza, G., L. Fukumoto, P. Delaquis, B. Girard, and B. Ewert. 1999. Anthocyanins, phenolics, and color of Cabernet franc, Merlot, and Pinot noir wines from British Columbia. J. Agric. Food Chem. 47:4009-4017.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-428.

Nicolini, G., and F. Mattivi. 1995. Utilizzo di enzimi nella vinificazione in rosso. Vignevini 10:44-48.

Nicolini, G., G. Versini, F. Mattivi, and A. Dalla Serra. 1994. Glicosidasi in mosti e vini. Vignevini 7/8:26-32.

Pocalyko, D.J., and M. Tallman. 1998. Effect of amphipaths on the activity and stability of *Fusarium solani pisi* cutinase. Enzyme Microb. Tech. 22:647-651.

Revilla, I., and M.L. González-San José. 2003. Addition of pectinolytic enzymes: An enological practice which improves the chromaticity and stability of red wines. Int. J. Food Sci. Tech. 38:29-36.

Revilla, E., J.M. Ryan, and G. Martín-Ortega. 1998. Comparison of several procedures used for the extraction of anthocyanins from red grapes. J. Agric. Food Chem. 46:4592-4597.

Rice-Evans, C.A., N.J. Miller, and G. Paganga. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2:152-159.

Sabaté, D.C., L. Carrillo, and M.C. Audisio. 2009. Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Res. Microbiol. 160:163-169.

Singleton, V.L., and J.A. Rossi, Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-158.

Sudraud, P. 1958. Interprétation des courbes d'absorption des vins rouges. Ann. Technol. Agric. 7:203-208.

Wightman, J.D., S.F. Price, B.T. Watson, and R.E. Wrolstad. 1997. Some effects of processing enzymes on anthocyanins and phenolics in Pinot noir and Cabernet Sauvignon wines. Am. J. Enol. Vitic. 48:39-48.

Yahya, A.R.M., W.A. Anderson, and M. Moo-Young. 1998. Ester synthesis in lipase-catalyzed reactions. Enzyme Microb. Tech. 23:438-450.

Youssef, N.H., K.E. Duncan, D.P. Nagle, K.N. Savage, R.M. Knapp, and M.J. McInerney. 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Meth. 56:339-347.