



UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS
DEPARTAMENTO DE CIÊNCIA DE ALIMENTOS

PRODUÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO E APLICAÇÃO INDUSTRIAL DE LACASE FÚNGICA

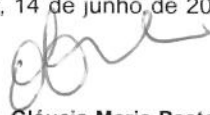
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Este exemplar corresponde à redação final da tese defendida por **Rosana Cristina Minussi**, aprovada pela Comissão Julgadora em 14 de junho de 2002.

Rosana Cristina Minussi
(Engenheira de Alimentos)

Campinas, 14 de junho de 2002

Prof.^a. Dr.^a. Gláucia Maria Pastore
(Orientadora)


Prof.^a. Dra. Gláucia Maria Pastore
Presidente da Banca

Tese apresentada à Faculdade de Engenharia de Alimentos na Universidade Estadual de Campinas como parte dos requisitos para a obtenção do título de Doutor em Ciência de Alimentos.

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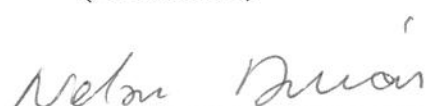
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BANCA EXAMINADORA

Aprovada em ____/____/____



Prof.^a. Dr.^a. Gláucia Maria Pastore
(DCA-FEA-UNICAMP)
(Orientadora)



Prof. Dr. Nelson Eduardo Durán Caballero
(DFQ-IQ-UNICAMP)
(Membro)

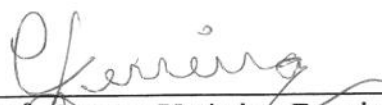


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(DCA-FEA-UNICAMP)
(Membro)

Prof. Dr. Hiroshi Ayoama
(DB-IB-UNICAMP)
(Membro)



Prof.^a. Dr.^a. Elisa Esposito
(NIB-UMC)
(Membro)



Prof.^a. Dr.^a. Carmen Veríssima Ferreira
(DB-IB-UNICAMP)
(Membro)

Prof.^a. Dr.^a. Nilce Correia Meirelles
(DB-IB-UNICAMP)
(Membro)

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RESUMO GERAL

As condições ótimas de cultivo em meio semi-sólido e líquido de *Trametes versicolor*, *Trametes villosa*, *Lentinus edodes* e *Botrytis cinerea* para produção de lacase foram estudadas. A maior atividade de lacase foi obtida em meio líquido com o fungo *T. versicolor* na presença de 2,5-xilidina e cobre como indutores. A atividade de lacase presente no extrato bruto foi então purificada e caracterizada. Foram encontradas duas formas de lacases (L1 e L2) com a mesma massa molecular de 66 kDa. As lacases purificadas apresentaram propriedades catalíticas e físicoquímicas similares as lacases de outros fungos basidiomicetos. A aplicação de lacase de *T. versicolor* em efluentes provenientes da indústria papelreira e da produção de óleo de oliva mostrou o alto potencial biotecnológico desta enzima em presença e ausência de mediadores. A presença do mediador entendeu os substratos oxidados pela enzima.

Fungos foram selecionados com base na sua habilidade em descolorir efluente têxtil e corantes comerciais em meio sólido. Atividade de lacase foi observada em placas descoradas por *Trametes versicolor* e *Trametes villosa* de corante Azul Reativo 19. *Lentinus edodes* mostrou a maior habilidade em termos de extensão e rapidez de descoloração nos diferentes corantes. Os resultados obtidos indicam uma possível relação entre a produção de sideróforos e a descoloração de corantes utilizados na indústria têxtil.

Uma grande correlação entre potencial antioxidante total e teor de fenóis totais em vinhos comerciais foi observada. Ácido gálico foi o mais abundante dos ácidos fenólicos em vinhos tintos, seguido de (+)-catequina e (-)-epicatequina. Estes compostos também foram correlacionados com o potencial total de antioxidantes dos vinhos. A determinação do potencial total de antioxidantes pela descoloração de radicais cátions do ácido 2,2'-azino-bis(3-etilbenztiazoline-6-sulfônico) (ABTS), usando ácido gálico como padrão, mostrou-se eficiente na determinação de características de diferentes vinhos. A utilização de lacase de *Trametes versicolor* na remoção de fenóis em mosto visando estabilização de vinhos indicou que o tratamento com o mosto tinto afeta principalmente os compostos fenólicos responsáveis pelas propriedades antioxidantes. Entretanto, o tratamento de mostos brancos com lacase mostrou maior redução em fenóis totais que no potencial antioxidante. A degradação de fenóis foi mais rápida para catequinas, seguida pelos estilbenos (*cis* e *trans* resveratrol) e derivados de ácidos cinâmicos (ferúlico e cafeico) e benzóicos (siringico, vanílico e gálico).

GENERAL ABSTRACT

The optimum cultivation conditions in semi-solid and liquid medium of *Trametes versicolor*, *Trametes villosa*, *Lentinus edodes* and *Botrytis cinerea* for laccase production were studied. A high laccase activity was obtained in a liquid culture of *T. versicolor* in the presence of 2,5-xylydine and copper as inducers. The laccase activity present in the crude extract was then purified and characterized. Two forms of laccases (L1 and L2) with the same molecular mass of 66 kDa were found. The purified laccases physicochemical and catalytic properties were similar to the analogous enzymes of other basidiomycetes. The application of this laccase in E₁ (pulp and paper) and olive oil wastewaters showed high biotechnology potential in the presence and absence of mediators. The mediator presence extended the oxidized compounds by laccases in these wastewaters.

Four selected fungi were screened for their ability to decolorize a textile effluent and commercial reactive dyes in a solid medium. Laccase activity was observed in Reactive Blue 19 decolorized plates by *Trametes versicolor* and *Trametes villosa*. *Lentinus edodes* presented the greatest decolorization ability both in terms of extent and rapidity of decolorization. The results showed a possible relation between siderophores production and decolorization of textile dyes.

A high relationship between the total antioxidant potential and the phenolic content of commercial wines was found. In red wines, gallic acid was the major of the phenolic acids and (+)-catechin and (-)-epicatechin were the next most abundant phenolics. Also, these compounds are strictly correlated with the total antioxidant potential of wines. Total antioxidant potential by bleaching of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cations, using gallic acid as standard, could be a successful tool for evaluating the characteristics of different wines. Laccase utilization in must aiming wine stabilization indicated that the treatment of a red must with laccase affect mainly the phenolic compounds responsible for the must antioxidant properties. Although, the treatment of white musts with laccase showed a greater reduction in total phenol than in total antioxidant potential. Phenol degradation was very rapid for catechins, and less rapid for stilbenes (*cis* and *trans* resveratrol) and derivatives of cinnamic (ferulic and caffeic) and benzoic (syringic, vanillic, and gallic) acids.

INTRODUÇÃO GERAL

Lacase é uma cuproproteína do pequeno grupo de enzimas chamadas cupro-proteínas azuis, cuprooxidasas azuis ou ainda apenas oxidasas azuis. As lacases geralmente apresentam quatro átomos de cobre circunvizinhos, os quais estão distribuídos entre diferentes sítios de ligação e são classificados em três tipos: cobre tipo 1, 2 e 3 diferenciados por possuírem propriedades características específicas que os permitem desempenhar papel importante no mecanismo catalítico da enzima.

Lacase é uma fenol oxidase (*p* - difenol oxidase, EC 1.10.3.2) que catalisa a oxidação de várias substâncias aromáticas e inorgânicas (particularmente fenóis) com a concomitante redução de oxigênio para água. Compostos fenólicos estão amplamente distribuídos na natureza e a oxidação é importante em processos tais como oxidação celular, proteção da parede celular, escurecimento em frutas, processamento de sucos e vinhos, deslignificação de polpas, descolorimento de tecidos, detoxificação de solos e águas contaminadas, entre outros.

Lacase têm sido muito estudada devido ao seu potencial uso em diversas áreas como nas indústrias têxteis, de alimentos e de papel. O principal objetivo deste trabalho centrou-se no estudo de uma atividade fenoloxidásica, particularmente lacase, produzida por um fungo selecionado.

Os objetivos específicos deste estudo foram:

- Selecionar um fungo com alta atividade de lacase.
- Estudar a produção de lacase em cultura líquida e semi-sólida. Determinar alguns parâmetros físico-químicos que conduzem à maior produção de lacase, tais como utilização de um indutor sintético.
- Purificação e caracterização da fração contendo a atividade fenoloxidásica a partir do extrato bruto do fungo selecionado.
- Avaliar o potencial biotecnológico da enzima semipurificada em presença e ausência de mediador químico, aplicados em processos de descontaminação de efluentes industriais e na remoção de compostos fenólicos em mostos visando estabilização em vinhos na indústria de alimentos.

A presente tese está sendo apresentada na forma de artigos (de revisão e de pesquisa) publicados e submetidos.

ARTIGOS DE REVISÃO

POTENTIAL APPLICATIONS OF LACCASE IN THE FOOD INDUSTRY: A REVIEW

Rosana C. Minussi^{1,2}, Gláucia M. Pastore¹ and Nelson Durán^{2,3*}

Food Biochemistry Laboratory, Faculdade de Engenharia de Alimentos¹ and Biological Chemistry Laboratory, Instituto de Química², Universidade Estadual de Campinas (UNICAMP), C.P. 6154, Campinas - São Paulo, Brazil, CEP 13083-970, and Núcleo de Ciências Ambientais³ - Universidade de Mogi das Cruzes.

Abstract

Laccase is an enzyme that has been widely studied because its potential use in several areas such as textile, and paper and pulp industries. This review presents the potential application of this enzyme in the food industry. Laccase can be used in bioremediation, beverage (wine, fruit juice and beer) processing, ascorbic acid determination, sugar beet pectin gelation, baking, and as biosensors and to improve food sensory parameters. Laccase could increase productivity, efficiency and quality of food products without a costly investment and has the advantage of being a mild technology.

Introduction

The use of enzymes in the food industry is well known (Christen & López-Munguía, 1994; Linko, Javanainen & Linko, 1998; Gibbs, Kermasha, Alli & Mulligan, 1999; Niehaus, Bertoldo, Kahler & Antranikian, 1999; Pandey, Selvakumar, Soccol & Nigam, 1999; Venugopal, Lakshmanan, Doke & Bongirwar, 2000). Enzymes are specific biological catalysts effective in small amounts and that react under mild conditions of temperature and pH. Once they are derived from plants, animals, or microbial sources, enzymes are perceived as natural, non-toxic food components and are preferred over chemicals as food-processing aids by consumers (James & Simpson, 1996). The use of enzymes may lead to upgrading agro-industrial processes, reduction of energy costs associated with processing, improvement in food nutritional quality and safety, new products generation and alternative applications for several agricultural products (Rolle, 1998).

Laccase is a type of copper-containing polyphenol oxidase (*p*-diphenol oxidase, EC 1.10.3.2) that oxidizes polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, but does not oxidize tyrosine (as do the tyrosinases) (Thurston, 1994). Figure 1 shows a typical laccase reaction, where a

phenol is submitted to oxidation by loss of an electron to form a free radical. This active oxygen specie can be transformed into a quinone in a second oxidation stage. The quinone as the free radical product can undergo polymerisation. Laccases can be obtained from bacteria, fungi and plant origins (Gianfreda, Xu & Bollag, 1999). The aim of this review is to present in a summary of the potential areas where laccase is applied and where it could be extended in the food industry.

Potential applications of laccase in the food industry

Bioremediation

Laccase is a well-known enzyme studied in bioremediation because of its ability to degrade phenolic compounds (Gianfreda et al., 1999; Durán & Esposito, 2000). Aromatic compounds, including phenols and aromatic amines, constitute one of the major classes of pollutants and are heavily regulated in many countries (Karam & Nicell, 1997). According to Crecchio, Ruggiero and Pizzigallo (1995), the presence of these compounds in drinking and irrigation water or in cultivated land represent a significant health hazard. These authors reported that laccase immobilized on organogel supports was capable of removing naturally occurring and xenobiotic aromatic compounds from

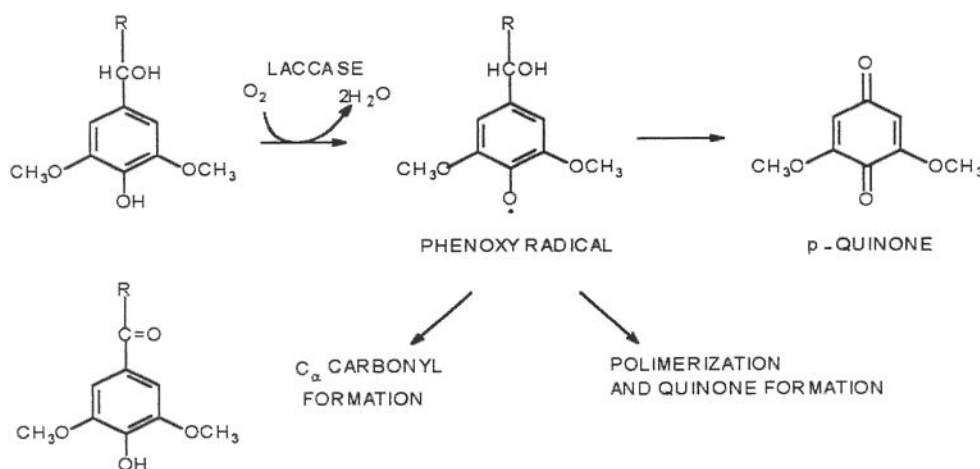


Figure 1. Typical reaction of laccase: phenols oxidation (Higuchi, 1989).

aqueous suspensions. Laccase immobilized by adsorption on polyethersulphone showed chemical and physical properties potentially useful for decreasing phenol concentration in a model wastewater solution (Lante, Crapisi, Krastanov & Spettoli, 2000). Laccase from *Pleurotus ostreatus* immobilized on Eupergit®C was capable of continuously elimination of 2,6-dimethoxyphenol in a packed bed reactor followed by filtration of the precipitate formed. The precipitates, which resulted from oxidative coupling of laccase substrates, were found to be insoluble under the conditions predominating in industrial wastewaters (Hublik & Schinner, 2000). Another studies of laccase application for phenolic compounds removal have been published (Cho, Nam, Choi & Leonowicz, 1998; Schlosser, Grey, Hoefer, Schneegass, Guenther & Fassler, 1999; Kadhim, Graham, Barrat, Evans & Rastall, 1999; Krastanov, 2000; Michizoe, Goto & Furusaki, 2001; Burton, 2001).

Some fractions of beer-factory wastewaters represent an important environmental concern owing to their high content of polyphenols and dark-blown colour. Yague et al. (2000) studied the ability of *Coriopsis gallica*, a white-rot fungus, to degrade this high-tannin-containing wastewater. Pyrolysis/gas chromatography/mass spectrometry

results showed a decrease of polyphenols pyrolysis products, mainly phenol and guaiacol, with the incubation time.

A *Trametes* sp, another white-rot fungus, was tested in bioremediation of a distillery wastewater (Gonzalez, Terron, Yague, Zapico, Galletti & Gonzalez, 2000). This effluent is generated during ethanol production from fermentation of sugar-cane molasses, called vinasses, and produces an important ecological impact due to its high content of soluble organic matter and its intense dark-brown colour. Maximum effluent decolourisation values and chemical oxygen demand reduction attained after seven days of fungal treatment were 73.3 and 61.7%, respectively, when 20% (v/v) of distillery vinasses were added to the culture medium. Under these conditions, a 35-fold increase in laccase production by this fungus was observed.

Olive mill wastewaters (OMW) are a characteristic by-product of olive-oil production and a major environmental problem in the Mediterranean area. Two opposite approaches can be conducted with this effluent: sewage disposal and spreading on soils. However, a drastic reduction in phenol concentration is a prerequisite for both techniques. Greco, Toscano, Cioffi, Gianfreda & Sannino (1999) compared the

utilization of a polyphenol oxidase naturally immobilized on olive husk and purified laccase from *Trametes versicolor* in bioremediation of this kind of effluent. Both enzymatic systems showed relevant activity towards phenol polymerisation. Immobilized laccase from *Lentinus edodes* led to a partial decolourisation of OMW effluent as well as to significant abatements in its content in polyphenols and *o*-diphenols combined with a decreased toxicity of the effluent (D'Annibale, Stazi, Vinciguerra, Di Mattia & Giovanozzi Sermanni, 1999; D'Annibale, Stazi, Vinciguerra & Giovanozzi Sermanni, 2000). Martirani, Giardina, Marzullo and Sannia (1996) reported that the treatment of OMW effluent with purified laccase from *Pleurotus ostreatus* significantly decreased the phenolic content (up to 90%) but no reduction of its toxicity was observed. Other studies of OMW biodegradation have been reported with laccase (Capasso, 1997) and white-rot fungi (Vinciguerra, D'Annibale, Dellemonache & Giovanozzi Sermanni, 1995; D'Annibale, Crestini, Vinciguerra & Giovanozzi Sermanni, 1998; Yesilada, Sik & Sam, 1998; Kahraman & Yesilada, 1999).

Olive oil mill wastewater can also be used in laccase production by fungi (Sanjust, Pompei, Rescigno, Rinaldi & Ballero, 1991; Tomati, Galli, Dilella & Buffone, 1991; Pérez, De La Rubia, Ben Hamman & Martínéz, 1998; Kahraman & Yesilada, 2001). Other agricultural products have been studied in this area (Pandey & Soccol, 2000).

Beverages

Wine stabilization

Wine stabilization is one of the main applications of laccase in the food industry (Minussi, Pastore and Durán, 1999). Musts and wines are complex mixtures of different chemical compounds, such as ethanol, organic acids, salts

and phenolic compounds. While the alcohol and organic acids are responsible for the wine aroma, the colour and the taste depend particularly on the phenolic compounds present in different types of wines (Brenna & Bianchi, 1994). Many groups of phenolic compounds are found in wines; cinnamic acid derivatives and catechins are present in different quantities in all the wines, while rose and red wines are characterized by anthocyanidins. The percentage and the composition of phenolic compounds in wine depend on many factors from the grape used to the processing variations (Macheix, Sapis & Fleuriet, 1991; Cheynier, Hidalgo Arellano, Souquet & Moutounet, 1997). The major colour source in grapes is malvidin, an anthocyanidin monoglucoside (Gibson, 1997).

The sensorial properties of fresh wines should remain constant until consumption; in other words, they should be sufficiently stable at least during the first year of storage. Due to a complex sequence of events, where the polyphenols (derived of the coumaric acid derivatives, flavans and anthocyanidins) play an important role, oxidative reactions in musts and wines catalysed by iron, copper and enzymes, which involve aldehydes, amino acids and proteins, cause turbidity, colour intensification, and aroma and flavour alterations. This oxidation phenomenon has been called madeirization (Zamorani, Spettoli, Lante, Crapisi & Pasini, 1993).

Different methods have been used in order to prevent the decolourisation and flavour alteration in wines, such as the removal of phenolic groups with polyvinylpyrrolidone (PVPP), and the use of sulphur dioxide to block oxidizers, among others.

Polyphenol removal in must should be selective to avoid an undesirable alteration in the wine's organoleptic characteristics. An alternative for the physical-chemical adsorbents could be the use of

enzymes that selectively target specific polyphenols during the madeirization process. The polyphenolic substances would be oxidized by the enzyme, polymerised and then removed by clarification (Zamorani, 1989). One enzyme studied for this purpose is laccase (Cantarelli, 1986).

This treatment is interesting for its specific action and as a "mild technology", with less drastic effects in the characteristics of the wine. Laccase presents some important requirements when used for the treatment of polyphenol elimination in wines, such as optimum pH around 2.5-4.0, stability in acid medium and reversible inhibition with sulphite, among others. Many publications in the literature report studies showing that laccase treatment promotes wine stabilization (Cantarelli & Giovanelli, 1990a; Plank & Zent, 1993; Servili, De Stefano, Piacquadio & Sciancalepore, 2000).

