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Phenols removal in musts: Strategy for wine stabilization by laccase

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Abstract

The potential of laccase from *Trametes versicolor* for phenolic removal in must for wine stabilization was evaluated through a combination of an analytical methodology (capillary zone electrophoresis) and kinetics of phenols removal as the total antioxidant potential variation. Total phenolic content, total antioxidant potential and polyphenols were monitored from 0 to 3 h of must treatment. The results indicated that the treatment of a red must with laccase affect mainly the phenolic compounds responsible for the must antioxidant properties. The treatment of white musts with laccase showed higher reduction in total phenol than in the total antioxidant potential. Phenol degradation by laccase was very fast for catechins, and slowly for stilbenes (*cis*- and *trans*-resveratrol) and derivatives of cinnamic (ferulic and caffeic) and benzoic (syringic, vanillic, and gallic) acids. It is possible to conclude in this case that the use of laccase in white wines is perfectly feasible. This would allow softer and ecologically correct treatments, which would diminish the cost of processing and avoid deterioration of wines for long storage times.

Keywords: Laccase; Phenols; Wine; Must; Capillary zone electrophoresis

1. Introduction

Wine makers has to make choices at crushing and pressing stage that will affect the style of wine. Both colour and astringency derive from polyphenols which are concentrated particularly in the stems, seeds, and skins. Wine phenolic composition depends on the grapes used to make the wine and on the vinification conditions [1]. Polyphenolic components of wine fall into one of two major classes. Non-flavonoids comprise hydroxybenzoates and hydroxycinnamates. Flavonoids include flavonols (e.g., quercetin, myricetin), flavan-3-ols (e.g., catechin and epicatechin), as well as polymers of the latter defined as procyanidins, and anthocyanins that are the pigments responsible for the colour of red wines; collectively they are 20-fold higher in red than in white wines [2]. Some of these phenol derivatives from not only in red wine but also in white ones [3] are important as antioxidant in the human diet. Therefore, their optimal organoleptic properties should remain unchanged until consumption.

Due to a complex sequence of events, where the polyphenols (coumaric acid derivatives, flavans, and anthocyanins) play an important role, oxidative reactions stimulated by iron, copper and enzymes, and that also involve aldehydes, amino acids and proteins, can occur in musts and wines causing flavour alterations, and in red wines colour intensification. This phenomenon of oxidation is known as madeirization [4].

In traditional wine technology, the madeirization prevention can avail itself of stabilizing procedures that either act on catalytic factors, block oxidizers, or remove of polyphenols. Proteinaceous, clarification, use of polyamides and high doses of sulphur dioxide have been used for this purpose. An alternative for the physical–chemical adsorbents involves the use of enzymes that act on the polyphenols responsible for the madeirization process.

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Enzymatic preparations have been studied in the wine industry for about 60 years, beginning in the decade of the 1930s with preparations for juice clarification. Today, the wine industries have available technology for the application of several industrial enzymes. Pectinases, β-glucosidases, β-glucanases, polyphenol oxidases, ureases, proteases and lysozyme are important examples of enzymatic preparations that can be used in wine production [5]. Although the use of enzyme preparations in the food industry is well established and expanding rapidly, enzyme processing in enology is less common. Some reasons why enzymatic techniques are not commonly used are: the 'classic' wine industry is still based on traditional methods, low-grade purity of enzyme preparations, possible enzyme persistence in wine, legal restrictions and high cost. For wine stabilization, the enzymatic preparations that are available contain enzymes active for polyphenolic substances such as laccases, tannases and peroxidases [6]. This treatment is interesting for its specific action and is a mild technology with less drastic effects than the chemical treatment on the characteristics of the wine.

Laccases (*p*-diphenol oxidase, EC 1.10.3.2) are multi-copper-containing enzymes that catalyse the oxidation of various aromatic compounds [7–10]. The literature reports many studies on the use of laccase for fruit juice and wine stabilization [4,6,11–14]. Maier et al. [15] evaluated the polyphenols percentage, colouration, stability and sensorial quality of Riesling wines prepared with and without oxidation of the must, or with oxidation of the must and the treatment with laccase. The results showed that the wines made with the forced oxidation/laccase treatment were the best, which suggests that stable wines and of high quality can be made with little or no addition of SO_2 [15]. Cantarelli and Giovanelli [16] carried out assays in order to determine if the enzymatic preparations could be used in white wines production for polyphenols reduction in musts (and consequent stabilization of the wine colour) instead of oxidation. The results demonstrated that the enzymatic treatment coupled with filtration with polyvinylpolypyrrolodone (PVPP) reduced the quantity of oxidized polyphenols.