According to Cantarelli (1986), laccase from *Polyporus versicolor* (optimum pH 2.7) eliminated up to 70% catechin and 90% of anthocyanidins of a model solutions in 3 h of treatment. The laccase action on black grape juice showed a removal of 50% of total polyphenols and the analysis by HPLC of the must treated with the active and inactive extract of the enzyme demonstrated the efficacy of this laccase. The method was superior in action specificity and stabilization when compared to the physical-chemical treatment. The use of the laccase in a must resulted in a stable wine and good flavour. The results showed that it is possible to use a laccase from *Polyporus* as a fining agent, followed by addition of silica solution or a thermal treatment. Finally, an ultrafiltration for the removal of the oxidized products and of the enzyme can be applied. Zamorani (1989) compared the use of different enzymatic extracts containing tannase, phenolase, laccase or anthocyanase in wine production from Pinot grapes. The results showed

that laccase was more effective than other enzymes in polyphenol removal, but the author alerted that sulphur treatment, clarification and filtration must be done to remove the oxidized products (Figure 2). The results obtained with Trebbiano wine processed by the above-mentioned sequence (laccase + silica solution) were compared with the data obtained by clarification (caseinate + active carbon + bentonite) in presence and absence of sulphur dioxide. The use of the laccase was shown to be highly effective, preferable or practically identical to those obtained by the traditional processing. The organoleptic analyses confirmed that wines treated with laccase and silica solution or sulphur dioxide showed better organoleptic madeirization-resistant features. Cantarelli, Brenna, Giovanelli and Rossi (1989) also confirmed the potential of laccase in the pre-fermentative treatment when coupled with conventional clarifiers such as proteins and PVPP. Maier, Dietrich and Wucherpfenning (1990) evaluated the polyphenol content, colour, haze stability and sensorial quality of Riesling wines prepared with and without oxidation of the must, or with must oxidation and laccase treatment. The results showed that wines made by laccase treatment were the best, suggesting that a stable and high quality wine can be made with little or no added SO₂. Cantarelli and Giovanelli (1991) used laccase (5-20 units/mL to the must), which was then filtered (with and without PVPP) or clarified with bentonite or gelatin + silica solution, or sulphited. The results demonstrated that the enzymatic treatment offers a promising avenue for future research.

Preliminary studies with laccase of *Trametes versicolor* demonstrated that this enzyme has great potential for phenolic compound removal from wines. More than 90% of ferulic acid was removed from a model solution (Minussi, Pereira, Pastore &

Durán, 1998) and 34% removal of phenolic compounds from wines was obtained (Minussi, Pastore and Duran, 1998).

Since the use of laccase as a food additive is still not allowed (JECFA, FAO/WHO Food Additives Systems Dates), this enzyme has been used in wines production in an immobilized form to insure their elimination from the must and for reutilization (Brenna et al., 1994; Zamorani et al., 1993; Lante, Crapisi, Pasini, Zamorani & Spettoli, 1992, 1996; De Stefano, Piacquadio & Sciancalepore, 1996). Laccases from *Pyricularia oryzae* and *Botrytis cinerea* were immobilized by physical and chemical methods on organic and inorganic supports and tested for phenolic removal in model solution, must and wine. Laccase, adsorbed on molecular sieves and silica gel, and immobilized by adsorption and covalent binding, gave immobilization yields of 90%, while the best activity yields varied from 22 to 38%. Laccase immobilized on silica gel and glutaraldehyde resulted in a decrease of the catechin percentage in the model solution, must and wine, and was reusable for up to 5 times (Zamorani et al., 1993). Brenna and Bianchi (1994) studied laccase immobilization on an activated matrix based on agarose and they tested its efficacy with a model solution of catechin and in must produced from Riesling grapes. The immobilized enzyme was compacted in chromatographic columns of several sizes, and equilibrated with air, N₂ and O₂. The solutions were collected using different flow rates in the reactor. The diluted samples were treated with PVPP. The oxidation of the phenolics varied mainly with the flow in the reactor. The increase of the colour with the decrease in total polyphenols was maximal at low flow rates in the reactor (samples saturated with O₂). The immobilized

laccase of *Trametes versicolor* was stable and could be reused at least 8 times, with a minimum loss of activity after a period of 6 months when conserved under controlled conditions. Activity yields of 51 to 63% with immobilized laccase by covalent binding in polysaccharides supports were obtained by Lante et al. (1996). In this case, phenolic content in must and wine decreased when treated with the immobilized laccase. De Stefano et al. (1996) reported that Sepharose 4B-Epi-IDA-Cu²⁺ was successfully used for the immobilization of laccase and the removal of phenolic compounds. The results obtained suggest the potential of this immobilized enzyme for continuous enzymatic catalysis in food processing.

In recent years, the moderate consumption of red wines has been shown to benefit human health (Fremont, 2000). Investigators have certified the antioxidant properties of polyphenols that are present in red wines; however, the specific role of individual compounds remains elusive (Brouillard, George & Fougereuse, 1997). In view of this, it is necessary to analyse the possible interactions of the laccase with these components that are so important in wine and which in principle, are the substrates for the enzyme. According to Minussi, Rossi, Bologna, Rotilio, Pastore and Durán (2002), the treatment of a red must with laccase from *Trametes versicolor* mainly affects the phenolic compounds responsible for the must antioxidant properties. However, the treatment of white musts with the same laccase showed higher reduction in total phenol than in total antioxidant potential, so the treatment of white musts is perfectly feasible. An interesting approach for the selectivity of laccase also could be done, by choosing the laccase isoenzymes that least affect the important phenolic components of the red wine with antioxidant

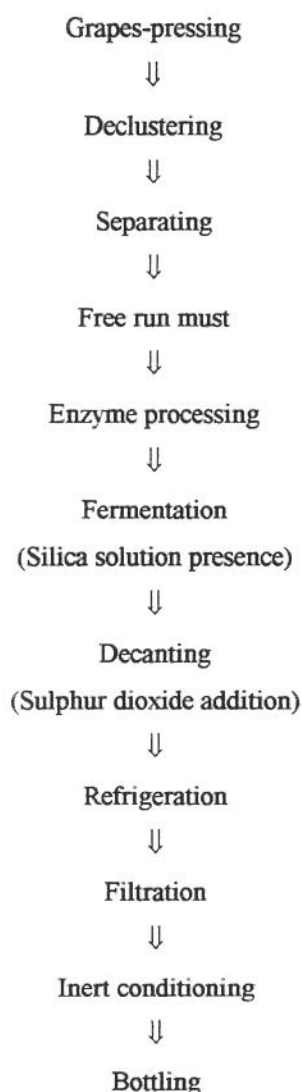


Figure 2. Process diagram of wine production with laccase treatment (Zamorani, 1989).

properties. The transformation of these compounds by laccase 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.

Laccase as indicator of Botrytis cinerea infection in must

Laccase is an enzyme secreted by *Botrytis cinerea* that causes various forms of rot in grapes (Macheix et al., 1991). This enzyme is present only in musts and wines made with grapes infected by the fungus and is not present in musts and wines

made from sound grapes. Several methods have been developed to determine laccase activity in must as an indicator of *Botrytis cinerea* infection in grapes (Fregoni & Iacono, 1987a,b; Zouari, Romette & Thomas, 1987; Zouari, Romette & Thomas, 1988; Grassin & Dubourdieu, 1989; Cameira-dos-Santos, Silvestre, Godinho & Curvelo-Garcia, 1992; Angele & Degre, 1993; Silvestre, Cameira-dos-Santos & Curvelo-Garcia, 1996).

Although the presence of laccase can be considered as the sign of grapes contamination by *Botrytis cinerea*, there is a controversy about laccase determination and degree of *Botrytis*

cinerea infection. According to Macheix et al. (1991) and Perino, Vercesi and Fregoni (1994), it is not reliable to measure the proportion of contamination by using the laccase activity of the corresponded must because the increase in laccase activity is not always proportional to an increase of *Botrytis*-infected berries. These variations in laccase activity, which involve the cultivars, may be caused by *Botrytis* itself, which is a very variable fungus. Redl and Kobler (1992) reported that determination of laccase activity in vines or plots were not as close as that previously observed for single fruit. Yaropolov, Ghidilis, Gaina, Nedov, Zamaru and David (1988) showed that there was a direct linear correlation between laccase activity in must and the intensity of infection of grapes. A positive correlation was also found between the total phenol concentration of must and the extent of grey rot in grapes. Another reports demonstrated correlation between the laccase activity in the must and the percentage of berries infected by *Botrytis cinerea* (Roudet, Prudet & Dubos, 1992; Meneguzzo, Rizzon, Miele & Ayub, 1999).

Fruit juice processing

Naturally occurring phenolics and their oxidation products are important constituents of natural beverages, contributing to colour and taste. The use of enzymes, in particular those involved in the breakdown of carbohydrates and proteins, has long been established in the extraction and stabilization of juices. For apple and grape juices, excessive oxidation of phenolics has almost always been considered detrimental to the organoleptic quality of the product. Thus, a measure of the phenolic content of a natural beverage is necessary both for an adequate organoleptic description and as an indicator of stability on storage. Cliffe, Fawer, Maier, Takata and Ritter (1994) developed two

enzymes assays for determination of phenols in beverages by *Trametes versicolor* laccase. Using apple juices, a linear correlation was observable between these enzyme assays and the Folin-Ciocalteu wet chemical assay for total phenol content. These enzyme assays may prove helpful to predict the stability of natural beverages.

Hazes or sediments in beer, wine, and clear fruit juices result from a number of different causes, but the most frequent is protein-polyphenol interaction. These beverages are typically stabilized to delay the onset of protein-polyphenol haze formation (Siebert, 1999). Prevention of physical-chemical deterioration of fruit juices is a major problem related to the evolution of conditioning and distribution systems and to the rising awareness of the consumer for product's intrinsic quality. It is well known that browning, both enzymatic and chemical, is one of the major faults in beverages (Giovanelli and Ravasini, 1993). Various pre- and post treatments are available to avoid post-turbidity and discoloration of fruit juices. Stabilization of beverages by gelatin, bentonite, silica gel and PVPP are widespread conventional treatments (Gökmen, Borneman & Nijhuis, 1998). Various enzymatic treatments have been proposed for fruit juice stabilization, among them the use of laccase (Cantarelli, Brenna, Pavesi & Rossi, 1986; Dietrich, Wucherpfenning & Maier, 1990; Maier & Dietrich, 1990; Maier, Mayer, Dietrich & Wucherpfenning, 1990; Goessinger & Vogl, 1996a,b; Piacquadio, De Stefano, Sammartino & Sciancalepore, 1997). The results of laccase apple juice treatments reported are contradictory. Giovanelli et al. (1993) and Gökmen et al. (1998) reported that the stability tests of ultrafiltrated samples showed that laccase treatment increased the susceptibility of browning during storage. Golden Delicious apple juice was treated with conventional method (SO₂ added as

metabisulfite, PVPP, bentonite) and laccase free or immobilized on metal chelate regenerable carrier. The enzymatic treated apple juice was less stable than the conventionally treated juice (Sammartino, Piacquadio, De Stefano & Sciancalepore, 1998). However, Maier, Mayer and Dietrich (1990) and Ritter, Maier, Schoepplein and Dietrich (1992) showed that the application of laccase in conjunction with cross-flow-filtration (ultrafiltration) in a continuous process without the addition of fining agents lead to a stable and clear apple juice. Stutz (1993) proved that it is possible to produce clear and stable juices/concentrates with a light colour by means of ultrafiltration and the use of laccase, without any large additional investment. Ritter and Dietrich (1996) and Piacquadio, De Stefano, Sammartino and Sciancalepore (1998) results also show that the use of laccase improves stability in apple juice. Cantarelli and Giovanelli (1990b) tested laccase oxidation followed by different alternative operations, such as pasteurisation, conventional fining (gelatin and silicates), filtration with "active" filter aid and ultrafiltration. Treatment with laccase acting at a compatible pH followed by "active" filtration or ultrafiltration improved colour and flavour stability relative to conventional treatments by addition of ascorbic acid and sulphites.

Beer stabilization

The tendency for hazes to develop in some beers during long-term storage, even in products that are haze-free at time of packaging, is a persistent problem in the brewing industry (MacMurrough, Madigan, Kelly & O'Rourke, 1999). Storage life of a beer depends on many factors, e.g. temperature, haze forming potential and oxygen content. Classical haze formation in beers is a result of protein precipitation that is usually

stimulated by small quantities of naturally occurring proanthocyanidins polyphenols (Mathiasen, 1995). This type of complex is frequently manifested as chill-haze, which appears on cooling but which may re-dissolve at room temperature or above. This is generally ascribed to hydrogen bonding or to hydrophobic interactions with proline residues. At a later stage, nucleophilic substitution of phenolic rings by protein sulphhydryl groups may lead to a permanent haze that does not re-dissolve when warmed. The excess polyphenols are traditionally removed by treatment with insoluble PVPP, which is difficult to handle without creating an unhealthy environmental for the workers and is also a problem in the wastewater due to its low biodegradability. As an alternative to the traditional treatment, a polyphenol oxidase (e.g. laccase) could be added to the wort. According to Mathiasen (1995), laccase could be added at the end of the process, because oxygen is unwanted in the finished beer, so addition of laccase may remove any excess oxygen whereby storage life of beer is enhanced, and at the same time the laccase will remove some of the polyphenols that may still remain in the beer. The polyphenol complexes formed by laccase may be removed by filtration or other means of separating. In addition to laccase a reduced amount of PVPP may also be added to the fermented beer. Rossi, Giovanelli, Cantarelli and Brenna (1988) and Giovanelli (1989) also confirmed the potential of laccase utilization in beer stabilization.

Ascorbic acid determination

The total ascorbic acid determination has gained increased significance in several areas of analytical chemistry such as in pharmaceutical, clinical and food applications. A flow-injection enzymatic kinetic spectrofluorimetric procedure for determining total ascorbic acid in solution by

combining the merging zones principle with the stopped-flow technique is described by Huang, Cai, Du and Zeng (1995). This study indicates that this procedure is simple, fast and economic and that the enzyme laccase is suitable for the selective determination of total ascorbic acid in a range of samples (wine, beer, urine and pharmaceutical preparations). The spectrofluorimetric determination of L-ascorbic acid has been enhanced by micelle (Huang et al., 1997) and organic media (Huang, Huang, Cai & Zeng, 1997) and could be applied in pharmaceuticals and milk powder. These methods are based on the inhibition of L-ascorbic acid on the formation of 2,3-diaminophenazine, which is an oxidation product of *o*-phenylenediamine catalysed by laccase.

Improvement of food sensory parameters

According to Petersen, Mathiasen, Peelen and Andersen (1996), oil or product comprising oil may be deoxygenated by adding an effective amount of laccase. Many food items as salad dressings, e.g., French dressings and mayonnaise are prepared with vegetable oils, e.g., soybean oil. Soybean oil contains a large amount of linoleic and linolenic acids that readily react with dissolved oxygen in the product whereby undesirable volatile compounds are produced. Laccase is characterized by being a group of multi-copper proteins of low specificity acting on both *o*- and *p*-quinones, whereby oxygen is reduced to water. Therefore, the flavour quality of oils could be improved by eliminating dissolved oxygen in the oils. Petersen and Mathiasen (1996) also showed that laccase could deoxygenate food items derived partly or wholly from extracts of plant materials. The food item may be a juice, a soup or a concentrate (in the form of a pulp, a puree, a catsup or a paste). Useful extracts may also

be in the form of hydrolysates, e.g., wheat gluten and maize gluten (corn gluten).

Cacao nib was soaked in solutions containing crude laccase of *Coriolus versicolor*, dried and roasted in order to improve the flavour and taste of cacao nib and its products (Takemori, Ito, Ito & Yoshima, 1992). Bitterness and other unpleasant tastes are removed by the treatment and chocolate manufactured from the cacao mass tasted well than the control.

Off-flavours reduction was related in the literature. Addition of *Myceliophthora thermophilum* laccase to soy protein hydrolysates improved flavour notes without affecting the level of hydrolysis (anonymous, 1999). Tsuchiya, Petersen and Christensen (2000) showed that cysteine treated with recombinant *Myceliophthora thermophilum* laccase and chlorogenic acid gave only very weak odour (malodour), while non-treated cysteine gave a strong characteristic odour of H₂S. More than 50% reduction of cysteine was confirmed with an HPLC analysis.

Laccase from *Trametes villosa* was added to chopped olives in olive-water mixture. Bitterness was considerably reduced as compared with controls and the test samples were also darker than the controls (Novo Nordisk A/S, 1995). According to Bouwens, Trivedi, Van Vliet and Winkel (1997, 1999), treatment with a fungal laccase from a *Pleurotus* species can be performed to enhance the colour of a tea-based product.

Sugar beet pectin gelation

There is a considerable interest in the food industry for finding new functional ingredients. Sugar beet pectin is a food ingredient with specific functional properties. It may form gels by an oxidative cross-linking of ferulic acid (Norsker, Jensen & Adler-Nissen, 2000). Micard & Thibault

(1999) showed that is possible to cross-link the beet pectin through the oxidative coupling of the feruloyl groups to a new gelation process using laccase. The degree of cross-linking, which can be modulated by the amount of pectin added and the reaction time, was found to be very critical for the hydration properties of the powders. Beet pulp has for a long time been investigate as an alternative source of pectins, to apple pomace or citrus peels. Peroxidase or laccase can perform the oxidative gelation. Peroxidase requires addition of hydrogen peroxide whereas laccase is capable of using oxygen present in the sample (Norsker et al., 2000). The obtained gel is thermo-irreversible. This makes it very interesting for the food industry as the product can be heated while maintaining a gel structure. The enzymatic gelation was studied in three food products with added sugar beet pectin. In luncheon meat it is a cohesive gel which binds the meat pieces together thereby making the products sliceable. This observation could open up news ways of manufacturing of restructured meat products in the food or pet industries. In black currant juice and milk some unwanted side effects were observed. The enzymes do not only catalyse the cross-linking but they oxidise anthocyanins in black currant juice and in milk lipids are oxidised resulting in off-flavour. In luncheon meat no unwanted side effects were found. It was also found that the laccases were more efficient in luncheon meat and milk products than peroxidase.

Baking

In the bread-making process it is known to add bread and/or dough-improvement additives to the bread dough, the action of which results in improved texture, volume, flavour and freshness of the bread as well as improved machinability of the dough. Enzymes have been used as dough and/or

bread improving agents, in particular enzymes that act on components present in large amounts in the dough. Examples of such enzymes are found within the groups of amylases, proteases and cellulases, including pentosanases. According to Si (1994), when a laccase enzyme is added to dough used for producing baked products, it may exert an oxidizing effect on the dough constituents and thereby serve to improve the strength of gluten structures in dough and/or baked products. In particular, the use of laccase results in an increased volume, an improved crumb structure and softness of the baked product, as well as increased strength, stability and reduced stickiness and thereby improved machinability of the dough. The effect on the dough has been found to be particularly good when poor quality flour has been used. The improved machinability is of particular importance for doughs that are processed industrially.

The viscoelastic properties of wheat flour dough depend primarily on the dough's protein constituents and prolamins are recognized as the most important functional proteins. The glutenins provide elasticity, whereas gliadin provides viscosity and extensibility in a dough system. Arabinoxylans, despite their low content in wheat flour, are also important in determining dough-handling properties and bread quality (Labat, Morel & Rouau, 2000). Previous reports showed that laccase catalyses gelation by dimerization of feruloylated esters in feruloylated arabinoxylans (Figuerola-Espinoza & Rouau, 1998; Figuerola-Espinoza, Morel & Rouau, 1998; Figuerola-Espinoza et al., 1999; Figuerola-Espinoza & Rouau, 1999). Labat, Morel and Rouau (2000) reported the effects of laccase and ferulic acid on wheat flour dough. Laccase accelerated dough formation and dough breakdown and the effects on mixing properties were enhanced with added ferulic acid.

Laccase used alone decreased arabinoxylans water extractability. When used in combination with added ferulic acid, laccase increased the oxidation of sulphhydryl groups and the rate of protein depolymerization during mixing. Wheat gluten and water extractable pentosans were mixed in a batch-mixer in the presence of laccase or manganese peroxidase (Labat, Morel & Rouau, 2001). The oxidative enzymes increased dough consistency, accelerated the loss in total ferulic acid, decreased arabinoxylans water extractability and water extractable arabinoxylans apparent intrinsic viscosity in the dough water extracts. The effects of laccase and manganese peroxidase were similar on feruloylated arabinoxylans when compared to a reaction time basis. No covalent complex between arabinoxylans and protein was detected in mixed and oxidised gluten/water extractable pentosans. A recent review showed several examples on the trends in the development of food enzymes and on food enzyme applications, and the recent advances for the baking industry exemplified by laccase application (Heldt-Hansen, 1997).

Biosensors

A biosensor is a device that detects, transmits and records information regarding a physiological or biochemical change. Technically, it is a probe that integrates a biological component with an electronic transducer thereby converting a biochemical signal into a quantifiable electrical response. The function of a biosensor depends on the biochemical specificity of the biologically active material. Enzymes, antibodies, DNA, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, real-

time analysis and operation simplicity (D'Souza, 2001).