Published data suggest that white and red wines acutely improves endothelial functions in patients with CAD [17]. This fact pointed out to the importance of phenolic components in both wines.

The aim of this study was to evaluate the potential of laccase from *Trametes versicolor* in must phenolics removal for wine stabilization (red and white wines). Total phenolic content, total antioxidant potential and polyphenols were monitored from must treatment.

2. Experimental

2.1. Enzyme production

Laccase was obtained from *T. versicolor* CCT 4521 (Fundação Tropical de Pesquisa e Tecnologia-Andre Tosello, Campinas, SP, Brazil) and was grown for 20 days at 30 °C and 240 rpm in a liquid medium containing (g/L): peptone, 10; malt extract, 5; $CuSO_4{\cdot}5H_2O$, 0.005 and glucose, 20; at pH 5.4. Laccase induction consisted in the addition of 0.5 mM 2,5-xylidine at 96 h of growth. The culture filtrate (Millipore 0.45 μm) was lyophilised, resuspended in 50 mM citrate–phosphate buffer (pH 5.0) and precipitated with 90% saturated ammonium sulphate. The enzyme was eluted in a gel chromatography column (Sephacryl S-200, Sigma) and lyophilised. The semi-purified laccase activity in a liquid stock solution was around 100 U/mL [18].

2.2. Enzyme assay

Laccase activity was assayed by measuring oxidation of syringaldazine [18]. The assay mixture contained 0.1 mL of 1.0 mM syringaldazine, 0.3 mL of 50 mM citrate–phosphate buffer (pH 5.0) and 0.6 mL of culture filtrate. The increase of A_{525} for 5 min was used to monitor syringaldazine oxidation. Enzyme activity was expressed in units of 1 U which was defined as 1 μmol of syringaldazine oxidized per minute.

2.3. Must treatments

Montepulciano d'Abruzzo (red), Montonico (white) and Moscato (white) musts were tested (20 mL). Laccase activities of 1 and 5 U/mL were added to the Montepulciano must, and in the case of Montonico and Moscato musts were treated with 1 U/mL of laccase. Total phenolic content, total antioxidant potential and polyphenols were monitored from 0 to 3 h of must treatment with laccase. The treatment was conducted in the dark at 25 °C with slow agitation.

2.4. Total phenolic content

The total phenolic contents of the must samples were determined with the Folin-Ciocalteu reagent, using gallic acid as standard. To 1000 μL of must sample (adequately diluted), 250 μL of carbonate–tartrate solution (200 g of Na_2CO_3 and 12 g of $Na_2C_4H_4O_6{\cdot}2H_2O$ in 1 L of distilled water) and 25 μL of Folin-Ciocalteu reagent were added. No interference of wine components as proteins was found. The absorbance of the sample was measured at 700 nm after 30 min of reaction. The results were expressed as mg of gallic acid equivalents (GAE)/L (at a range of 0.1–60 mg/mL gallic acid) [19,20].

2.5. Total antioxidant potential (bleaching of 2,2′-azinobis(3-ethylnenzotriazoline-6-sulfonate) (ABTS) radical cations)

ABTS radical cations were prepared by incubation of 150 μM (50 mL) with 2 M potassium persulfate (1.25 mL) for 2 h at 50 °C in 0.02 M phosphate buffer pH 7.0 [21]. To 996 μL of the ABTS radical cation, 4 μL of the must sample adequately diluted were added [20]. The absorbance of the sample was measured after 15 min at 734 nm. Gallic acid was used as standard and the results were expressed as mg of gallic acid equivalents (GAE)/L [19,20].

2.6. Assay of polyphenols by capillary zone electrophoresis (CZE)

CZE was employed to quantify polyphenols in musts according to Rossi et al. [22]. For the liquid–liquid extraction, 1 mL of red must (2 mL in case of white must) was extracted with 1 mL (2 mL for white must) of diethyl ether (twice). The organic phases were completely dried in the dark under nitrogen flux and resuspended with 100 μL of 10% methanol in electrophoretic buffer. Electrophoretic buffer composition was 25 mM phosphate and 10 mM borate at pH 8.8. Capillary electrophoresis analyses were performed using a P/ACE 5500 electrophoreser (Beckman Instruments Inc., Fullerton, CA, USA), equipped with a diode-array detector (DAD), and interfaced with Beckman P/ACE Station 5000 software, on an Epson Endeavour XL personal computer. The column used was an uncoated fused silica capillary tube of 75 μm i.d. (Beckman) with effective and total lengths of 50 and 57 cm, respectively. Electrophoretic analyses were performed at an applied voltage of 15 kV at 20 °C. Moreover, the silica column was pre-rinsed with bidistilled water (1.5 min) and separation buffer (1.5 min), and after each cycle the column was rinsed with a solution of 0.1 M HCl (1.5 min), 0.1 M NaOH (1.5 min) and bidistilled water (1.5 min). Samples were hydrodynamically injected at 3.45×10^3 Pa pressure for 7 s. This buffer was obtained by mixing solutions of H_3BO_3 (100 mM) and Na_2HPO_4 (100 mM), and NaOH (2 M) to reach the desired pH value. Calibration curves were obtained by hydrodynamic injection of concentrations, from 1 to 50 mg/L, of each compound for 7 s, at a pressure of 3.45×10^3 Pa (data not shown).