A number of biosensors containing laccase have been developed for immunoassays (Scheller, Wollenberger & Makower, 1994; Ghindilis, Makower & Scheller, 1995; Bauer et al., 1999; Huang, Warsinke, Koroljova-Skorobogat'ko, Makower, Kuwana & Scheller, 1999; Ghindilis, 2000), glucose determination (Scheller et al., 1986; Wollenberger, Scheller, Pfeiffer, Bogdanovskaya, Tarasevich & Hanke, 1986), aromatic amines (Simkus & Laurinavicius, 1995) and phenolic compound determinations (Ghindilis, Gabrilova & Yaropolov, 1992; Wang, Lin, Eremenko, Ghindilis and Kurochkin, 1993; Papkovsky, Ghindilis & Kurochkin, 1993; Guo, Hu, Wang & Zhou, 1994; Zouari, Romette & Thomas, 1994; Yaropolov, Kharybin, Emnéus, Marko-Varga & Gorton, 1995; Simkus et al., 1996; Freire, Durán & Kubota, 2001; Freire, Durán, Wang & Kubota, 2002).

Others

Johansen (1996) reported a bactericidal, bacteriostatic, fungicidal and/or fungistatic composition comprising a basic protein or peptide capable of killing microbial cells in combination with a cell-wall degrading enzyme or an oxidoreductase, like laccase. Such compositions have a pH in the alkaline range and it has been found that basic proteins such as protamine and protamine sulphate exhibit their optimum antimicrobial effect at alkaline pH, thus making such proteins very suitable for incorporation in compositions for cleaning purposes. The composition is useful as antimicrobial ingredient wherever such an ingredient is needed, for example for the preservation of food, beverages, cosmetics, contact lens products, food ingredients or enzyme

Table 1. Some prices of commercially available laccases.

	Quantity (Units)	Price
1) Jülich Fine Chemicals		
(www.juelich-chemicals.com)		
From <i>Agaricus bisporus</i>	10 KU	305.00 (US\$)
	100 KU	1,560.00 (US\$)
From <i>Coriolus versicolor</i>	10 KU	250.00 (US\$)
	100 KU	1,290.00 (US\$)
2) Tienzyme™		
(www.tienzyme.com)		
From <i>Pleurotus ostreatus</i>	10,000 (concentrate)	150.00 (US\$)
	10,000 (purified)	400.00 (US\$)
	100,000 (concentrate)	650.00 (US\$)
	100,000 (purified)	1,600.00 (US\$)
3) Sigma-Aldrich		
(www.sigma-aldrich.com)		
From <i>Rhus vernificera</i>	10,000	72.30 (US\$)
From <i>Agaricus bisporus</i> (≥ 1.5 U/mg)	1 g	30.50 (US\$)
	5 g	120.90 (US\$)
From <i>Coriolus versicolor</i> (≥ 1 U/mg)	1 g	44.00 (US\$)
	10 g	358.20 (US\$)
4) Jena BioScience		
(www.jenabioscience.com)		
From <i>Trametes versicolor</i> , <i>Coprinus</i>	100 U	15.00 (EUR)
<i>cinereus</i> , and <i>Pycnoporus cinnabarinus</i>	1000 U	75.00 (EUR)

* The methodology and expression of laccase activity (Units) are different among the companies.

composition; as a disinfectant for use on human or animal skin, mucous membranes, wounds, bruises or in the eye, for killing microbial cells in laundry and for incorporation into cleaning compositions for hard surface cleaning.

The treatment of canola meal with an enzyme by *Trametes versicolor* (a polyphenoloxidase) reduced around 90% of phenolics content in 3 h of processing (Lacki & Duvnjak, 1996). Canola meal is used as a supplementary source of nutrient in livestock and poultry diets. In addition, if the concentration of phenolics in canola meal can be

reduced, then this kind of meal and/or canola proteins could fully replace the more expensive soybean products.

Conclusions

This review has shown that laccase is a promising enzyme with a great potential application in several areas of food industry. The use of this enzyme could improve productivity, efficiency and quality of food products without high investment costs and has the advantage of being a mild technology. Studies of laccase production,

purification and immobilization techniques at lower costs (Table 1) are also needed to improve the industrial application of this enzyme. Recombinant DNA methods are a powerful tool in this area, offering new possibilities of raw materials and microorganisms' improvement for use in the food industry. Also laccase with improved characteristic could be produced, which has ability to act under drastic conditions of pH and temperature.

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PRODUÇÃO DE LACASE E SUAS APLICAÇÕES AMBIENTAIS NA PRESENÇA DE MEDIADORES (LACCASE PRODUCTION AND ITS INDUSTRIAL APPLICATIONS IN THE PRESENCE OF MEDIATORS)

N. Durán¹, R.C. Minussi², G.M. Pastore², O.L. Alves³, I.F. Gimenes³, P. Peralta-Zamora⁴, S.G. de Moraes¹

Lab. Quím. Biol.¹ e Lab. Novos Materiais³, Inst. Quím.; Lab. Bioq. Alim.², Fac. Eng. Alimentos, Universidade Estadual de Campinas, C.P. 6154, Campinas, CEP 13083-870, S.P., Brasil. E-Mail: duran@iqm.unicamp.br, e Lab. Quím. Aplic.⁴, UFPR, Brasil.

ABSTRACT

Laccases are phenoloxidases produced by fungi and found in plants, belonging to the oxidase group which complex copper. Laccases catalyzes oxidations of phenolic or non-phenolic subunits of lignin in the presence of mediators as ABTS or syringaldazine in a similar way as peroxidases. 1-Hydroxybenzotriazole (HBT) was recently suggested to degrade lignin models. The state of art of laccase-mediator system is discussed. New results on the action of fungi as *Trametes* and *Lentinula* in the presence of mediators on the pulp and paper effluents are described. The interaction of fungi/ABTS or HBT and laccase/ABTS or HBT showed the importance of the fungi/enzyme immobilization in adequate supports. In this study the use of glass-ceramic and resins in order to immobilize laccase or fungi are discussed. These systems were found as extremely promising in the industrial applications.

Aspectos Bioquímicos e Microbiológicos

Lacases são produzidas principalmente por basidiomicetos de degradação branca, mas são também detectadas em fungos de degradação parda e em fungos de degradação mole (Durán e Esposito, 1997). As massas molares relativas estão em torno de 60-100 kDa. As lacases catalisam oxidação por extração de um elétron de um substrato fenólico gerando um radical fenoxila (Higuchi, 1990):

Os radicais formados atuam em reações não catalíticas como acoplamento de radicais, desprotonação e ataque nucleofílico por água. Logo, estas reações múltiplas produzem: a) polimerização, b) quebras alquil-aryl, c) oxidação Cα e desmetilação (Katase & Bollag, 1991).

Produção de Lacases

Foi publicado recentemente (Durán and Esposito, 1997) uma relação com os produtores mais importantes de lacases selecionados de madeiras degradadas (Tabela 1).

Sítio Ativo da Lacase

A lacase, como uma oxidase multicobre (p-difenil:dioxigênio oxidoreductase, EC 1.10.3.2), catalisa uma redução de quatro elétrons de dioxigênio à água. Lacase contém quatro átomos de Cu os quais tem sido classificados de acordo com seu aspecto no EPR: Tipo 1 ou azul, tipo 2 ou normal e tipo 3 ou sítio de acoplamento de cobre binuclear onde os cobres estão antiferromagneticamente acoplados por meio de uma ponte de ligação (não detectado por EPR) (Sundaran et al. 1997). A espectroscopia combinada com cristalografia, dicroísmo circular magnético e raios-X tem dado uma descrição detalhada do sítio ativo da lacase (Cole et al. 1990; Sundaran et al. 1997). Os modelos de ponte entre o tipo 2 e tipo 3 (Fig. 1A,B) (Sundaran et al. 1997) explicam a redução catalítica de O₂ a H₂O. O cobre tipo 2 é requerido para a redução de O₂ uma vez que a ponte a este centro está envolvida na estabilização do intermediário peróxido. A estrutura geométrica da ponte entre os sítios tipo 2

- a) $2 \text{ (Cu[II])}-\text{Lacase} + \text{fenol} \longrightarrow 2 \text{ (Cu[I])}-\text{Lacase} + \text{radical fenolato} + 2\text{H}^+$
 b) $2 \text{ (Cu[I])}-\text{Lacase} + \text{O}_2 + 2\text{H}^+ \longrightarrow 2 \text{ (Cu[II])}-\text{Lacase} + \text{H}_2\text{O}$

e tipo 3 no sítio trinuclear da oxidase multicobre é diferente ao modelo previamente sugerido (Magnus et al. 1993).

A redução de O_2 pela lacase ocorre em passos de $2e^-$. O primeiro é um passo que é controlado pela velocidade da reação. Este modo de ponte do tipo 2/tipo 3 para os $2e^-$ reduzidos, os níveis do intermediário peróxido poderiam facilitar a redução dos segundos $2e^-$ (de centros de tipo 2 e tipo 1) em que o peróxido é diretamente coordenado para reduzir o cobre tipo 2, e o tipo 1 reduzido é acoplado ao tipo 3 por ligação covalente de Cys-His.

Estudos prévios (Cole et al. 1990) relataram que 40% do tipo 1 e tipo 3 rapidamente reagem com dioxigênio correspondente a lacase nativa (Fig. 2). É claro que o cobre tipo 2 é para a reatividade do dioxigênio na lacase e que a redução do dioxigênio ocorra na ausência do cobre tipo 1. Isto demonstra que o tipo 2-tipo 3 do sítio de cobre trinuclear

representa o sítio ativo para a ligação e redução multieletrôn do dioxigênio. O Cu tipo 1 é claramente não necessário para a reatividade com o dioxigênio, e em sua ausência, um intermediário é formado que compartilha algumas das propriedades com o intermediário de oxigênio previamente descrito na lacase nativa.

Lacase e mediadores

Lacase de *T. versicolor* é capaz de oxidar modelos de lignina não fenólicos na presença de 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS) ou siringaldazina em forma similar a lignina peroxidase (LiP) (Bourbonnais e col. 1995). Estudos em branqueamento de polpas têm sido feitos (Archibald et al. 1997).

Na lignina somente subunidades fenólicas são atacadas por lacase, gerando radicais centrados no oxigênio que conseqüentemente polimerizam ou despolimerizam (Archibald et al. 1997). Como

Tabela 1. Produtores de Lacase no Período 1996-1997.

MICROORGANISMOS	REFERÊNCIA
<i>Trametes villosa</i>	(Soares, 1997; Soares et al. 1997)
<i>Cerrena unicolor</i>	(Luterek et al. 1997)
<i>Coriolus versicolor</i>	(Aso and Oda, 1996)
<i>Pycnoporus coccineus</i>	(Aso and Oda, 1996)
<i>Phlebia radiata</i>	(Gayazov e Rodakiewicz, 1996)
<i>Pycnoporus cinnabarinus</i>	(Eggert et al. 1996)
<i>Phanerochaete chrysosporium</i>	(Rodriguez et al. 1997; Dittmer et al. 1997)
<i>Botrytis cinerea</i>	(Fortina et al. 1996; Xu et al. 1997)
<i>Botryosphaeria sp.</i>	(Barbosa et al. 1996)
<i>Pleurotus sajor-caju</i>	(Fu et al. 1997)
<i>Trametes Hal</i>	(Shimizu e Nakaya, 1997)
<i>Pleurotus ostreatus</i>	(Homolka et al. 1997)
<i>Myceliophthora thermophila</i>	(Berka et al. 1997)
<i>Polyporus pinsitus</i>	(Yaber et al. 1997)
<i>Ganoderma lucidum</i>	(Peruma e Kalaichelvan, 1996)

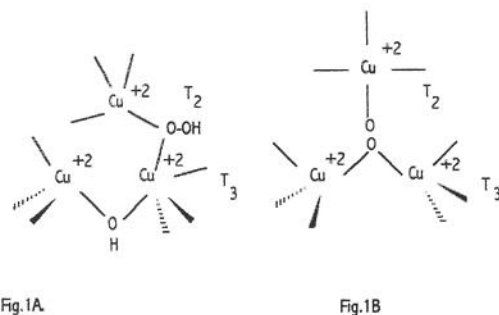


Fig. 1. Dois possíveis modelos espectroscópicos efetivos para a ponte peróxido e o sítio “cluster” trinuclear: (A) ponte entre tipo 2 e um de cobre tipo 3 no modo μ -1,1-hidroperóxido; (B) ponte entre os três cobres no modo μ_3 , $(\eta^1)_3$ (Sundaran et al. 1997).

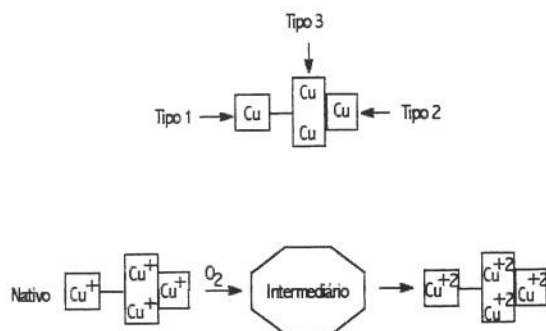


Fig. 2. Reatividade de derivados da lacase com oxigênio (Cole et al. 1990).

descrito anteriormente, lacase pode estender sua reatividade a não fenólicos por inclusão de mediadores como ABTS (Bourbonnais and Paice, 1996).

Logo, álcoois são oxidados a aldeídos e modelos diméricos não fenólicos β -1 e β -O-4 são quebrados ou oxidados na posição C α pela dupla lacase-ABTS (Archibald et al. 1997). Recentemente outro mediador foi sugerido, 1-hidroxibenzotriazol (HBT) que permite 50% de deslignificação em polpas na presença de lacase (Call and Mucke, 1995). Neste caso a oxidação do substrato por um

elétron está acoplada a uma redução do dioxigênio. O HBT ao consumir oxigênio transforma-se num intermediário reativo que é oxidado pela enzima (Call and Mucke, 1997).

Os produtos caracterizados após a oxidação com HBT foram 2H-benzotriazol, 1H-benzotriazol e 1-hidroxibenzotriazol (Chen et al. 1997).

A deslignificação de polpas kraft usando lacase catalisada por oxigênio oferece algum potencial na substituição de branqueamentos convencionais e tem a vantagem de exigir menos pressão e temperatura. Mediadores como ABTS e HBT são

os mais eficientes. Entretanto outros mediadores foram estudados: ácido 1-nitroso-2-naftol-3,6-disulfônico, ácido 4-hidroxi-3-nitroso-1-naftalensulfônico, promazine, clorpromazine e Remazol Brilliant Blue (Bourbonnais et al. 1997). Oxidação de corantes pela lacase é grandemente aumentada por Naftol verde B, 1-nitroso-2-naftol ou sal de nitroso-R em comparação aos mediadores conhecidos como o HBT (Zing et al. 1997). Estudos com compostos diméricos modelos indicaram que os mecanismos de oxidação por ABTS e HBT são diferentes.

Estudos eletroquímicos e cinéticos de HBT mostraram que a conversão do HBT era irreversível e que devido a isto não seria prático o uso deste mediador nos processos catalisados por lacase (Krikstopaitis et al. 1996).

Aplicação do sistema lacase-mediador

Lacase com acetosiringone, alquilsiringatos, 10-fenotiazinas-substituídos e 10-fenoxazines substituídos como mediadores para preparações alimentares e coloração de cabelos foram usados (Henriksen e Likke, 1997). Descoloração enzimática de corantes usando lacase microbiana e mediador (Ishihara et al. 1997), aplicação na lavagem de roupa tingida de algodão na presença de mediadores e co-mediadores (Call, 1997ab; IBV Ind. Biover. 1996), em composição de detergentes industriais na presença de lacase e siringatos e fenotiazinas (Herbots et al. 1997), degradação de antraceno na presença de ABTS e HBT (Johannes et al. 1996), oxidação de fenantreno por lacase na presença de HBT e de lipídeos insaturados (Bohmer et al. 1998), branqueamentos com lacase e ácido fenotiazine-10-propiónico (Damhus et al. 1997), oxidação de iodeto por lacase e ABTS (Xu,

1996), são algumas das novas aplicações do sistema lacase-mediador.

Em termos de polpas ou de degradação de ligninas, várias novas pesquisas foram realizadas. Oxidação de compostos contendo nitrogênio pela lacase na presença de ABTS (D'Annibale et al. 1996), deslignificação de polpas kraft usando lacase e ABTS (Bourbonnais et al. 1996, Paice et al. 1996), degradação ou branqueamento de lignina com lacase e ácido violúrico (Call, 1997), oxidação mediada por lacase da lignina na presença de um mediador natural, ácido 3-hidroxi antranílico (Eggert et al. 1995, 1996), biobranqueamento de xilanase associado a lacase-HBT (Oksanen et al. 1997), tratamentos de polpas com lacase/HBT e ABTS (Potthast et al. 1997; Bourbonnais et al. 1998; Tamminen et al. 1998; Ander et al. 1998), foram recentemente publicados.

A indução de atividade de lacase e de sua ação sobre efluentes kraft na presença simultânea de mediadores era desconhecido até o momento. Nossos estudos mostraram um efeito importante na presença de ABTS e HBT. *L. edodes* (CCT-4519), *T. versicolor* (CCT-4521) e *T. villosa* (CCT-5567) foram cultivados em ágar extrato de malte e em extrato de malte na presença de 20% de efluente (condições induzidas) na presença de ABTS e HBT. *T. versicolor* reduziu 50% dos fenóis totais na presença de ABTS sem redução da cor. *T. villosa* (CCT-5567) induzido e não induzido na presença de ABTS atingiu 70% e 25% de redução de fenóis totais, respectivamente. Neste caso existiu uma correlação entre a atividade da lacase e a descontaminação. Quando os fungos estudados e/ou suas enzimas foram imobilizadas em resinas ou em vitrocerâmicas os resultados de descontaminação foram ainda mais significativos. Estudos detalhados nesta área estão sendo realizados.

Em resumo, o sistema lacase-mediador aparece como um importante sistema enzimático com grande aplicação em várias áreas.

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ENZIMA DE INTERES EN ENOLOGIA: LACASAS

Rosana C. Minussi^{*}, Glaucia M. Pastore^{*} y Nelson Durán^{**}

Instituto de Química, Laboratório de Química Biológica^{**}, Faculdade de Engenharia de Alimentos^{*},
Universidade Estadual de Campinas, C.P. 6154, Campinas, CEP 13083-970, S.P., Brasil. E-mail:
duran@iqm.unicamp.br

RESUMEN

En este trabajo se analizan los aspectos básicos de química del vino, la bioquímica de lacasas y las implicaciones de su utilización en el mejoramiento de vinos blancos y las posibilidades de utilizarlas en vinos tintos. Se discuten las interacciones de las lacasas con polifenoles antioxidantes en el caso de los vinos tintos y su posible estrategia para aumentar su selectividad.

ABSTRACT

Chemical and biochemical basic aspects of laccases and their implications are analyzed in order to improve the white wines and the possibilities of using them in red wines. Comments are made relating to the activity of laccases with polyphenol antioxidants in the case of the red wines and their possible strategies for its selectivity.

ASPECTOS GENERALES

De acuerdo con las reglas de la Unión Europea, el vino es definido como un producto obtenido exclusivamente de la fermentación de uvas frescas o del líquido, conocido como mosto, el cual es obtenido de las uvas por presión (Gibson, 1997). Todas las uvas son verdes y duras en las primeras fases de su crecimiento, presentando bajos porcentajes de azúcar y altas concentraciones de ácidos. El inicio de la maduración es marcado por el rápido cambio en la coloración, como resultado del aumento en la concentración de polifenoles, en particular de las antocianidinas (Bucelli et al., 1991). Tanto el color como la astringencia se derivan de la concentración de polifenoles particularmente en los tallos, semillas y cáscara. Estos están presentes en concentraciones hasta alrededor de 3 g dm^{-3} en jugos tintos, pero cerca de un décimo de este valor en jugos de uvas blancas. La mayor fuente de color en uvas es la malvidina, un monoglucósido de antocianidina (Gibson, 1997).

Hay dos formas en equilibrio del monoglucósido, el ión flavilium (rojo) y la forma carbinol (incoloro).

En realidad estos dos componentes están en equilibrio con otras formas que son violeta, amarillo, incolora y el resultado de la mezcla muestra una dependencia compleja del pH (Allen, 1996; Aruoma, 1996). Sin embargo, polimerización oxidativa y condensaciones de estos flavonoides ocurre eventualmente dando lugar a la producción de moléculas grandes (taninos) que son característicamente más amarillos o marrones, de tal manera que los vinos tintos jóvenes pierden su tono púrpura, llegando a ser más rojo ladrillo y luego un color pálido café con su madurez (Iland et al., 1993). Este proceso es acelerado en la presencia de aire por tirosinasa, una polifenol oxidasa natural de las uvas, pero esta enzima no es muy soluble y es fácilmente inhibida por dióxido de azufre. La actividad de una enzima similar pero más poderosa, la lacasa, es más difícil de controlar. Sin embargo, no está normalmente presente en uvas sanas, pero esta es excretada por hongos como

Botrytis cinerea y es introducido con la uva como contaminación, su control yace esencialmente en la selección de las frutas sanas.

Los polifenoles, son importantes y contribuyen al sabor de los vinos. Los pigmentos coloreados por si mismos contribuyen muy poco y mientras taninos de masa molecular menor dan un gusto amargo, los de masa molecular mayor tienen un efecto mas dramático. Estos interaccionan con proteínas en la lengua y en la boca, dando la sensación astringente y seca encontrada en algunos de los vinos tintos (Gibson, 1997).

QUÍMICA Y BIOQUÍMICA DE LACASA

Lacasa es una cuproproteína perteneciente al pequeño grupo de enzimas denominadas oxidases azules (Thurston, 1994). La lacasa es una fenol oxidasa (p-difenol oxidasa, EC 1.10.3.2) que cataliza la oxidación de varias sustancias aromáticas e inorgánicas (particularmente fenoles) con la concomitante reducción de oxígeno para agua (Thurston, 1996; Xu, 1996). En general las lacasas presentan cuatro átomos de cobre vecinos, los cuales estan distribuidos entre diferentes sitios de ligación y son clasificados en tres tipos: cobre tipo 1, 2 y 3 diferenciados por tener propiedades características específicas que les permiten desempeñar un papel importante en el mecanismo catalítico de la enzima (Malmstron et al., 1975; Yaropolov et al., 1994; Mcmillin y Eggleston, 1997; Durán et al., 1998).

Lacasa ha sido bastante estudiada en los aspectos de genética molecular (Cullen, 1997), expresión genética (Ong et al., 1997), transcripción genética (Collins y Dowson, 1997) y clonaje (Giardina et al., 1996).

Compuestos fenólicos estan ampliamente distribuidos en la naturaleza y su oxidación es

importante en procesos tales como la oxidación celular, protección de la pared celular, oscurecimiento en frutas, procesamiento de jugos y vinos, deslignificación de pulpas, fortalecimiento de productos compuestos, descoloración de tejidos, desintoxicación de suelos y aguas contaminadas (Xu, 1996; Smith et al., 1997). La típica reacción de lacasa, es donde un fenol es sometido a oxidación por pérdida de un electron para formar un radical libre. Esta especie activa de oxígeno puede ser convertida a una quinona en una segunda etapa de oxidación. Tanto la quinona cuanto el producto radical libre pueden sufrir polimerización (Higuchi, 1989).

Las lacasas son notablemente no específicas en relación a su sustrato reducido y la serie de sustratos oxidados cambia de una lacasa para otra. Difenoles simples como dihidroquinona y catecoles son buenos sustratos para la mayoría de las lacasas, pero guaicol y 2,6-dimetoxifenol generalmente son mejores. Luego, la lacasa es una oxidasa que oxida polifenoles, metoxifenoles substituidos, diaminas y una serie de otros compuestos, pero no oxida tirosina (como las tirosinasas lo hacen). Esta enzima está ampliamente distribuida em plantas superiores (Mayer y Harel, 1979) y en hongos. Hasta ahora existen solamente dos bacterias, la *Azospirillum lipoferum* (Givaudan et al., 1993) y la *Alteromonas sp* (Sanchez-Amat y Solano, 1997), en la cual una oxidasa fenólica tipo lacasa ha sido demostrada.

Las lacasas producidas por diferentes hongos difieren significativamente en relación a su inducibilidad, número de isoformas, peso molecular, pH óptimo, especificidad por el sustrato (Bollag y Leonowicz, 1984). En algunas especies de hongos, la introducción de sustancias inductoras en el medio del cultivo inicia la

biosíntesis de una nueva forma extracelular, mientras que las formas constitutivas son sintetizadas continuamente.

ASPECTOS BIOTECNOLÓGICOS DE LA LACASA EN VINOS

Los mostos y vinos son mezclas complejas de diferentes compuestos químicos, tales como etanol, ácidos orgánicos, sales y compuestos fenólicos. Mientras el alcohol y ácidos orgánicos son responsables por el aroma del vino, el color y el sabor dependen particularmente de los compuestos fenólicos presentes en los diferentes tipos de vinos (Brenna y Bianchi, 1994). Muchos grupos de compuestos fenólicos son encontrados en vinos: Derivados del ácido cinámico y catequinas están presentes en diferentes cantidades en todos los vinos, mientras que los vinos rosados y tintos son caracterizados por la presencia de antocianidinas. El porcentaje y la composición de compuestos fenólicos de un vino dependen de muchos factores, desde la uva empleada hasta variaciones en el procesamiento (Macheix et al., 1991; Cheynier et al., 1997). Las propiedades sensoriales de los vinos frescos deberían permanecer constantes hasta el consumo, o sea, deberían ser suficientemente estables al menos en el primer año de almacenaje. Debido a una compleja secuencia de eventos, donde los polifenoles (derivados del ácido cumárico, flavanas y antocianidinas) desempeñan un importante papel, reacciones oxidativas catalizadas por hierro, cobre y enzimas y que también pueden incluir aldehídos, aminoácidos y proteínas, pueden ocurrir en mostos y vinos ocasionando turbidez, intensificación en la coloración y alteraciones en el aroma y en el sabor. Este fenómeno de oxidación ha sido denominado de *maderización* (Zamorani et al., 1993). Diferentes métodos han sido testados

tratando de prevenir la descoloración y la alteración del sabor en vinos, tales como la remoción de grupos fenólicos con polivinilpolipirrolidona (PVPP), adición de dióxido de azufre, entre otros.

En los tratamientos que eliminan polifenoles se desea que esta remoción sea selectiva una vez que si esta fuese indiscriminada el vino tendrá una alteración indeseable en las características organolépticas. Una alternativa para los adsorbentes físico-químicos podría ser el uso de enzimas que actúan en los polifenoles responsables por el inicio de la *maderización*. Estas sustancias polifenólicas serían hidrolizadas y oxidadas por tales enzimas, siendo previamente desestabilizadas por polimerización y floculación o sea fácilmente eliminadas por clarificación (Zamorani, 1989). Las preparaciones enzimáticas que están disponibles contienen enzimas activas en sustancias polifenólicas y entre estas enzimas se encuentran lacasa, peroxidasas, difenoloxidasas, catecol-1,2-oxigenasa, ácido protocatechuico oxigenasa y otras hidrolasas y transferasas (Cantarelli, 1986). Este tipo de tratamiento es interesante por su especificidad de acción y por ser una tecnología no agresiva ("mild technology"), con efectos menos drásticos en las características del vino. Preparaciones enzimáticas han sido usadas en la industria de vinos por más de 60 años, iniciándose en la década del 30 con preparaciones para clarificación del jugo. Hoy la industria de vinos tiene tecnología disponible para la aplicación de varias enzimas industriales. Pectinasas, β -glucosidasas, β -glucanasas, oxidasas, ureasas, proteasas y lisozima son los mayores ejemplos de preparaciones enzimáticas que puede ser usadas en la fabricación de vinos (Bisson y Butzke, 1996).

La lacasa presenta algunos requisitos importantes para ser utilizada como tratamiento de eliminación de polifenoles en vinos, tales como pH óptimo en torno de 2,5-4,0, estabilidad en ambiente ácido, inhibición reversible con sulfito, entre otros. Varios trabajos en la literatura reportan estudios con la utilización de lacasa visualizando estabilización de vinos (Cantarelli, 1986; Cantarelli et al., 1989; Zamorani, 1989; Maier et al., 1990; Cantarelli y Giovanelli, 1991; Zamorani et al., 1993; Brenna e Bianchi, 1994; Lante et al., 1992, 1996; Minussi et al., 1998a,b).

Segun Cantarelli (1986), lacasa de una mutante de *Polyporus versicolor* (pH optimo 2,7) eliminó alrededor de 70% de catequina y 90% de antocianidinas de las soluciones modelo en 3 horas de tratamiento. La acción de la lacasa en un jugo de uvas negras mostró una remoción de 50% de polifenoles totales y el análisis por HPLC de un mosto tratado con el extracto activo e inactivo de la enzima demostró la eficacia de esta lacasa, superior en especificidad de acción y estabilización cuando comparada al tratamiento físico-químico. La utilización de la lacasa en el mosto resultó en un vino estable y de buen gusto. Los resultados mostraron que es posible la utilización de la lacasa como un agente clarificante, seguido de la adición de solución de sílica, o de un tratamiento térmico, y una ultrafiltración para la remoción de los productos oxidados y de la proteína enzimática. Zamorani (1989) comparó la utilización de diferentes extractos enzimáticos conteniendo tanasa, fenolasa, lacasa o antocianasa en la fabricación de vinos obtenidos de uvas Pinot. Los resultados mostraron que lacasa fue mas efectiva que las otras enzimas en la eliminación de los polifenoles, pero el autor alerta que la sulfitación, clarificación y filtración tambien deben ser hechos

visualizando remoción de los productos oxidados. Los resultados obtenidos con vino Trebbiano tratado con lacasa y solución de sílica cuando comparados con los datos obtenidos por clarificación (caseinato+carbono activo+bentonita) en presencia y ausencia de dióxido de azufre, mostraron que el uso de lacasa fue altamente efectivo y practicamente identicos a los obtenidos por el método tradicional. Los análisis organolépticos confirmaron que los vinos tratados con lacasa y solución de sílica o dióxido de azufre poseen mejores características organolépticas resistentes a la maderización. Cantarelli et al. (1989) también confirmaron el potencial de la lacasa como tratamiento pré-fermentativo cuando acoplado con agentes clarificantes convencionales tales como proteínas y PVPP. Maier et al. (1990) evaluaron el porcentaje de polifenoles, coloración, estabilidad y calidad sensorial de vinos Riesling preparados con y sin oxidación del mosto, o con oxidación del mosto y el tratamiento con lacasa. Los resultados mostraron que los vinos hechos con la oxidación forzada/tratamiento con lacasa fueron los mejores, sugiriendo que vinos estables y de alta calidad pueden ser hechos con poca o ninguna adición de SO₂. Cantarelli e Giovanelli (1991) realizaron ensayos tentando determinar si las preparaciones enzimáticas podrian ser usadas en la fabricación de vinos blancos para la reducción de polifenoles en mostos (y consecuente estabilización de la coloración del vino) en el lugar de la hiperoxidación, que es considerado tecnológicamente no confiable. La enzima utilizada fue lacasa, adicionada a 5-20 unidades/mL al mosto, el cual fue filtrado (con y sin PVPP) o clarificado con bentonita o gelatina + solución de sílica, o sulfitado (5 g SO₂/hL). Los resultados demostraron que el tratamiento enzimático

acoplado a filtración con PVPP redujo la cantidad de polifenoles oxidables y de los pigmentos formados. Un análisis comparativo entre los tratamientos de hiperoxidación y enzimático mostró compatibilidad en términos de coloración pero sensibles diferencias en las características organolépticas, relacionados a la variedad de la uva utilizada. Estudios preliminares con lacasa de *Trametes versicolor* demostraron que esta enzima tiene gran potencial para degradación de compuestos fenólicos en vinos. Fueron obtenidas degradaciones mayores de 90% de ácido ferúlico en una solución modelo (Minussi et al, 1998a) y 34% de compuestos fenólicos en vinos (Minussi et al., 1998b).

Como la utilización de la lacasa como aditivo alimentar todavía no es permitida (JECFA, FAO/WHO Food Additives Data Systems), esta ha sido testada en vinos en la forma inmovilizada (Zamorani et al., 1993; Brenna e Bianchi, 1994; Lante et al., 1992, 1996), facilitando su eliminación del mosto y posibilidad de reutilización. Lacasa de *Pyricularia oryzae* y *Botrytis cinerea* fué inmovilizada por métodos físicos y químicos en soportes orgánicos e inorgánicos y testada en cuanto la eliminación de fenólicos en solución modelo, mosto y vino. Lacasa adsorbida en tamizes moleculares y sílica gel, inmovilizada por adsorción y ligaciones covalentes, dio rendimientos de inmovilización sobre 90%, mientras que los mejores rendimientos de actividad variaron de 22 a 38%. Lacasa inmovilizada en sílica gel y glutaraldehído causó una disminución en el porcentaje de catequina en la solución modelo, en el mosto y en el vino, pudiendo ser reutilizada por hasta 5 veces. Brenna e Bianchi (1994) estudiaron la inmovilización de lacasa en matrices activadas basadas en agarosa y testaron su

eficacia en una solución modelo de catequina y en mosto proveniente de uvas Riesling. La enzima inmovilizada fue compactada en columnas cromatográficas de varios tamaños, equilibrada con aire, N₂ y O₂. Las soluciones fueron colectadas utilizando diferentes flujos en el reactor. Las muestras eluidas fueron tratadas con PVPP. La oxidación de los fenólicos varió principalmente con el flujo en el reactor. Tanto el aumento de la coloración como el decrecimiento en polifenoles totales fueron máximos en flujos bajos en el reactor (muestras saturadas con O₂). La lacasa inmovilizada de *Trametes versicolor* se mostró bastante estable, pudiendo ser reutilizada por lo menos 8 veces, con pérdidas mínimas de actividad después de un período de 6 meses cuando conservada en condiciones controladas. Rendimientos de la actividad de 51 a 63% con lacasa inmovilizada por enlaces covalentes en soportes polisacarídicos fueron obtenidos por Lante et al. (1996). En este caso también fue observado decrecimiento en fenólicos presentes en mostos y vinos tratados con la lacasa inmovilizada.

ANTIOXIDANTES-LACASAS.

En los últimos años ha sido aceptado que el consumo de vinos tintos en forma moderada es un factor benéfico para la salud humana. Investigadores han calificado las sustancias antioxidantes como polifenoles que están en grandes cantidades en los vinos tintos, sin embargo, han tenido grandes dificultades para determinar el papel de cada uno de estos compuestos en esa actividad (Brouillard et al., 1997). En vista de esto, es necesario analizar las posibles interacciones de la lacasa con estos componentes tan importantes en el vino tinto (no sería problema en los vinos blancos el uso de la

lacasa) que en principio serian substratos para la enzima. Luego, haremos una breve revisión de las características y tipos de compuestos con esta propiedad antioxidante y como podrian interaccionar en forma negativa para la calidad del vino con la lacasa.

Varios métodos han sido discutidos ultimamente en la determinación de la actividad antioxidante. El método de radical cation del ABTS fue aplicado en vinos tintos, rosados y blancos (Henn y Stehle, 1998). En este trabajo fue mostrado que existe una excelente correlación ($r = 0.97$) del contenido de fenoles totales con la actividad antioxidante.

El mismo método fue aplicado para vinos blancos y tintos y como también en vinos durante la maduración encontrándose también una correlación entre los fenoles totales y la capacidad antioxidante con el radical cation del ABTS (Campodonico et al., 1998). Método similar al anterior con ABTS fue aplicado a vinos y cerveza (Bartosz et al., 1998; Cano-Lario et al., 1998).

Los vinos tintos fueron mas activos como antioxidantes en humanos que vinos blancos, aparentemente debido al contenido de antocianidinas. Acidos fenólicos, catequinas, monómeros de antocianidina, flavonoles y antocianidinas poliméricas de los vinos fueron analizadas en función de su contribución en los efectos antioxidantes. La intensidad del color y los vinos fue directamente relacionada con la capacidad antioxidante, de acuerdo con la alta contribución de las antocianidinas (Ursini et al., 1997).

El vino tinto conservado en dos diferentes maneras sobre dos condiciones oxidativas, fue estudiado para determinar de las diferencias de las actividades biológicas de las fracciones

polifenólicas. Estudios analíticos mostraran que los compuestos antocianidinicos estaban relacionados con la actividad antioxidante de los vinos estudiados (Baldi, 1997; Ghiselli et al. 1998).

Cultivos de *Vitis vinifera* L producen varios tipos glucosidos estilbénicos en vino tinto. En culturas de células estudiadas recientemente tres glucosidos estilbénicos fueron encontrados conteniendo estructuras tetra- e trihidroxil-estilbénicas (Teguo et al., 1998).

Numerosos vinos de diferentes orígenes, variedades y cosechas fueron analizados segun su contenido de fenoles totales, contenido de dióxido de azufre, color y su capacidad de capturar radicales superóxidos (SOSA). El fraccionamiento de los fenoles mostró que no existe correlación entre SOSA y fenoles totales en una fracción (A) conteniendo ácidos fenólicos, azucar, ácidos orgánicos, aminoácidos y sales. Una buena correlación fue encontrada con una fracción (B) conteniendo procianidinas, flavonoles y catequina; y una excelente correlación con una fracción (C) conteniendo antocianidinas y taninos. El contenido de fenoles de la fracción C fue alrededor de 2 veces mayor que el de la fracción B. La fracción C contribuye para la mayoría de la actividad SOSA en el vino. Los fenoles extraídos del vino Merlot de esta forma fueron eficientes en la actividad antioxidante (Sato et al., 1997).

La modificación oxidativa de las lipoproteínas de baja densidad (LDL) ha sido implicada en la arterioesclerosis. El vino tinto fue fraccionado en ácidos fenólicos (fracción 1), catequinas y antocianidinas monoméricas (fracción 2), flavonoles (fracción 3) y antocianidinas poliméricas (fracción 4). La fracción 2 mostró un valor de antioxidante significativamente mayor que las fracciones 3 e 4, pero no mayor que la fracción 1.

Luego, particularmente las catequinas, antocianidinas y ácidos fenólicos são los mas eficientes antioxidantes en vinos tintos (Kerry y Abbey, 1997).

En este mismo sentido fueron recientemente medidos 15 polifenoles (los ácidos fenólicos gentísico, vanílico, ferúlico, m-cumárico, p-cumárico, cafeico y gálico; los trihidroxiestilbenos cis- y trans-resveratrol, cis- y trans-polidatin; y los flavonoides, catequina, epicatequina, quercetina y morina) en vinos blancos (Chardonnay, Riesling, Seyval Blanc, Vidal) y de tintos (Pinot Noir, Cabernet Sauvignon, Cabernet Franc, Merlot, Gamay Noir). El ácido ferúlico fue el mas alto de todos los ácidos fenólicos en vino Riesling; en el resto de los vinos blancos, ácido caféico y p-cumárico fueron los mas altos. En los vinos tintos el ácido gálico fue el mayor y el segundo en mayor proporción el cafeico. De los hidroxiestilbenos el polidatin fue el mas importante. Pinot Noir fue el que mostró el valor mas alto de catequinas y epicatequina, sin embargo, el Cabernet Sauvignon fue el mas bajo en estos flavonoides, pero el mas alto en quercetina (Soleas et al., 1997a).

Las concentraciones de 17 constituyentes fenólicos de vino tinto fueron estudiados por analisis de regresión lineal multiple para su contribución con el estado de antioxidante total (TAS). La ecuación deste modelo puede predecir casi 100% del TAS para los contenidos de ácido vanílico, trans-polidatin, catequina, ácido m-cumárico, epicatequina, quercetina, cis-polidatin y trans-resveratrol con un alto grado de confianza (Fig.1).

A pesar que ácidos siringico y gálico correlacionaron bien con TAS en un analisis univariado, estos no contribuyeron en la descripción estadística de los parámetros en la

extensión de los 8 constituyentes ya identificados. Los otros constituyentes parecen no contribuir significantivamente al TAS o no tienen correlación con TAS en analisis univariado (Soleas et al., 1997b).

Despues de haber visto los componentes importantes del vino tinto que dan las caraterísticas de un buen antioxidante, podemos analizar su posible comportamiento con la lacasa.

No fueron encontradas reacciones de epicatequina, quercetina, cis- e trans-polidatin con lacasas en la literatura actual. Sin embargo, el ácido siringico es rápidamente oxidado a 2,6-dimetoxi-1,4-benzoquinona por la lacasa de *Azospirillum lipoferum* (Faure et al., 1996).

El ácido gálico es oxidado a quinona por la lacasa de *Botrytis cinerea* (Viterbo et al., 1993). Infelizmente lacase inmovilizada degrada 75% de la catequina en una solución modelo, pero a pH mayores de 4,0, luego tendria una degradación parcial en el vino que posee pHs menores (De Stefano et al., 1996). El resveratrol es oxidado a epsilon-viniferin por dos isoenzimas de la lacase de *Botrytis cinerea* (punto isoelectrico de 4,35 e 4,30) (Pezet, 1998).