3. Results and discussion

Investigators have certified the antioxidant properties of polyphenols that are present in red wines; however, the specific role of individual compounds remains elusive [23]. In view of this, it is necessary to analyse the possible interactions of laccase with these components, which are so important in red wines and which in principle, are the substrates for the enzyme. The red must Montepulciano d'Abruzzo treatment resulted in high total antioxidant potential reduction (around 70%) with the two activities of laccase tested (1 and 5 U/mL) (Fig. 1). The total phenol reductions after 3 h of treatment were 37.4 and 69.4% with laccase activities of 1 and 5 U/mL, respectively. These results indicated that the treatment of this red must with laccase affect mainly the phenolic compounds responsible for the must antioxidant properties. Therefore, the treatment of this type of must with this kind of laccase is not recommended. A more careful approach for the selectivity of laccase should be used. Stutz [24] showed the dependence of haze reduction and browning reaction with the type of laccase used in apple juice stabilization. Then, based on a study of the isoenzymes of the laccase and of their selectivities, one can select those that have the least effect on the important components of red wine with antioxidant properties. The transformation of these compounds by laccase also could be retarded or inhibited by the presence of large quantities of a phenolic compound without antioxidants properties in the mixture that has fast degradation kinetic by this enzyme. More

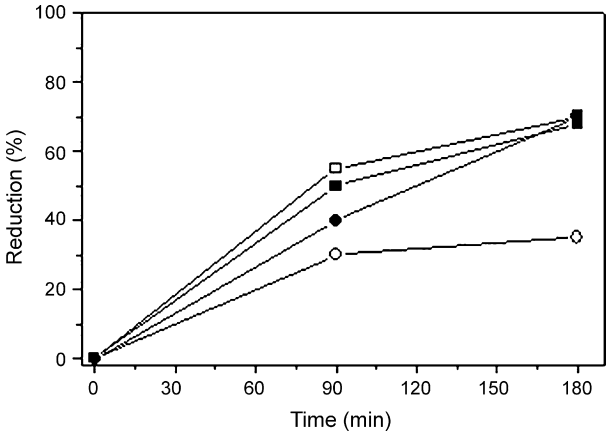

Fig. 1. Red must (Montepulciano d'Abruzzo) treated with laccase from *Trametes versicolor*: total phenols (◯, ●) and total reactive antioxidant potential (□, ■) reduction (%). The open symbols are those treated with 1 U/L and solid symbols are those treated with 5 U/mL (average value from three measurements, 2–3% deviation error).

detailed studies in this area are in progress with the purified laccase isoenzymes from *T. vesicolor*.

The white must Moscato treatment resulted in 9.2 and 32.1% of total antioxidant and total phenols reductions, respectively. The results with white must Montonico treatment indicated reductions of 13.9 and 33.4% in total antioxidant potential and total phenol, respectively (Fig. 2). In this way, the treatment of these must types with laccase is indicated in order to have an improved quality, stability and with all the phenolic properties as described above.

The polyphenol removal in musts should be selective, since indiscriminate removal will have an undesirable alteration in their organoleptic characteristics. Optimal analytical conditions have been obtained by a very rapid liquid–liquid extraction of few millilitres of sample with diethyl ether and an exhaustive capillary electrophoretic run by using a phosphate–borate electrophoretic buffer. CZE performance, coupled with diode-array detection, allowed a very good resolution and gave us the pos-