Ácido vanílico reacciona lentamente con lacasa formando dímeros (Tatsumi et al., 1994). La lacasa fúngica oxida los derivados de hidroxi-cinámicos en el orden del ácido sinápico>ácido ferúlico>ácido cumárico (Takahama, 1995). Todas estas transformaciones de los derivados de ácidos cinámicos ocurren a pH \geq 4,0 y temperaturas de 50°C (Lacki y Duvnjak, 1996).

Luego, la presencia de grandes cantidades de ácido gálico en la mezcla, llevará a este compuesto a ser el mas facilmente degradado que los de mayor interés como antioxidantes (Takahama, 1995). En otras palabras tendremos una reacción competitiva

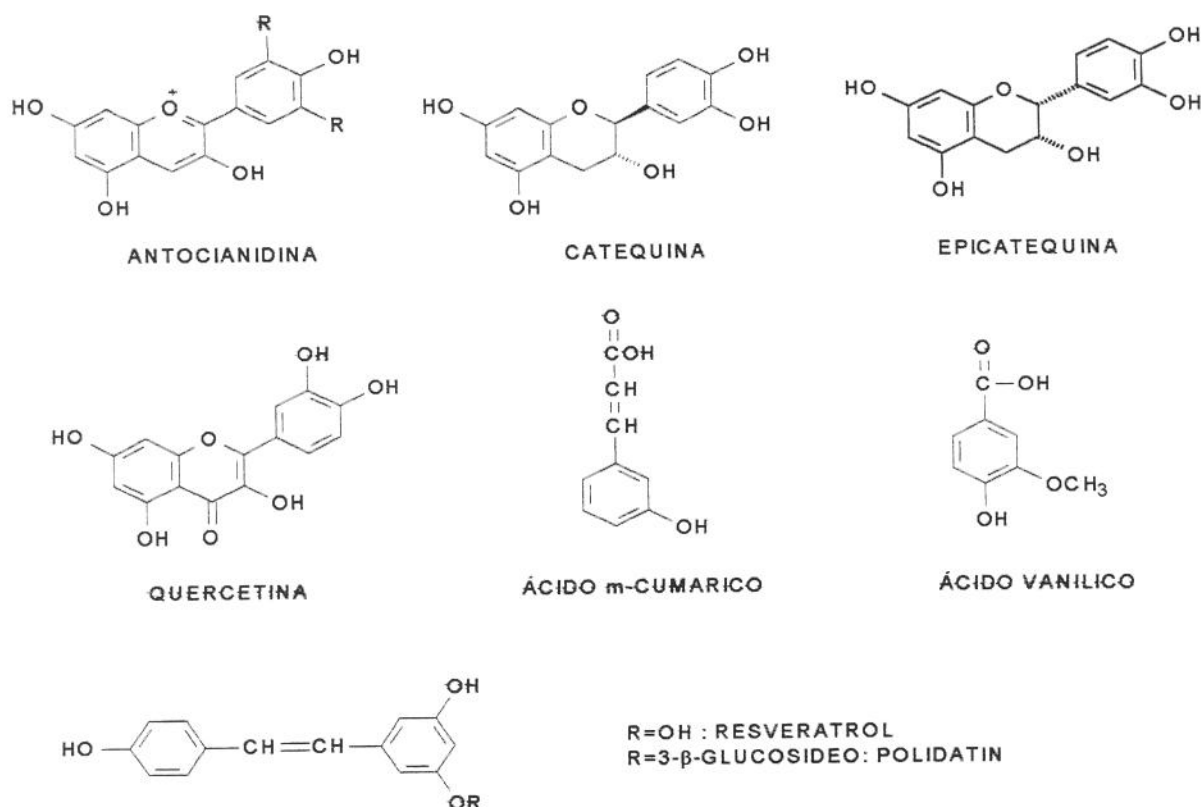


FIG.1. Estructuras de compuestos importantes como antioxidantes en vinos tintos.

que podría ser de interés en las lacasas para aumentar su selectividad en fenoles no importantes como antioxidantes.

Teniendo un estudio de las isoenzimas de la lacasa y de sus selectividades, podrían ser escogidas aquellas que afecten menos a los componentes importantes del vino tinto con propiedades antioxidantes.

CONCLUSIONES

Esta revisión ha mostrado que la utilización de lacasa en vinos blancos es perfectamente posible. Esto permitiría tratamientos mas suaves y ecológicamente mas correctos, disminuyendo los costos del procesamiento y evitando el deterioro de vinos en tiempos largos de almacenamiento. En el caso de los vinos tintos, la utilización de las lacasas debe ser mas cuidadosa, ya que la selectividad en

este caso debe ser máxima. Los fenoles con grandes propiedades antioxidantes deben ser preservados. Estudios de acción de isoenzimas de lacasa de *Trametes versicolor*, estan en estudio en nuestros laboratorios. Como la inmovilización de estas enzimas seria de gran importancia en su desarrollo nuevos sistemas de inmovilización de enzimas oxidativas estan en estudio con soportes vitrocerámicos y resinas (Peralta-Zamora et al., 1998a-d).

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ARTIGOS DE PESQUISA

LACCASE INDUCTION IN FUNGI AND LACCASE-MEDIATOR SYSTEM APPLIED IN PAPER MILL EFFLUENT

Rosana C. Minussi^{1,2}, Gláucia M. Pastore¹, Nelson Durán^{2,3*}

Food Biochemistry Laboratory, Faculdade de Engenharia de Alimentos¹ and Biological Chemistry Laboratory, Instituto de Química², Universidade Estadual de Campinas (UNICAMP), C.P. 6154, Campinas - São Paulo, Brazil, CEP 13083-970, and Núcleo de Ciências Ambientais³ - Universidade de Mogi das Cruzes.

Abstract

The optimum cultivation conditions in semi-solid and liquid medium of *Trametes versicolor*, *Trametes villosa*, *Lentinus edodes* and *Botrytis cinerea* for laccase production and the effects of laccase-mediator system in E₁ effluent were studied. All the fungi tested showed laccase production capacity under the culture conditions. A high laccase activity was obtained in a liquid culture of *T. versicolor* in the presence of 2,5-xylidine and copper as inducers. The effluent biotreatments were not efficient in decolorization. At high laccase activity, higher total phenol degradation was observed. The best value for total phenol reduction was around 23% in the absence of mediators. The presence of 1-hydroxybenzotriazole did not increase the phenol reduction. The 3-hydroxyanthranilic acid was rapidly degraded by the laccase, which shows that this acid is not an efficient mediator for this system. However, acetohydroxamic acid, which was not degraded by laccase, acted very efficiently on E₁ effluent, reducing 70% and 73% the total phenol and total organic carbon, respectively. The calculations considered the phenol and organic charge given by the controls. Therefore, acetohydroxamic acid appears as the best mediator for laccase bioremediation in E₁ effluent and a more detailed study is in progress.

Keywords: Laccase; Polyphenol oxidase; White rot fungi; Inducers; Mediators; Lignin

1. Introduction

Lignin is a complex aromatic biopolymer degradable by a few organisms like white-rot basidiomycetes. Industry has an increasing interest in extracellular enzymes from white rot fungi, such as lignin and manganese peroxidases, and laccases, due to their potential to degrade both highly toxic phenolic compounds and lignin (Mansur et al., 1998).

Laccases (EC 1.10.3.1) are multi copper enzymes that catalyze the oxidation of a variety of phenolic compounds, with concomitant reduction of O₂ to H₂O. These polyphenol oxidases are widely distributed among plant and fungal species; however, their biological significance is unclear. Interest in laccases has been stimulated by their potential use in detoxification of environmental pollutants, wine stabilization, paper processing, enzymatic conversion of chemical intermediates

and production of useful chemicals from lignin (Berka et al., 1997; Gianfreda et al., 1999; Hublik and Schinner, 2000).

Many studies have been made to find the favorable conditions for laccase production by fungi (Arora and Gill, 2000; Koroleva et al., 2000; Ullah et al., 2000; Arora and Gill, 2001; Dekker and Barbosa, 2001). Fungal laccase production depends on the growth media and conditions. Laccases from various fungal cultures differed markedly in their inducibility, number of enzyme forms, molecular mass, optimum pH, and substrate specificity with methoxyphenolic acids (Bollag and Leonowicz, 1984). Different compounds like veratryl alcohol, benzyl alcohol, 2,5-xylidine, liginosulfonate, ferulic and gallic acids have been studied as laccase inducers. Many producers of laccase secrete isoenzymes that differ in stability and catalytic features (Heinzkill et al., 1998).

Laccase alone has a limited effect on bioremediation due to its specificity for phenolic subunits in lignin. Bourbonnais & Paice (1990) founded that the substrate range of laccase can be extended to nonphenolic subunits of lignin by inclusion of a mediator, such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS). Furthermore, the laccase-ABTS couple has also been shown to effectively demethylate and delignify kraft pulp (Bourbonnais & Paice, 1992). More recently, 1-hydroxybenzotriazole (HBT) has been found to mediate pulp delignification in the presence of laccase (Call, 1994), phenothiazines have been found to bleach and prevent redeposition of azo dyes in textile manufacturing (Schneider & Pedersen, 1995), and 3-hydroxyanthranilic acid was observed to oxidize nonphenolic lignin model compounds (Eggert et al., 1996a).

Laccase has been studied recently in both the pure form and in the presence of a mediator described for the degradation of lignin in wood, pulp and model compounds (Bermek et al., 1998; Goodell et al., 1998; Sealey & Ragauskas, 1998; Li et al., 1999). Potthast et. al. (1999) investigated the polymerizing and depolymerizing properties of the laccase-mediator system. The polymerizing action of laccase and the ABTS cation radical on phenols was demonstrated in addition to the depolymerizing effect of the system. The laccase-mediator system causes polymerization of phenolic substrates in the initial phase of the reaction, but then degrades the initially formed polymers. The extensive breakdown of this oligomeric and polymeric material occurs exclusively in the presence of both components, mediator and laccase. The use of a laccase-mediator system is one of the promising possibilities for an environmentally benign pulp-bleaching process (Li et al., 1998). The University of Georgia has recently opened a pilot plant for

deinking of recycled paper. Pulp bleaching with a laccase-mediator system has reached pilot plant stage and it is expected to be commercialized soon (Bajpai, 1999).

Chlorine is an effective and widely used bleaching agent for chemically produced wood pulps. However, chlorination followed by alkaline extraction results in large volumes of effluents containing substantial levels of absorbable organic halogens (AOX), primarily in the form of chlorophenols, chloroguaiacols, chloroaliphatics, chlorocatechols, chlorosyringols, and large polymerized chloroaromatics. The environmental impact of these compounds should be reduced.

The aim of this work was to determine optimal conditions for laccase production by selected fungi and to study the effects of laccase-mediator system on the treatment of E₁ effluent, since no study in this direction was published before.

2. Material and methods

2.1. Fungal strains

The cultures of *Lentinus edodes* CCT 4519, *Trametes versicolor* CCT 4521, *Trametes villosa* CCT 5567 and *Botrytis cinerea* 2802 were maintained on malt extract agar plates at 28°C.

All analyses were carried out in duplicate and the results represent mean values of at least two experimental repetitions.

2.2. Semi-solid culture conditions

The fungal strains were grown in Petri plates containing malt extract agar supplemented with 0.8 mM gallic acid, 2,5-xylidine or ferulic acid. The plates were incubated at 28°C for 20 days. Laccase activity was measured at 7, 14 and 21 days of cultivation. Laccase was extracted from the semi-solid medium using 20 mM citrate-phosphate buffer (pH 5.0). Cultures were filtered through a Millipore

membrane (0.45 μm) and the enzymatic activity was determined in the culture filtrate.

2.3. Liquid culture conditions

The liquid growth medium (modified by Von Hunolstein et al. (1986)) consisted of (g/L): malt extract, 5; peptone, 10; glucose (added after autoclaving), 20 and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.005, pH 5.4. Laccase induction consisted of the addition to the flasks of 0.5 mM 2,5-xyldine, gallic acid or ferulic acid at 96 hr of growth. Different concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.0 – 4.0 mM) and 2,5-xyldine (0.0 – 1.0 mM) were also tested in *Trametes versicolor* strain. The 250 ml Erlenmeyer flasks containing 50 ml of culture medium were inoculated with 4 fungal discs (7 mm diameter) and incubated for 20 days at 28°C and 240 rpm on a rotary shaker. Laccase activity was measured at 4, 8, 12, 16 and 20 days of cultivation.

2.4. Enzyme assay

Laccase activity was assayed by measuring oxidation of syringaldazine. 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. Oxidation of syringaldazine was monitored by measuring the increase in A_{525} for 5 minutes. Enzyme activity was expressed in units (U); 1 U was defined as 1 μmol of syringaldazine oxidized per min per liter of the culture filtrate.

2.5. Effluent Treatment

Laccase (activity in a culture filtrate was around 10000 U) was obtained by *Trametes versicolor* CCT 4521 grown in a liquid medium containing 0.5 mM 2,5-xyldine as inducer. Laccase was added in different activities (25, 50 and 100 U) to the E_1 effluent. The mediators used were 1-

hydroxybenzotriazole (HBT), 3-hydroxyanthranilic acid (3-HAA) and acetohydroxamic acid (AHA) at 0.34 mM concentration. Total phenols (APHA, 1989), total organic carbon and color (Livernoche et al., 1983) were monitored from 0 to 5 h of treatment.

3. Results

3.1. Laccase production in a semi-solid medium

The effect of gallic and ferulic acids on the laccase production by *Lentinus edodes*, *Trametes versicolor*, *Trametes villosa* and *Botrytis cinerea* in malt extract agar can be seen in Fig. 1. The maximum value for laccase activity was obtained with *T. versicolor* at 21 days of growth in the presence of ferulic acid (587.5 U) (Fig. 1B). Ferulic acid also showed an inducing effect in *T. villosa*, but up to 14 days of growth, reaching the maximum value at 7 days (95.4 U) (Fig. 1C). Low amounts of laccase were detected in the medium of *L. edodes* (Fig. 1A) and *B. cinerea* (Fig. 1D) in these growth conditions. The highest laccase activities were observed at 7 days for *L. edodes* in the presence of ferulic acid (2.4 U) and at 14 days for *B. cinerea* in the presence of gallic acid (8.1 U).

Different 2,5-xyldine concentrations were tested in a semi-solid medium and the results are shown in Fig. 2. The highest laccase activity was obtained with *T. versicolor* cultured in the presence of 0.8 mM 2,5-xyldine (557.5 U) at 21 days of growth (Fig. 2B). *Trametes villosa* and *L. edodes* were not inhibited with the 2,5-xyldine concentration of 8 mM. Laccase activities of 304.2 U at 14 days and 2.3 U at 7 days of growth were obtained under these conditions (Fig. 2C and 2A), respectively. The 2,5-xyldine concentrations up to 0.8 mM improved laccase activity in cultures of *B. cinerea* (Fig. 2D). At 14 days of growth, the laccase activity found in the presence of 0.3 mM

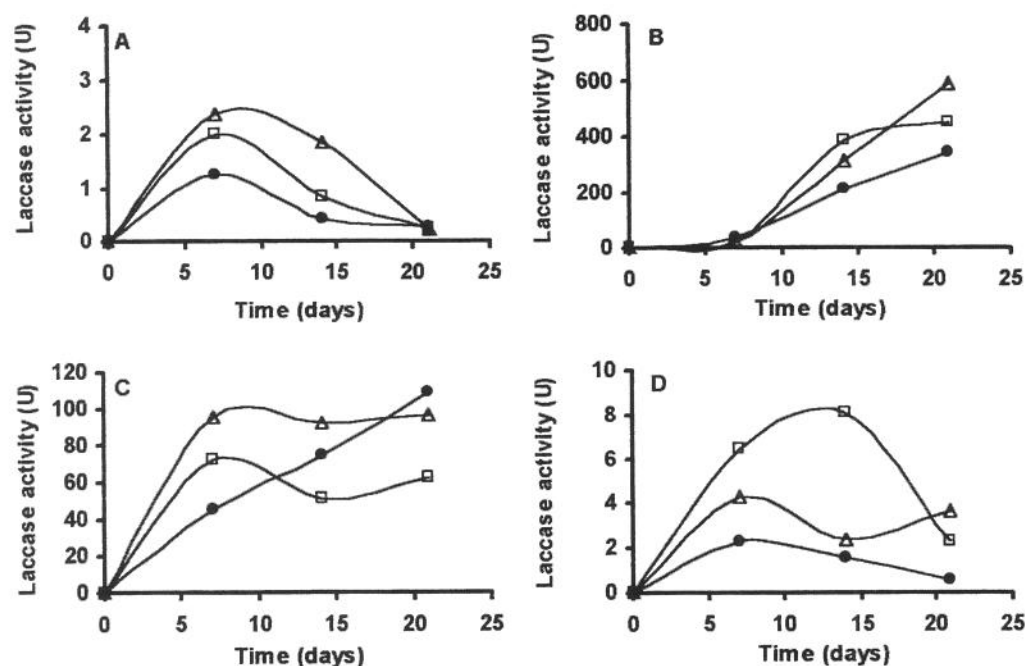


Fig. 1. Laccase activity (U) of A) *Lentinus edodes*, B) *Trametes versicolor*, C) *Trametes villosa* and D) *Botrytis cinerea* cultured for 21 days at 28°C on malt extract agar, in the absence (●) or presence of 0.8 mM ferulic acid (Δ) or gallic acid (□).

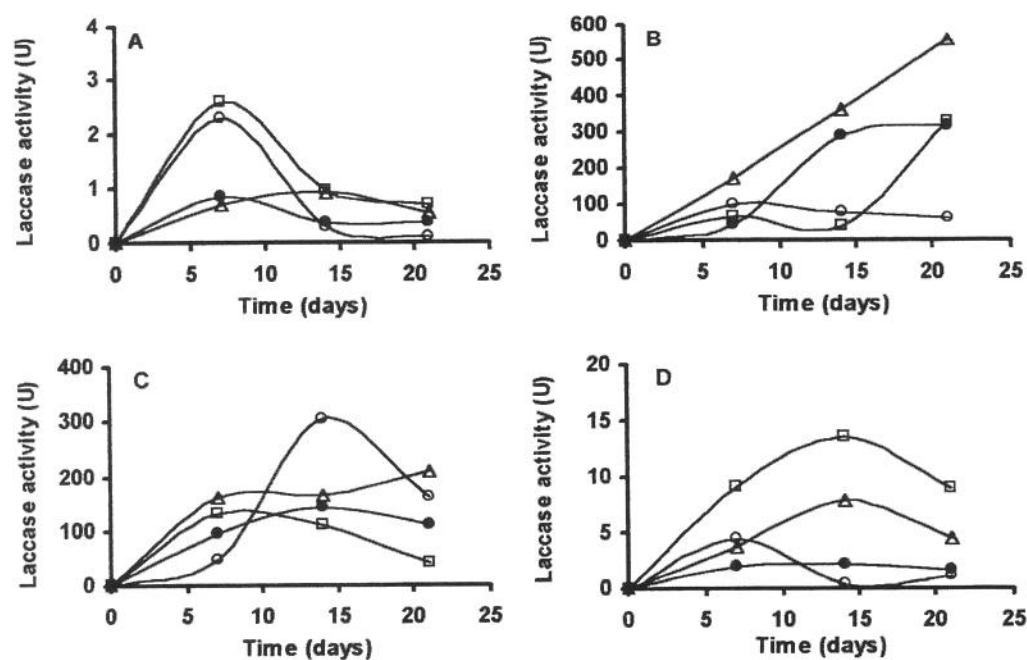


Fig. 2. Laccase activity (U) of A) *Lentinus edodes*, B) *Trametes versicolor*, C) *Trametes villosa* and D) *Botrytis cinerea* cultured for 21 days at 28°C on malt extract agar, in the absence (●) or presence of 2,5-xylidine (0.3 mM (□), 0.8 mM (Δ) and 8 mM (○)).

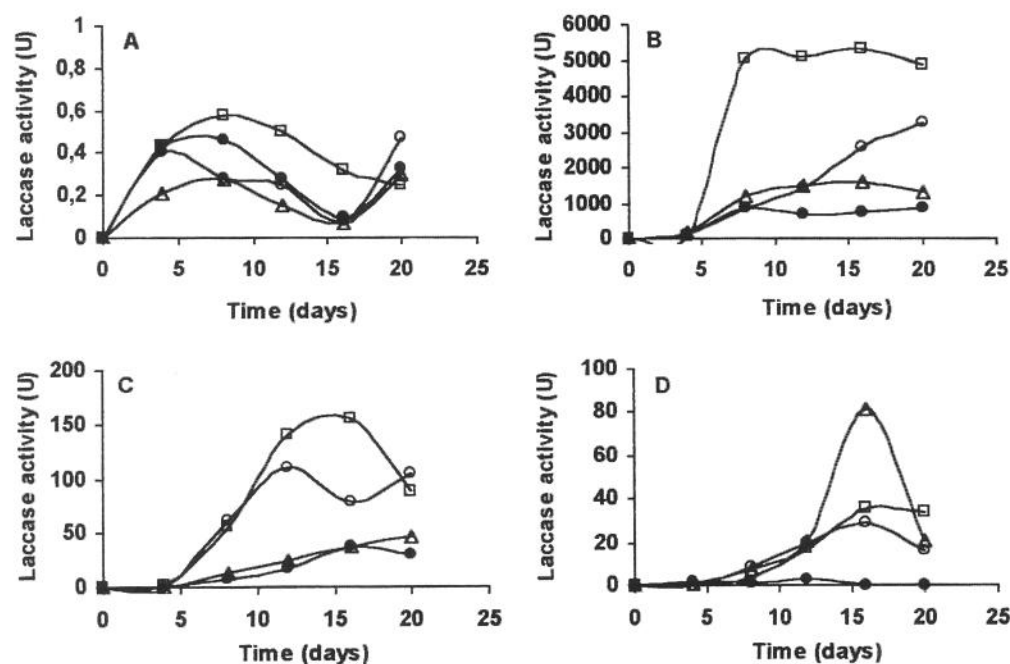


Fig. 3. Laccase activity (U) of A) *Lentinus edodes*, B) *Trametes versicolor*, C) *Trametes villosa* and D) *Botrytis cinerea* cultured for 20 days at 28°C and 240 rpm in a liquid medium in the absence (●) or presence of 0.5 mM 2,5-xylydine (□), acid gallic (△) or ferulic acid (○).

2,5-xylydine was 6.4 times higher than that obtained without this compound (13.5 and 2.1 U, respectively).

3.2. Laccase production in a liquid medium

Inducers (2,5-xylydine, ferulic and gallic acids) added at 96 h of growth were tested for laccase production in fungi at a concentration of 0.5 mM. The best results were obtained with *T. versicolor* in the presence of 2,5-xylydine at 16 days (5317.8 U) and ferulic acid at 20 days of growth (3238.5 U) (Fig. 3B). 2,5-xylydine also showed to be the best inducer in *T. villosa* and *L. edodes* cultures. The highest laccase activities obtained under these conditions were 154.8 U at 16 days and 0.6 U at 8 days of growth (Fig. 3C and 3A), respectively. All inducers tested improved laccase activity in *B. cinerea* cultures (Fig. 3D). The high laccase activity with this fungus was observed in the presence of

gallic acid at 16 days of growth (81.1 U), a value 289.6 times higher than that observed without inducers (0.28 U).

Increasing concentrations of 2,5-xylydine (0.0 – 1.0 mM) were tested for laccase activity in *T. versicolor*. The best 2,5-xylydine concentration was 1.0 mM, reaching a laccase activity of 7741.2 U at 21 days of fungal growth (9.2-fold) (Fig. 4). *Trametes versicolor* cultured in the presence of 1.0 mM 2,5-xylydine showed that the higher the copper sulphate concentration (0.0 – 0.4 mM), the higher the laccase obtained (Fig. 5). This demonstrates the effect of copper on laccase induction. The best result was observed for 0.4 mM at 20 days of growth (12756.4 U), a laccase activity 200.3 times higher than that obtained without copper (63.7 U).

3.3. Kraft E_1 effluent treatment with laccase-mediator system

The results of this work showed that the effluent biotreatments were not efficient in decolorization (data not shown). As higher laccase activity, higher the total phenol reduction observed (Fig. 6). The best value for phenol reduction was around 23% in the absence of mediators. The presence of HBT did not increase the phenol reduction.

We observed that the 3-HAA was rapidly degraded by the laccase, showing that this acid was not a good mediator for this system. However, the AHA, which was not degraded by laccase, acted very efficiently on E_1 effluent reducing 70% and 73% of the total phenol and total organic carbon, respectively (Durán et al., 2001). The calculations considered the phenol and organic charge given by the controls (data not shown).

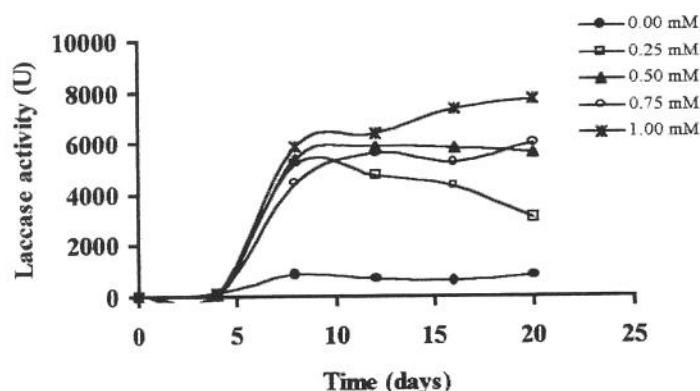


Fig. 4. Laccase activity (U) of *Trametes versicolor* cultured in a liquid medium in the presence of different concentrations of 2,5-xylydine (0.0 – 1.0 mM).

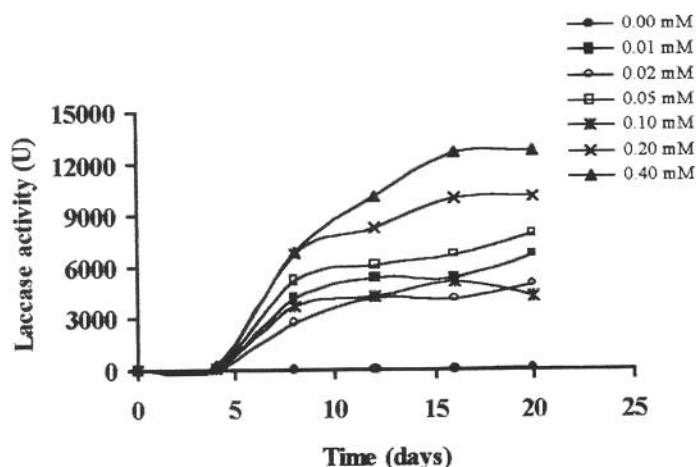


Fig. 5. Laccase activity (U) of *Trametes versicolor* cultured in a liquid medium in the presence of different concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.0 – 1.0 mM).

4. Discussion

The optimum cultivation conditions in semi-solid and liquid medium of *T. versicolor*, *T. villosa*, *L. edodes* and *B. cinerea* for laccase production were studied. All the fungi tested showed the capacity of laccase production under the culture conditions. The high laccase activity was obtained in a liquid culture of *T. versicolor* in the presence of 2,5-xyldine and copper as inducers. The action of 2,5-xyldine as an efficient inducer of laccase has been reported in *T. versicolor* (*Polyporus*, *Coriolus* or *Polystictus versicolor* are synonyms) (Fahraeus and Reinhammar, 1967; Bollag and Leonowicz, 1984; Von Hunolstein et al., 1986; Collins and Dobson, 1997), *T. villosa* (*Polyporus* or *Coriolus pinsitus*) (Yaver et al., 1996), *Pycnoporus*

sanguineus (Pointing et al., 2000), *Pycnoporus cinnabarinus* (Eggert et al., 1996b) and *Phlebia radiata* (Rogalski et al., 1991). This phenolic compound also showed inducer effect in semi-solid and liquid cultures of *L. edodes* and *B. cinerea*. This induction of laccase in *B. cinerea* in the presence of 2,5-xyldine is in contrast to the literature (Gigi et al., 1980; Bollag and Leonowicz, 1984).

According to Collins and Dobson (1997), the expression of laccase in the white-rot fungus *T. versicolor* is regulated at the level of gene transcription by copper and nitrogen. When copper, 2,5-xyldine or both compounds were added to cultures grown in the absence of copper, increased laccase transcript levels were detected within 15 minutes. Corresponding increases in

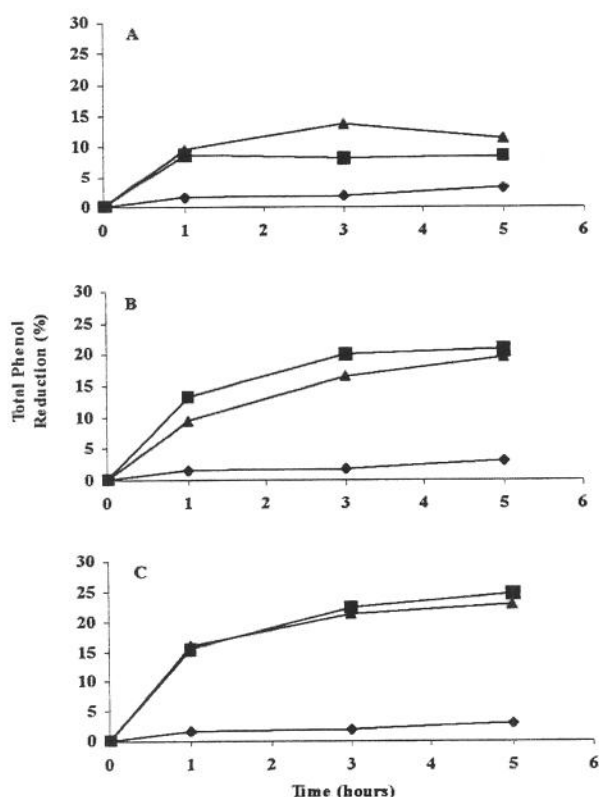


Fig. 6. Total phenol reduction of E₁ effluent (control (●)) treated for 5 hours with laccase (▲), laccase and HBT (■). A) Laccase activity: 25 U; B) Laccase activity: 50 U; C) Laccase activity: 100 U.

laccase activities were observed after 24-h incubation only when copper was present. This finding is in agreement with Palmieri et al. (2000), which show that copper was the most efficient inducer of laccase activity in *Pleurotus ostreatus* and the expression of laccase isoenzymes was regulated at the level of gene transcription. Copper is also reported as laccase inducer in *Phanerochaete chrysosporium* (Dittmer et al., 1997), *Neurospora crassa* (Huber and Lerch, 1987) and *Marasmius quercophilus* (Farnet et al., 1999).

Our results indicate that ferulic acid induced laccase in the fungi tested, mainly in *T. versicolor*. This finding is in contrast to the results of Collins and Dobson (1997) but in agreement with another reports (Leonowicz and Trojanowski, 1978; Leonowicz et al., 1978). Leonowicz and Trojanowski (1975) also show inducer effect of laccase activity by ferulic acid in *Pleurotus ostreatus*. The presence of gallic acid in liquid cultures of *Botrytis cinerea* showed an increase of 289.6 fold in laccase activity, confirming that gallic acid is the best inducer for this fungus (Gigi et al., 1980; Fortina et al., 1996).

Laccases are believed to play an important role in lignin biodegradation. The treatment of kraft E₁ effluent with laccase showed a phenol reduction around 23% in the absence of mediators. Kadhim et al. (1999) showed that culture filtrate extract from *Coriolus versicolor* grown on wheat bran supplemented with yeast extract was used to remove a range of phenolic compounds and the removal was due to the presence of laccase. The effect of the position of chlorine substitution on the benzene ring and its influence on the removal was examined. Phenolic compounds with chlorine substitutions in ortho and para positions were easily attacked by the enzyme unlike compounds with substitutions in the meta position. Jolivald et al.

(1999) also observed that the para- and ortho-hydroxyphenyl derivatives were enzymatically transformed, whereas the meta derivative was not. The performance of laccase depended on the pH, with an optimum for the para-derivative degradation rate at pH 5.

Laccase alone has a limited effect on the degradation of nonphenolic structures in lignin. However, laccase may catalyze reactions in conjunction with several redox mediators to oxidize nonphenolic lignin structures (Call and Mucke, 1997). One of the most intensively studied mediators is 1-hydroxybenzotriazole (HBT), which is oxidized to its nitroxide radical by laccase (Srebotnik and Hammel, 2000). Although, our results showed that the presence of HBT did not increase the phenol reduction in the effluent biotreatment. Recently, Xu et al. (2000) demonstrated that the oxidation rate is dependent on the redox potential difference between the N-hydroxy substrate (HBT for example) and laccase. A laccase with a higher redox potential or an N-hydroxy compound with a lower redox potential tended to have a higher oxidation rate. Bourbonnais et al. (1997) found significant differences in reactivity between laccases from different fungi when they were tested for pulp delignification in the presence of the mediators ABTS and HBT. A more detailed study of *T. versicolor* laccase with ABTS and HBT showed that HBT gave the most extensive delignification over 2 h but deactivated the enzyme, and therefore a higher enzyme dosage was required. They tested seven crude preparations of laccase from various white-rot fungi and found that they were all equally capable of delignifying kraft pulps when they were coupled to ABTS. However, their performances with HBT were much more variable, which can be explained in part by their rates of activity loss in the presence of this mediator. Xu et

al. (1996) compared the reactivities and redox potentials of fungal laccases and concluded that higher redox potential correlates with higher activity. They concluded that structural differences in the substrate activation site (the blue, type I copper center) control the redox potentials, as well as the substrate specificities, of the laccases.

The 3-hydroxyanthranilic acid was rapidly degraded by the laccase showing that this acid was not a good mediator for this system. However, the acetohydroxamic acid, which was not degraded by laccase, acted very efficiently on E₁ effluent, reducing 70% and 73% the total phenol and total organic carbon, respectively. The calculations considered the phenol and organic charge given by the controls. Then, acetohydroxamic acid appears as the best mediator for laccase bioremediation in E₁ effluent and a more detailed study is in progress. The results obtained open the possibility to study laccase-mediator system in bioremediation of industrial effluents.

Acknowledgements

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PURIFICATION, CHARACTERIZATION AND APPLICATION OF LACCASE BY *Trametes versicolor* IN COLOR AND PHENOLIC REMOVAL OF OLIVE MILL WASTEWATER IN THE PRESENCE OF 1-HYDROXYBENZOTRIAZOLE

Rosana C. Minussi^{1,2}, Márcio A. Miranda³, José A. Silva⁴, Carmen V. Ferreira³, Hiroshi Aoyama³, Sérgio Marangoni⁴, Domenico Rotilio⁵, Gláucia M. Pastore¹ and Nelson Durán^{2,6*}

Faculdade de Engenharia de Alimentos¹, Instituto de Química² and Instituto de Biologia³, Universidade Estadual de Campinas (UNICAMP), Campinas - SP, Brazil; Consorzio Mario Negri Sud, "Gennaro Paone" Environmental Health Center⁵, Santa Maria Imbaro, Italy and Núcleo de Ciências Ambientais⁶, Universidade de Mogi das Cruzes, Mogi das Cruzes - SP, Brazil.

Abstract

Laccase forms (L1 and L2) from *Trametes versicolor* CCT 4521 showed a molecular mass of 66 kDa and optimum temperature around 40°C. The pH optimum (4.0 and 5.0) and K_m (28.6 and 5 μ M) values using syringaldazine as substrate for L1 and L2 were found, respectively. The enzymes were able to oxidize several compounds and were strongly inhibited by sodium azide, L-cysteine and dithiothreitol. The N-terminal sequences showed a 75% of identical residues among both forms and similarities around 40–60% to those laccases from wood-degrading fungi. The use of 1-hydroxybenzotriazole as a mediator extended the oxidized compounds by laccases in olive mill wastewater.

Keywords: laccase, *Trametes versicolor*, 1-hydroxybenzotriazole, mediator, olive mill wastewater

1. Introduction

Laccase (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) is an enzyme that oxidizes a large variety of organic substrates. The enzyme is widely distributed in fungi; however its biological function is still not fully clarified [1]. Fungal laccases have been implicated in sporulation, rhizomorph formation, pathogenesis, formation of fruity bodies and lignin degradation [2]. There has been great interest in using fungal laccases for biotechnological processes due to their chemical and catalytic features [3]. In recent years, interest in laccases has been fuelled by their potential uses in detoxification of environmental pollutants, prevention of wine decolouration, paper processing, enzymatic conversion of chemical intermediates, and production of useful chemicals from lignin [4].

Comparative studies of fungal laccases have shown that these enzymes are similar in their catalytic activity on phenolic compounds, regardless of their origin, but differ markedly in their inducibility, number of enzyme forms, molecular mass and pH optimum [5]. Laccase alone has a limited effect on bioremediation due to its

specificity for phenolic subunits in lignin. Bourbonnais and Paice [6] found that the substrate range of laccase can be extended to nonphenolic subunits of lignin by inclusion of a mediator, such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS). The laccase-ABTS couple has been shown to effectively demethylate and delignify kraft pulp [7]. Furthermore, 1-hydroxybenzotriazole (HBT) has been found to mediate pulp delignification in the presence of laccase [8], phenothiazines have been found to bleach and prevent redeposition of azo dyes in textile manufacturing [9], and 3-hydroxyanthranilic acid was observed to oxidize nonphenolic lignin model compounds [10]. The use of a laccase-mediator system is one of the promising possibilities for an environmentally benign pulp-bleaching process [11] and decolourisation of textile dyes [12].

In the extraction process of the olive oil production, the following two by-products are obtained along with the oil (which accounts for 20% of the total): a solid residue (30% of the total) and a black wastewater (50% of the total) called olive mill wastewater (OMW). Most of the solid

residue is used as fuel, but the OMW is an environmental problem for the Mediterranean countries [13]. An important step in the degradation of OMW is the breakdown of coloured polymeric phenolics (decolourisation) to monomers, which can subsequently be mineralised.

In a previous paper [14] we showed that *Trametes versicolor* CCT 4521 was selected as the best laccase producer in a liquid medium containing 2,5-xyldine as inducer. The aim of this work was to develop a procedure for the purification of extracellular laccases from *Trametes versicolor* CCT 4521, to determine the individual physicochemical and catalytic properties of these enzymes and to study the effects of laccase-mediator system on the treatment of olive mill wastewater, since no study in this direction was published before.

2. Materials and methods

2.1. Fungal strain, culture conditions and laccase assay

Trametes versicolor CCT 4521 is a white-rot fungus isolated in Brazil by Dr. Elisa Esposito. This fungal strain was maintained on malt extract agar plates at 28°C. Laccase was obtained growing the fungus in a liquid medium containing (g/l): peptone, 10; malt extract, 5; CuSO₄ .5H₂O, 0.005 and glucose, 20; at pH 5.4. Laccase induction consisted in the addition of 1.0 mM 2,5-xyldine at 96 hours of growth. The 1-liter Erlenmeyer flasks containing 200 ml of culture medium were inoculated with 8 fungal disks (7 mm diameter) and incubated for 20 days at 28°C and 240 rpm on a rotary shaker.

Laccase activity was assayed by measuring oxidation of syringaldazine at 525 nm for 5 minutes [15]. 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. One

unit of laccase activity was defined as 1 µmol of syringaldazine oxidized per min per litre of the culture filtrate. The effect of pH on laccase activity was measured over the range of 3.0 to 6.0 in 50 mM citrate-phosphate buffer and 7.0 to 8.0 in 50 mM Hepes buffer. The effects of temperature on enzyme activity and stability (after the incubation time of 20 minutes) were measured at pH optimum of each laccase isoform with 50 mM citrate-phosphate buffer at a range of 25 to 80°C. All spectrophotometric measurements were carried out on an Agilent 8453E UV-visible Spectrophotometer System coupled with temperature control. All values represent the mean of duplicate measurement with sample mean deviation of less than 5%.

2.2. Purification of extracellular laccase

The fungal culture filtrate (380 mesh sieve) was frozen, thawed, filtrated (Millipore 0.45 µm) and lyophilised. The concentrated crude extract was applied to an ion exchange DEAE Sephadex A-50 column (4 by 20 cm; Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.05 M citrate phosphate buffer, pH 5.0. Fractions containing laccase activity were eluted with the equilibrating buffer in two distinct peaks (L1 and L2). The fractions corresponded to L2 peak were lyophilised and loaded onto a gel filtration Sephacryl S-200-HR column (2.5 by 95 cm; Sigma) equilibrated with the same buffer containing 0.15 M NaCl in a FPLC System (Pharmacia). Laccase containing fractions were pooled, concentrated, dialysed and stored at -20°C until further use.

Protein concentrations were determined by the method of Lowry et al. [16] using bovine serum albumin as the standard. The protein concentration of the fractions collected during chromatography was estimated by the absorbance at 280 nm.