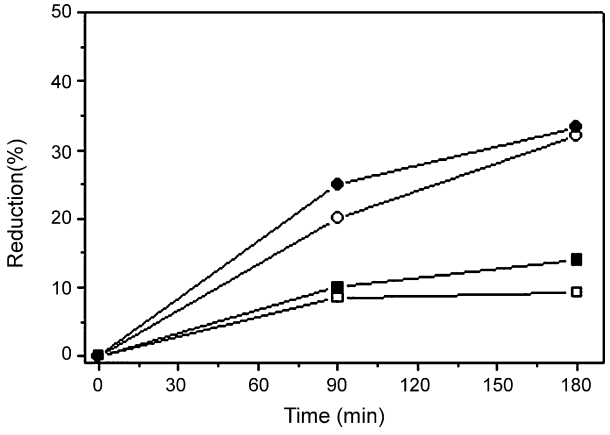

Fig. 2. Total phenols (◯, ●) and total reactive antioxidant potential (□, ■) reduction (%) of white musts Moscato (open symbols) and Montonico (solid symbols) treated for 180 min with 1 U/mL of laccase from *T. versicolor* (average value from three measurement, 2–3% deviation error).

Table 1
Must components determined by capillary zone electrophoresis

Peak no.	Name (structure)
1	*Cis*-resveratrol (*cis*-3,4′,5-trihydroxystilbene)
2	*Trans*-resveratrol (*trans*-3,4′,5-trihydroxystilbene)
3	(−)-Epicatechin
4	(+)-Catechin
5	Hydroxytyrosol (3,4-dihydroxyphenylethanol)
6	Syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid)
7	Ferulic acid (4-hydroxy-3-methoxycinnamic acid)
8	Vanillic acid (4-hydroxy-3-methoxybenzoic acid)
9	Caffeic acid (3,4-dihydroxycinnamic acid)
10	Gallic acid (3,4,5-trihydroxybenzoic acid)

sibility to study the specific enzymatic degradation. Analytical data showed different rate removal correlated to structures of the class of phenols present in must. Must polyphenols determined by CZE are listed in Table 1. Electropherograms of must phenolic degradation are shown in Figs. 3 and 4. These figures show the disappearance of wine components designed in Table 1 from 0 to 180 min during the laccase treatment. It is clear from these analyses that in both red (Montepulciano d'Abruzzo, Table 2) and white must (Montonico and Moscato, Table 3) that the phenol degradation was very rapid for catechins, and less for stilbenes (*cis*- and *trans*-resveratrol) and derivatives of cinnamic (ferulic and caffeic) and benzoic (syringic, vanillic and gallic) acids (Tables 2 and 3).

In a similar manner, a mutant laccase from *Polyporus versicolor* eliminated around 70% catechin and 90% of anthocyanidines from model solutions after 3 h of treatment [6] and a decrease of phenolics present in musts and wines was observed after treated with the immobilized laccase [13]. A high removal of (−)-epicatechin, ferulic and *o*-coumaric acids by immobilized laccase in white grape must was found [25].

Preliminary studies with laccase from *T. versicolor* demonstrated that this enzyme has great potential for degradation of phenolic compound in wines. Reductions higher than 90% of ferulic acid in a model solution and 34% of phenolic compounds in wines were obtained. Siringic acid is quickly oxidized to 2,6-dimethoxy-1,4-benzoquinone by laccase from *Azospirillum lipoferum* [26]. The gallic acid was oxidized to quinone by the laccase from *Botrytis cinerea* [27]. Resveratrol was oxidized to

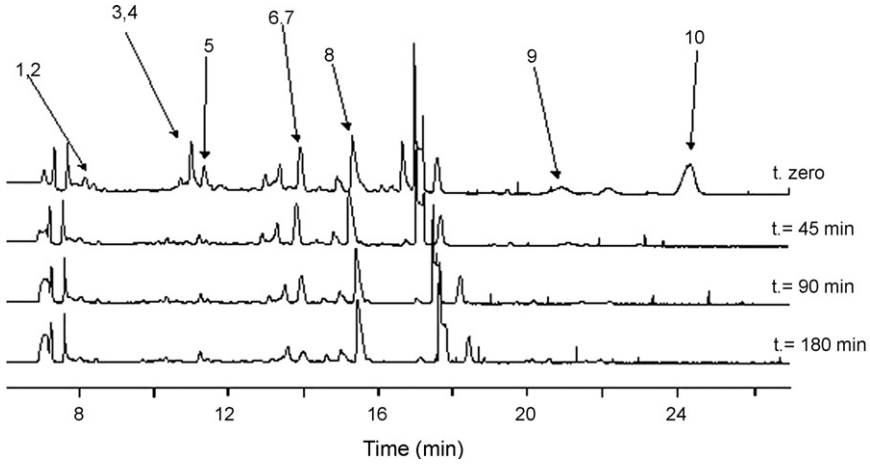

Fig. 3. Electropherograms of liquid–liquid extracted samples of red must (Montepulciano d'Abruzzo) treat with 1 U/L of laccase from *T. versicolor*.