2.3. Gel electrophoresis and molecular mass

The molecular mass under native conditions was estimated by gel filtration chromatography (Superdex 200, Pharmacia). Markers with molecular masses ranging from 14 to 94 kDa were used as standards. Apparent molecular mass under denaturing conditions and subunit composition of the laccase were estimated by 15% SDS-PAGE according to the method of Laemmli [18]. The laccase was subject to SDS-PAGE under reducing (0.1 M DTT) and non-reducing conditions. The gel was stained with Coomassie Blue R-250. Non-SDS 12.5% polyacrylamide gel was used for carbohydrate content determination using periodic acid-Schiff's reagent as described by Korn and Wright [19]. Albumin and asialofetuin were used as glycosylated controls (positive staining) and trypsin was used as non-glycosylated control.

2.4. Amino acid composition, N-terminal amino acid sequence and carbohydrate content

Reversed-phase HPLC on a μ -Bondapack C18 column (Waters System) [20] was also used to verify the purity of laccases and these samples were used in amino acid composition and N-terminal analyses. The column was preequilibrated with 0.1 (v/v) trifluoroacetic acid (solvent A) and initially eluted (2 ml/min) with this solution followed, after 80 min, by a gradient of acetonitrile in 0.1% TFA (0–45% in 10 min, 45–74% in 70 min, and 75–100% in 75 min). The elution profile was monitored at 280 nm.

Amino acid analysis was performed in a Pico-Tag amino acid analyser (Waters System). The purified sample was hydrolysed with 6N HCl containing 1% phenol (v/v) at 106°C for 24 hr. Hydrolysates reacted with 20 μ L of fresh derivatization solution (v/v, 7:11:1:1; ethanol: triethylamine: water: phenylisothiocyanate) for 1 hr

at room temperature. The phenylthiocarbamyl (PTC) amino acids were identified by HPLC.

A sample of 500 pmoles of the enzyme was used to determine its N-terminal sequence in an automatic Edman sequencer (Applied Biosystems model 477A sequencer). Phenylthiohydantoin (PTH) amino acids were identified in a model 120-A PTH-amino acid analyser, according to the retention times of a 20 PTH-amino acid standard. The sequence was submitted to automatic alignment, which was performed using the NCBI-Blast search system.

The neutral carbohydrate content of laccase was determined according to the method of Dubois et al. [17] using mannose as standard.

2.5. Substrate specificity and inhibition

Spectrophotometric measurements of substrate oxidation by *T. versicolor* laccases were carried out at 40°C in a 1-mL reaction volume containing the test substrates in 50 mM citrate-phosphate buffer (pH optimal of each laccase). The concentrations of both enzymes were adjusted to give the same oxidation rate of ABTS. The effect of potential inhibitors of the laccase activity was monitored with 0.1 mM syringaldazine as substrate in 0.05 mM citrate phosphate buffer (pH optimal of each laccase). Michaelis constant (K_m) was calculated using syringaldazine as substrate (concentrations between 4 and 100 μ M) from Lineweaver-Burk plot.

2.6. Olive mill wastewater treatment

The fungal culture filtrate (Millipore 0.45 μ m) was lyophilised, resuspended in 50 mM citrate-phosphate buffer (pH 5.0) and precipitated with 90% ammonium sulphate. The enzyme was eluted in a gel chromatography column (Sephacryl S-200,

Table 1

Purification of extracellular laccases by *Trametes versicolor* CCT 4521

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	200.0	762.9	2,227.7	2.9	100.0	
DEAE-Sephadex A-50 (L1)	58.0	5.4	127.3	23.6		8.1
DEAE-Sephadex A-50 (L2)	58.5	221.0	898.9	4.1	46.1 ^a	1.4
Sephacryl S-200 (L2)	61.0	7.2	727.3	101.0	38.4 ^b	34.8

^aThis value is the yield percentage for DEAE-Sephadex A-50 purification step (L1 and L2).^bThis value is the yield percentage for DEAE-Sephadex A-50 (L1) and Sephacryl S-200 (L2) purification steps.

Sigma) and lyophilised. The laccase activity in a liquid stock solution was around 100,000.0 U/l.

The olive mill wastewater (Abruzzo, Italy) was treated with laccase (2,000.0 U/l) in the presence and absence of the mediator 1-hydroxybenzotriazole (HBT) at 0.5 mM concentration. Total phenols [21] and colour [22] were monitored from 0 to 24 hr of treatment. During the treatment the effluent was kept in the dark and low agitation.

3. Results and discussion

Laccases were purified to homogeneity according to the procedure summarized in Table 1. Two chromatographic steps were required to purify the laccases. DEAE Sephadex A-50 chromatography removed a great amount of the brown-coloured pigments and yielded two distinct fractions with high laccase activity (L1 and L2). L1 fraction was absent of pigments or another enzymes. The L2 fraction with the presence of brown pigments was further purified to homogeneity with a gel filtration chromatography. The overall yield of the purification was around 38% with a purification fold of 35 for L2 and 8 for L1.

The molecular mass of purified laccases was found to be approximately 66 kDa, as determined by calibrated gel filtration and SDS-PAGE (Fig. 1).

This value is consistent with most fungal laccases. Laccases showed activity after SDS-PAGE. This was reported by another laccases and is maybe caused by the SDS protection [23]. The enzymes were found to be glycoproteins (data not shown) containing a carbohydrate content of approximately 7%.

A typical blue laccase normally contains three types of copper sites. The purified laccases had a UV-Visible spectrum with peaks of maximal absorption around 597 (L1) and 613 nm (L2), corresponding to the type-1 copper site, which is responsible for the deep blue colour of the enzymes.

The effect of pH value on the activity was examined in the range 3.0-8.0, using syringaldazine as substrate. L1 showed to be active in acidic pH range, with an optimum at pH 4.0, whereas retained 32.3 and 10.3% at pH 3.0 and 6.0, respectively. The pH optimum for L2 was 5.0, with residual activity of 68.7 and 29.0% at pH 4.0 and 6.0, respectively. Both forms showed to be inactive at pH 8.0. The temperature optimum was 40°C for both forms, showing remaining activity around 65.0 and 37.0% at 60 and 70°C, respectively. Although, L1 showed to be stable up to 60°C within 20 minutes (47.6% of residual activity at 70°C) whereas L2 was stable up to 50°C (28.1% of remaining activity at 60°C). Incubation of L1 at 80°C and L2 at 70°C for 20 minutes caused a complete loss of activity.

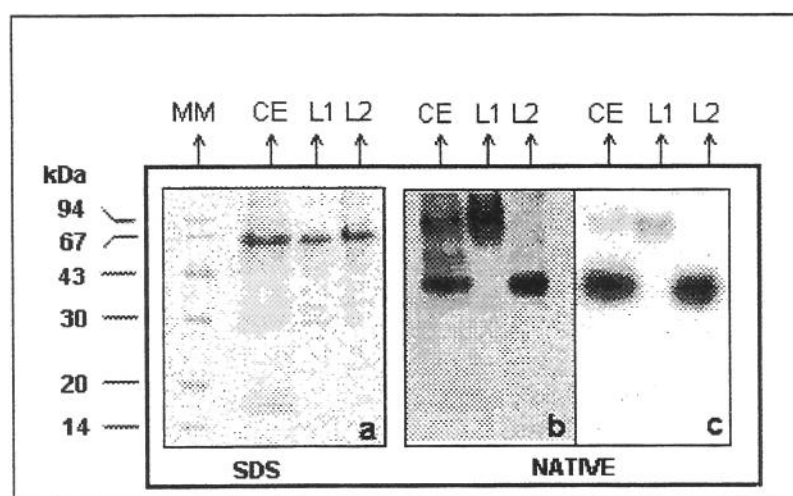


Fig. 1. Polyacrylamide gel electrophoresis of extracellular laccases by *T. versicolor* CCT 4521. SDS-PAGE (a) stained with Coomassie Blue R-250 for protein. MM, molecular mass markers; CE, crude extract. PAGE of crude extract and purified laccases stained with Coomassie Blue R-250 for proteins (b) and with 0.1 mM ABTS in citrate-phosphate buffer, pH 5.0, for laccase activity (c).

The substrate specificity of purified laccases was studied with several compounds. Like other laccases [24; 25], L1 and L2 were able to oxidize a variety of phenolic compounds, including simple diphenol (hydroquinone, catechol), methoxy-substituted monophenols (guaiacol, 2,6-dimethoxyphenol) and other substrates such as ABTS and o-dianisidine, including syringaldazine, which is considered a specific substrate for laccase. As expected for a laccase-like enzyme, no activity towards tyrosine was observed. The Lineweaver-Burk plot yielded a K_m value of 28.6 μ M for L1 and 5 μ M for L2 using syringaldazine as substrate.

The inactivation of laccases by various concentrations of potential inhibitors is shown in Table 3. The purified laccases were strongly inhibited by sodium azide, L-cysteine and dithiothreitol, whereas EDTA affected laccase activities to a lesser extent.

Table 4 presents the amino acid composition of laccases and it is in agreement with other laccases [24; 26]. In order to acquire information on the primary structure of the proteins, their N-terminal sequence was determined. L1 and L2 showed a 75% of identical residues (Table 5) and were non-redundant with another sequences. The N-terminal

Table 2
Substrate specificity of laccases by *Trametes versicolor* CCT 4521

Substrate	Conc. (mM)	E_{max} ($M^{-1}cm^{-1}$)	Wavelength (nm)	Laccase activity (%) ^a	
				L1	L2
ABTS	1.0	36,000	420	100.0	100.0
Hydroquinone	1.0	17,542	248	85.1	88.8
2,6-Dimethoxyphenol	1.0	35,645	470	44.1	40.3
Guaiacol	1.0	6,400	436	31.2	21.3
Syringaldazine	0.1	65,000	525	23.1	36.6
Catechol	1.0	2,211	392	22.3	17.3
o-Dianisidine	0.1	29,400	460	9.9	12.6
Tyrosine	1.0	-	280	ND ^b	ND

^aAll values represent the mean of duplicate measurements with a sample mean deviation of less than 5%.

^bND, not detected.

Table 3

Effect of inhibitors on the oxidation of syringaldazine by purified *T. versicolor* CCT 4521 laccases

Compound	Concentration (mM)	Inhibition (%) ^a	
		L1	L2
Sodium azide	0.0001	16.0	14.3
	0.0005	62.7	20.0
	0.0010	100.0	30.3
	0.0100	100.0	75.6
	0.1000	100.0	100.0
EDTA	50.0	8.4	25.3
L-Cysteine	0.05	73.0	88.1
	0.10	86.9	91.7
	0.50	100.0	100.0
Dithiothreitol	0.05	49.8	87.7
	0.10	66.4	95.0
	0.50	100.0	100.0

^aAll values represent the mean of duplicate measurements with a sample deviation of less than 5%.

sequences of purified laccases have a high similarity (40–60%) to those laccases from wood-degrading fungi.

Table 4

Amino acid composition of purified *T. versicolor* CCT4521 laccases

Amino acid	Number of amino acid residues/molecule of laccase	
	L1	L2
Asp	63	68
Glu	38	33
Ser	43	49
Gly	45	55
His	17	16
Arg	18	12
Thr	44	39
Ala	56	56
Pro	42	48
Tyr	17	6
Val	40	46
Met	6	3
Cys	3	0
Ile	31	31
Leu	51	28
Phe	49	94
Lys	18	9

Olive mill wastewaters originating from olive extraction units are the most polluted wastewaters of the food industry, which present toxicity towards plants as well as certain impacts to the hydro-flora

[34]. Two opposite approaches can be conducted with this effluent are sewage disposal and spreading on soils. However, a drastic reduction in phenol concentration is a prerequisite for both techniques. The treatment of OMW with semi-purified laccase by *T. versicolor* CCT 4521 indicated a significant total phenol reduction in the mediator (HBT) presence and absence, although the kinetic of phenolic reduction was faster and higher in the presence of HBT (Fig. 2A). In 3 hours of treatment, 50 % and 30% of phenolic reductions were observed in the presence and absence of HBT, respectively. Phenolic reductions around 67% and 55% were observed after 24 hours in the presence and absence of HBT, respectively. The presence of HBT also enhanced colour reduction of OMW, the results are shown in Fig. 2B. Colour removals of 27 and 40% were achieved in the presence of HBT, whereas only 7 and 15% were observed in the absence of this mediator after 5 and 24 hours of treatment, respectively.

The efficiency of laccase in polyphenol reduction of OMW is known [35; 36] but there is no report about the laccase-mediator system applied in this kind of effluent. In previous reports, laccases by *T. versicolor* CCT 4521 was able to decolourise

Table 5

Comparison of N-terminal amino acid sequences of *T. versicolor* CCT4521 and other fungal laccases

Microorganism	Reference	N-terminal amino acid sequence
<i>T. versicolor</i> CCT4521 (L1)		A I G P V A L L A T A P N A A V P D G F L R A A V V S V
(L2)		A I G P K A L L A D A P N A A D P D G I L R A A V F V N
<i>T. villosa</i> (L1)	[27]	G I G P V A D L T I T N A A V S P D G F S R Q A V V V N
(L2 and L3)		A I G P V A D L V V A N A P V S P D G F L R D A I V V N
<i>T. versicolor</i> (I)	[28]	A I G P V A S L V V A N A P V S P D G F L R D A I V V N
(II)		G I G P V A D L T I T D A A V S P D G F S R Q A V V V N
<i>Coriolus hirsutus</i>	[29]	A I G P T A D L T I S N A E V S P D G F A R Q A V V V N
<i>Pycnoporus cinnabarinus</i>	[30]	A I G P V A D L T L T N A A V S P D G F S
<i>Ceriporiopsis subvermispora</i>	[31]	A I G P V T D I E I T D A F V S P D G P
<i>Pleurotus ostreatus</i>	[32]	A I G P A G N M Y I V N E D V S P D G F
<i>Phlebia radiata</i>	[33]	S I G P V T D F H I V N A A V S P D G F

some reactive dyes in free and immobilized forms in the presence of HBT [37]. Although, the laccase-HBT system did not increase phenol and colour reduction in paper and pulp effluent (E_1 effluent) [38]. In this case, the laccase-acetohydroxamic acid appears as the best system.

Thus, laccases by *T. versicolor* CCT 4521 cultivated in the presence of 2,5-xyldine were purified by an easy and fast process. The purified laccases physicochemical and catalytic properties are similar to the analogous enzymes of other basidiomycetes and they have high potential for industrial application, mainly in the immobilised form. These previous results open the possibility to

apply laccase-mediator system in phenolic and colour reduction of olive mill wastewater.

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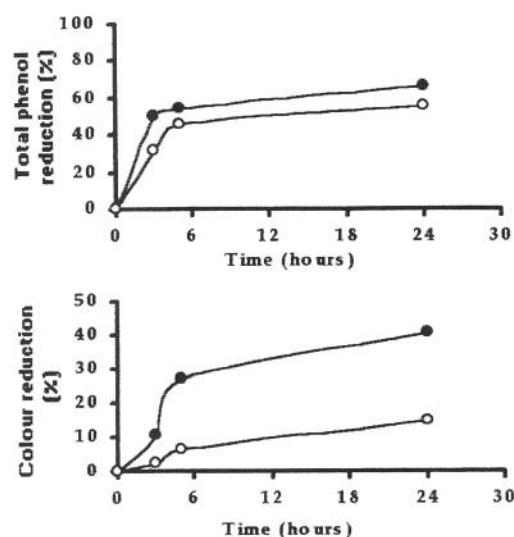


Fig. 2. Total phenol and colour reduction of OMW treated with laccase in the presence (●) and absence (○) of 0.5 mM HBT.

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BIODECOLORIZATION SCREENING OF SYNTHETIC DYES BY FOUR WHITE-ROT FUNGI IN A SOLID MEDIUM: POSSIBLE ROLE OF SIDEROPHORES

Rosana Cristina Minussi¹, Sandra Gomes de Moraes², Gláucia Maria Pastore¹ and Nelson Durán^{2,3}

Food Biochemistry Laboratory, Faculdade de Engenharia de Alimentos¹ and Biological Chemistry Laboratory, Instituto de Química² - Universidade Estadual de Campinas and Núcleo de Ciências Ambientais, Universidade de Mogi das Cruzes, SP, Brazil.

SUMMARY

Aims: Four selected fungi were screened for their ability to decolourise a textile effluent and commercial reactive dyes in a solid medium.

Methods: Ligninolytic enzymes activities (lignin peroxidase, manganese peroxidase and laccase) and siderophores presence were monitored in decolourized plates.

Results: The results showed low lignin peroxidase activity and no manganese peroxidase activity was detected for all fungi. Laccase activity was observed in Reactive Blue 19 decolourised plates by *Trametes versicolor* and *Trametes villosa*. Siderophores presence was observed in *Trametes versicolor*, *Phanerochaete chrysosporium* and *Lentinus edodes* decolourised plates.

Conclusions: *Lentinus edodes* displayed the greatest decolourization ability both in terms of extent and rapidity of decolourization.

Significance and Impact of the Study: The transformation observed for dyes open the possibility to study siderophores with a high oxido-reduction potential to treat dyes and textile effluents.

INTRODUCTION

The two major sources of dye release into the environment are the textile and dyestuff manufacturing industries (Nigam *et al.* 1996). Existing physical/chemical technologies for colour removal are very expensive and commercially unattractive (Beydilli *et al.*, 1998). Biological processes provide an alternative to existing technologies because they are more cost-effective, environmentally friendly, and do not produce large quantities of sludge (Azmi *et al.* 1998).

Synthetic dyes are not uniformly susceptible to biodegradation in conventional biological wastewater treatment processes because of their resistance to microbial attack (Wang and Yu 1998). Azo dyes, which are used extensively in many industries, are the largest class with a wide variety of colours and structure. White-rot fungi are attractive organisms for use in the decontamination of pollutant sites. They are capable of mineralising a wide variety of toxic xenobiotics (Knapp and Newby 1999), are ubiquitous in natural

environments and have the potential to oxidize substrates which low solubility because the key enzymes involved in the oxidation of several pollutants are extracellular (Reddy 1995).

According to Kim *et al.* (1995), the effectiveness of decolorization depends on the structure and complexity of each dye. Relatively small structural differences can markedly affect decolorization. These differences are presumably due, at least in part, to electron distribution and charge density, although steric factors may also contribute (Knapp *et al.* 1995).

The complex enzymatic systems responsible for organo-pollutant degradation and the conditions under which they are expressed vary among the white-rot fungi. The enzymatic systems of the white-rot fungi have not been fully characterized; *P. chrysosporium* and *L. edodes* are, in general, known to produce lignin peroxidase and manganese peroxidase, while *T. versicolor* produces laccases. Laccase shows broad specificity in the detoxification of a number of aquatic and terrestrial

xenobiotics, industrial wastewater and biotechnological industrial products (Rogalski *et al.* 1999). Another alternative for industrial effluent treatment is the use of natural chelating agents, siderophores, produced by fungi and bacteria. These substances show a high affinity constant for metals such as iron, and are able to reduce iron (III) to (II), exhibiting phenoloxidase-like activity (Goodell *et al.* 1997; Durán *et al.* 1999; Durán and Esposito 2000).

The aim of this work was to screen selected fungi for their ability to decolorize a textile effluent and the textile dyes Reactive Blue 19, Reactive Red 195, Reactive Yellow 145 and Reactive Black 5 in a solid medium.

MATERIALS AND METHODS

Fungal strains and solid-plate decoloration studies

The cultures of *Lentinus edodes* CCT 4519, *Trametes versicolor* CCT 4521, *Phanerochaete chrysosporium* ATCC 24725 and *Trametes villosa* CCT 5567 were maintained on malt extract agar plates at 28°C. The plates containing potato dextrose agar in the presence of each dye or the textile effluent were inoculated with the respective fungi at the centre (one agar disc punched from cultures growing on malt extract agar plates). Four commercial reactive dyes were used as substrates: Reactive Blue 19 (0.05%), Reactive Red 195 (0.025%), Reactive Yellow 145 (0.05%) and Reactive Black 5 (0.05%). These dyes are used in the textile industry, are of commercial quality and were tested without prior purification. Agar plates containing the dyes were all incubated at 28°C excepting those inoculated with *P. chrysosporium*, which were incubated at 37°C. The efficiency of biodecolorization was assessed by the visual

disappearance of colour from the plates, starting from the third to the 25th day of growth.

All analyses were carried out in duplicate and the results represent mean values of two experimental repetitions.

Enzyme assays

The enzymes were extracted from the solid medium using 20 mmol l⁻¹ citrate-phosphate buffer (pH 5.0). Cultures were filtered through a Millipore membrane (0.45 µm) and the enzymatic activities were determined in the culture filtrate.

Laccase activity was assayed by monitoring the oxidation of the syringaldazine at 525 nm. The reaction mixture contained 0.6 ml the culture filtrate, 0.3 ml 50 mmol l⁻¹ citrate-phosphate buffer (pH 5.0) and 0.1 ml 1.0 mmol l⁻¹ syringaldazine. One unit of laccase activity was defined as the amount of enzyme necessary to oxidize one micromole of substrate per minute per litre of the culture filtrate (Szklař *et al.* 1989).