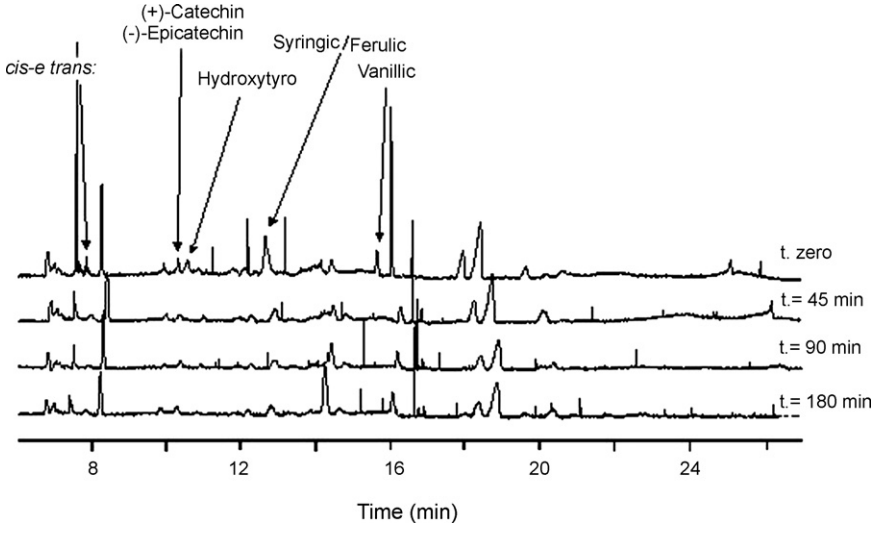

Fig. 4. Electropherograms of liquid–liquid extracted samples of white must (Montonico) treat with 1 U/L of laccase from *T. versicolor*.

Table 2
Percentages of polyphenol reductions by laccase action in Montepulciano d'Abruzzo (red must)

Substances	Laccase (5 U/L)			Laccase (1 U/L)			
	0 min	60 min	180 min	0 min	45 min	90 min	180 min
Cis-resveratrol	0	81	100	0	30	54	57
Trans-resveratrol	0	100	100	0	60	69	79
(+)-Catechin	0	100	100	0	84	100	100
(−)-Epicatechin	0	100	100	0	89	100	100
Hydroxytyrosol	0	61	63	0	57	58	60
Syringic/ferulic acids	0	100	100	0	0	26	69
Vanillic acid	0	25	52	0	7	12	12
Caffeic acid	0	100	100	0	90	100	100
Gallic acid	0	100	100	0	85	100	100

Table 3
Percentages of polyphenol reductions by laccase (1 U/L) action in Montonico and Moscato (white musts)

Substances	Montonico				Moscato			
	0 min	45 min	90 min	180 min	0 min	45 min	90 min	180 min
Cis-resveratrol	0	27	51	100	0	100	100	100
Trans-resveratrol	0	11	32	58	–	–	–	–
(+)-Catechin	0	21	100	100	0	100	100	100
(−)-Epicatechin	0	100	100	100	0	100	100	100
Hydroxytyrosol	0	100	100	100	–	–	–	–
Syringic/ferulic acids	0	60	68	76	–	–	–	–
Vanillic acid	0	47	60	60	–	–	–	–
Caffeic acid	–	–	–	–	0	43	100	100
Gallic acid	–	–	–	–	0	62	100	100

epsilon–viniferin by two isoenzymes of the laccase of *B. cinerea* (p*I* of 4.35 and 4.30) [28]. Vanillic acid reacts slowly with laccase forming dimers. According to Espin and Wichers [29], laccase did not modify the antiradical capacity of resveratrol. The fungal laccase oxidizes the hydroxy-cinnamic acid derivatives in the order: sinapic acid > ferulic acid > coumaric acid [30].

As the use of the laccase as additive in food is still not allowed (JECFA, FAO/WHO Food Additives Systems Dates) it should be applied in wines in the immobilized form [31], facilitating their elimination of the must and with reutilization possibility.

4. Conclusions

Previous and the actual data suggest that the use of laccase in white wines is perfectly feasible. This would allow a more soft and ecologically treatment, which could diminish processing costs and avoid deterioration of wines for long storage times. Other important fact was that the antioxidants were not removed to a great extent. In the case of red wines, the use of the laccases is not indicated at the studied conditions, because the phenols with large antioxidant properties should be preserved. Actually, a study of the action of immobilized laccases and laccase isoenzymes with specific activity on non-antioxidant phenols is in progress.

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