Lignin peroxidase (LiP) activity was determined by the oxidation of veratrylic alcohol at 310 nm. The reaction mixture was composed of 0.5 ml culture filtrate, 0.05 ml 10 mmol l⁻¹ H₂O₂, 0.375 ml 0.33 mol l⁻¹ sodium tartrate (pH 3.0), 0.45 ml distilled water and 0.125 ml 4 mmol l⁻¹ veratrylic alcohol. One unit of LiP corresponded to the amount of enzyme that oxidized one micromole of veratrylic alcohol per minute per litre of culture filtrate (Tien and Kirk 1984).

Manganese peroxidase (MnP) activity was determined by the oxidation of phenol red at 610 nm. The reaction mixture was composed of 0.5 ml culture filtrate, 0.1 ml 0.25 mol l⁻¹ sodium lactate, 0.2 ml 0.5% bovine albumin, 0.05 ml 2.0 mmol l⁻¹ MnSO₄, 0.05 ml 2.0 mmol l⁻¹ H₂O₂ (prepared in 0.2 mol l⁻¹ sodium succinate buffer, pH 4.5) and 0.1 ml 0.1% phenol red. The reaction mixture was

incubated for 5 minutes at 30°C and the reaction was interrupted by the addition of 40 µl 2.0 mol l⁻¹ NaOH. One unit of MnP was defined as the amount of enzyme that oxidized one micromole of phenol red per minute per litre of culture filtrate (Kuwahara *et al.* 1984).

Siderophores determination

Siderophore activity was determined with 0.5 ml Chrome Azurol S (CAS) mixed with 0.5 ml of the sample (broth extracted with acetate buffer, pH 4.0). The solution of Chrome Azurol S was prepared according to Schwyn and Neilands (1987). A reference sample was prepared with the same buffer used for the extracted sample. The absorbance at 630 nm was measured at time zero and after 20 h of reaction at room temperature. Positive reactions were qualitatively estimated by colour change (blue to orange). The percentage of iron-binding compounds of the siderophore type was calculated according to this equation:

$$[(Ar - As)/Ar] \times 100 = \% \text{ of CAS unit}$$

where: Ar = reference absorbance and As = sample absorbance. The assay was considered negative when no change in the blue colour was observed.

RESULTS

The ability of four white-rot fungi to decolorize four synthetic dyes and a textile effluent in a solid medium was evaluated. The plate surface appearance, such as halo fungal growth and clear halo formation, was observed visually daily. All fungi decolorized some dyes, and all dyes were decolorized to some extent (Fig. 1). Uninoculated controls showed no colour removal. The decolorization rates of all dyes were different for the white-rot fungi.

Lentinus edodes exhibited the highest decolorization rate against Reactive Blue 19, with all the dye decolorized after 7 days (Fig. 1A). *Trametes versicolor* and *T. villosa* showed 100% decolorization in 12 days. *Phanerochaete chrysosporium* started to decolorize Reactive Blue 19 after 5 days, and showed around 50% of colour removal at 10 days. Reactive Red 195 exhibited a similar initial decolorization rate to Reactive Blue 19 for *L. edodes* (total decolorization was observed in approximately 9 days), but was recalcitrant for *T. villosa* (Fig. 1B). The extent of decolorization for *P. chrysosporium* was 90% and for *T. versicolor* 50%, after 10 days.

Reactive Yellow 145 was totally decolorized after 9 days of cultivation with *P. chrysosporium* and 10 days with *L. edodes*. *Trametes versicolor* started to decolorize after 10 days and showed around 30% of colour removal after 25 days. Decolorization was not observed in the presence of *T. villosa* (Fig. 1C). The most recalcitrant dye was Reactive Black 5 (Fig. 1D). No decolorization was observed in the presence of *T. villosa* or *P. chrysosporium*. Maximum decolorization was obtained with *L. edodes* (75% at 22 days) and *T. versicolor* (25% at 16 days).

Decolorization of a textile effluent by the white-rot fungi is shown in Fig. 1E. Maximum decolorization (100%) was obtained with *L. edodes* at 18 days. After this time, *T. versicolor* reached around 30% of colour removal while *T. villosa* and *P. chrysosporium* did not show decolorization until 25 days.

The results showed low enzymatic activity for lignin peroxidase (less than 1 U l⁻¹). No manganese peroxidase activity could be detected in any of the fungal strains cultured under the growth conditions

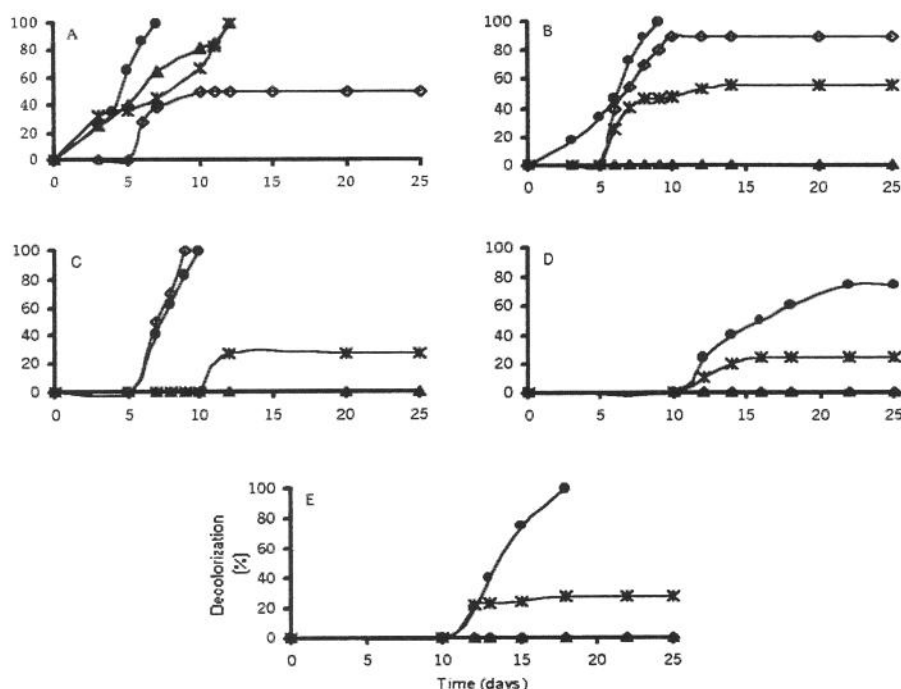


Figure 1. Decolorization (%) of A) Reactive Blue 19 (0.05%), B) Reactive Red 195 (0.025%), C) Reactive Yellow 145 (0.05%), D) Reactive Black 5 (0.05%) and E) textile effluent by fungi during 25 days of cultivation on potato dextrose agar plates. (●) *Lentinus edodes*, (*) *Trametes versicolor*, (▲) *Trametes villosa* and (◇) *Phanerochaete chrysosporium*

used. Laccase activities around 20-30 U l⁻¹ were observed in the Reactive Blue 19 decolorized plates by *T. versicolor* and *T. villosa*.

The production of siderophores by the white rot fungi was evaluated. The maximum production values are listed in Table 1.

The CAS universal assay for siderophore activity indicated that these compounds were produced by three fungi, *T. versicolor*, *P. chrysosporium* and *L. edodes* in the decolorized plates. The highest value was obtained in Reactive Red 195 decolorized plates by *T. versicolor* (68% of CAS unit). *Trametes villosa* did not show siderophore production under these growth conditions.

DISCUSSION

The ability of white-rot fungi to degrade synthetic dyes of diverse structures in agar plates

varied significantly. Heinfling *et al.* (1997) showed that from 18 fungal strains tested for their potential to decolorize commercial reactive textile dyes, only *Bjerkandera adusta*, *T. versicolor* and *P. chrysosporium* were able to decolorize all of the dyes. Of the five species of white-rot fungi tested for their ability to decolorize Amaranth, Remazol Black B, Remazol Orange, Remazol Brilliant Blue, Reactive Blue and Tropaeolin O, *Bjerkandera sp.* BOS55, *P. chrysosporium*, and *T. versicolor* displayed the greatest activity (Swamy and Ramsay 1999).

Knapp *et al.* (1995) reported that some wood-rotting fungi are also able to decolorize dyes, with several showing superior activity to *P. chrysosporium* with respect to both the rate and range of substrates degraded. Comparison of *P. chrysosporium* (ATCC 24725) and *Pleurotus ostreatus* (IE8) against 23 industrial dyes showed

that *P. ostreatus* was able to decolorize 12 while *P. chrysosporium* decolorized only 5 of these dyes (Rodríguez *et al.* 1999).

As previously observed (Knapp *et al.* 1995), we were unable to correlate dye decolorization with

Table 1. Siderophores production by white-rot fungi in the decolorized plates

Dyes/Fungi	<i>L. edodes</i>	<i>P. chrysosporium</i>	<i>T. versicolor</i>
Reactive Blue 19	49	20	21
Reactive Red 195	57	48	68
Reactive Yellow 145	56	42	ND
Reactive Black 5	30	ND	ND

Results are expressed as percentage of Chrome Azurol S (CAS) unit.

ND = not detected

lignin peroxidase and manganese peroxidase activities. We were only able to demonstrate laccase activities in Reactive Blue 19 inoculated with *T. versicolor* and *T. villosa*. Rodríguez *et al.* (1999) showed that several industrial dyes were decolorized by extracellular enzymes from various strains of white-rot fungi, and that this decolorization capacity correlated with the presence of laccase. Chivukula and Renganathan (1995) demonstrated that laccase from *Pyricularia oryzae* was capable of oxidizing phenolic azo dyes, presumably because this reaction releases azo linkages as molecular nitrogen, which prohibits aromatic amine formation.

Lentinus edodes was the only fungus showed siderophore production in the decolorized plates of the four dyes tested. Siderophores are specific chelants with high affinity constants for metals, forming complexes with high stability. They are produced by microorganisms for sequestering iron in environments with low bioavailability for this metal (Durán *et al.* 1999). Hydroxamate iron complexes and iron-binding catechols with phenoloxidase activity have been shown to act on lignin and chlorolignin (Parra *et al.* 1998a,b). Siderophore production has been demonstrated for *Gloeophyllum trabeum*, *T. versicolor*, *T. villosa*, *L. edodes*, *Azobacter vinelandii* and *Pseudomonas*

fluorescence (Durán *et al.* 1999). In a kraft E₁ effluent, the more effective fungi for decolorization were *G. trabeum* (54%), *T. versicolor* (43%) and *Ps. fluorescence* (10%). The same authors reported that an ethyl acetate extract from *L. edodes* broth containing mainly catechol-like siderophores was used in the biobleaching of *Pinus radiata* kraft pulp, resulting in a decrease of 3.5 units of kappa number. Until now, there have been no reports of these compounds acting on dye decolorization.

In summary, *Lentinus edodes* displayed the greatest decolorization ability, in terms of both extent and speed, in agar plates, and has potential for use in the treatment of dye-contaminated effluent. The transformation observed for dyes opens the possibility of studying siderophores to treat dyes and textile effluents.

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RELATIONSHIP BETWEEN TOTAL ANTIOXIDANT POTENTIAL AND TOTAL PHENOL CONTENT OF COMMERCIAL WINES

Rosana C Minussi^{1,2}, Massimo Rossi³, Luciano Bologna³, Livia Cordi², Domenico Rotilio³, Gláucia M Pastore¹ and Nelson Durán^{2*}

¹Food Biochemistry Laboratory, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970, Campinas - SP, Brazil; ²Biological Chemistry Laboratory, Instituto de Química, Universidade Estadual de Campinas (UNICAMP), C.P. 6154, CEP 13083-970, Campinas - SP, Brazil; ³Consorzio Mario Negri Sud, "G. Paone" Environmental Health Center, 66030 Santa Maria Imbaro, Italy.

ABSTRACT

Growing evidence of the role of free radicals and antioxidants in health and ageing has focused great interest on these compounds. As polyphenolic compounds, flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism (with the formation of less reactive flavonoid phenoxyl radicals) and metal ion chelating. The relationship between the total antioxidant potential and the phenolic content of commercial wines was evaluated. A close relationship between total phenolic content and total antioxidant potential for all wines in two sets of results ($r = 0.9878$ and $r = 0.992$) was observed. By using capillary zone electrophoresis was founded that in red wines, gallic acid was the highest of the phenolic acids and (+)-catechin and (-)-epicatechin were the next most abundant phenolics. Also, these compounds are strictly correlated with the total antioxidant potential of wines. Total antioxidant potential by bleaching of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cations, using gallic acid as standard, could be a practical and easy measurement to evaluate the characteristics of different wines. Furthermore capillary electrophoresis could be a powerful and high performing tool to evaluate principal antioxidant wine components.

Keywords: Wines; antioxidants; phenolic compounds; capillary zone electrophoresis

INTRODUCTION

Free radicals are extremely harmful to living organisms in that they attack different constituents of the cell which leads to acceleration of the ageing process and sometimes even its destruction, or if the DNA is affected, irreversible malfunctions (De Gaulejac, Glories & Vivas, 1999). Growing evidence of the role of free radicals and antioxidants in health and ageing has focused great interest on these compounds. Fruits and vegetables are natural sources of vitamins and antioxidants. Vitamins C, E and the various carotenoids are ubiquitous in plants. Flavonoids and other phenolic compounds found in plants have received much attention in the prevention of human degenerative diseases (Aruoma, 1996).

Phenolic compounds are responsible for some of the major organoleptic properties of wines, in particular colour and astringency. Wine phenolic composition depends on the grapes used to make the wine and on the vinification conditions (Cheynier, Hidalgo Arellano, Souquet & Moutounet, 1997). Polyphenolic components of wine fall into one of two major classes. Nonflavonoids 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 wine (Soleas & Goldberg, 1999).

The flavonoid content of red wine has been suggested as an explanation to the "French paradox", the fact that French people have low incidence of coronary heart disease despite having a diet high in fat and being heavy smokers (Aruoma, 1996). The mechanism of this protective action of the flavonoids is a subject of considerable debate. As polyphenolic compounds, flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism (with the formation of less reactive flavonoid phenoxyl radicals) and metal ion chelating (Arora, Nair & Strasburg, 1998). A wide range of studies has shown the antioxidative properties of these compounds in protection against arteriosclerosis and coronary heart disease (Estruch, 2000; Santos-Buelga & Scalbert, 2000; Visioli, Borsani & Galli, 2000). Other effects include modulation of eicosanoid synthesis toward a more antiatherogenic pattern and inhibition of tumour growth in vitro and in human cancer patients (Soleas, Dam, Carey & Goldberg, 1997).

The identification of the active phenolic compound or the phenol class that is responsible for red wine's antioxidant properties has raised much interest (Kerry & Abbey, 1997). These antioxidant properties provide a rationale for exploring the polyphenol content of commercial wines to define those that are specially abundant in these desirable compounds and to stimulate the development of enological techniques for their enrichment (Soleas et al., 1997a).

Total phenols and polyphenols are usually quantified employing Folin-Ciocalteu's reagent. This procedure is also employed in the wine industry, where gallic acid is usually selected as a standard. On the other hand, total antioxidant activity (TAA) values are measured from induction times in free radical mediated processes and/or from the bleaching of stable free radicals, such as 2,2'-

azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) derived radical cation (ABTS^{•+}) (Campodonico, Barbieri, Pizarro, Sotomayor & Lissi, 1998).

The aim of this work was to evaluate the relationship between the total antioxidant potential and the total phenol content of commercial wines, determined by bleaching of pre-formed ABTS radical cations and Folin-Ciocalteu's reagent, respectively. To identify phenolic compounds responsible for the antioxidant activity and to obtain a phenolic profile content of such wine, a very high performing analytical technique was used, as capillary zone electrophoresis. High efficiency and the capacity to resolve a very complex matrix of natural compounds as wine were the reasons of the choice to use capillary electrophoresis for the purpose (Pazourek, Gonzalez, Revilla & Havel, 2000). The useful application of this technique for wine and polyphenols analyses has yet been well demonstrated (Chu, O'Dwyer & Zeece, 1998; Mellenthin & Galensa, 1999) so quantitative data were easily obtained by using CZE.

EXPERIMENTAL

Wines. The different commercial types of red, rosé and white wines, differing in their vintages, variety, and region of production are listed in Tables 1 and 2. The samples (50 ml aliquots of freshly opened wine bottles) were stored at 4°C and were protected against direct light until the assays were completed.

Total Phenolic Content. The total phenolic contents of the wine samples were determined with the Folin-Ciocalteu's reagent, using gallic acid as standard. To 1000 µl of wine sample (adequately diluted), 250 µl of carbonate-tartrate solution (200 g of Na₂CO₃ and 12 g of Na₂C₄H₄O₆·2H₂O in 1 l of

distilled water) and 25 μl of Folin-Ciocalteu's reagent were added. The absorbance of the sample was measured at 700 nm after 30 minutes of reaction. The results were expressed as mg of gallic acid equivalents (GAE) l^{-1} .

Assay of Polyphenols.

Chemicals

Boric acid, sodium hydroxide and diethyl ether were obtained from Pro Analysis Merck (Darmstadt, Germany); RPE methanol and RPE hydrochloric acid were bought from Carlo Erba (Milan, Italy). All solutions were prepared with deionised water (Milli-Q, Millipore, MA, USA) and filtered with 0.2 μm Minisart filters from Sartorius (Göttingen, Germany). Drying procedure of the extraction step was performed on Safe-Lock 2.0 ml vials (Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany).

Sample Preparation

For the liquid/liquid extraction, 1 ml of red wine (2 ml in case of white or rosé wine) was extracted with 1 ml (2 ml for white or rosé wine) of diethyl ether (twice). The organic phases were completely dried in the dark under nitrogen flux and resuspended with 100 μl of methanol 10 % in electrophoretic buffer.

Capillary Electrophoresis Procedure

Capillary zone electrophoresis was employed to quantify the polyphenols listed in Table 3 according to Rossi, Di Tommaso & Rotilio (1998). Capillary electrophoresis analyses were performed using a P/ACE 5500 (Beckman Instruments Inc., Fullerton, CA, USA), equipped with a diode-array detector, and elaborated with Beckman P/ACE Station 5000 software, on an Epson Endeavor XL personal computer. The column used was an uncoated fused

silica capillary tube of 75 μm ID (Beckman) with effective and total lengths of 50 cm 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 HCl 0.1 mol l^{-1} (1.5 min), NaOH 0.1 mol l^{-1} (1.5 min) and bidistilled water (1.5 min). Samples were hydrodynamically injected at $3.45 \cdot 10^3$ Pa pressure for 7 seconds. Electrophoretic buffer composition was phosphate 25 mmol l^{-1} , borate 10 mmol l^{-1} , at pH 8.8. This buffer was obtained by mixing solutions of H_3BO_3 (100 mmol l^{-1}) and Na_2HPO_4 (100 mmol l^{-1}), and NaOH (2 mol l^{-1}) to reach the desired pH value. Calibration curves were obtained by hydrodynamic injection of concentrations, from 1 to 50 mg l^{-1} , of each compound (Sigma, Italy) for 7 seconds, at a pressure of $3.45 \cdot 10^3$ Pa. For qualitative peak recognition we use spectra performed by DAD and, in order to improve method sensitivity, peak integration has been calculated at different wavelengths for each compound. DAD parameters, calibration and recovery data are shown in Table 1.

Total Antioxidant Potential (TAP) The total antioxidant potential was measured by bleaching of ABTS radical cations. ABTS radical cations were prepared by incubation of 150 $\mu\text{mol l}^{-1}$ (50 ml) with 2 mol l^{-1} potassium persulfate (1.25 ml) for 2 hours at 50°C in 0.02 mol l^{-1} phosphate buffer pH 7.0 (Campodonico et al., 1998). To 996 μl of the ABTS radical cation, 4 μl of the wine sample adequately diluted were added. The absorbance of the sample was measured after 15 minutes at 734 nm. Gallic acid was used as standard and the results were expressed as mg of gallic acid equivalents (GAE) l^{-1} .

Table 1. Calibration and recovery data by capillary zone electrophoresis

Substance	Regression Equation ^{a)}	λ_{Abs}	Correlation Coefficient	Recovery (%)	L.O.D. (ng/ml)
Tyrosol	$y = 5.25 \cdot 10^{-4}x - 2.49 \cdot 10^{-1}$	206	1.000	100	200
<i>cis</i> -Resveratrol	$y = 1.15 \cdot 10^{-4}x + 1.52 \cdot 10^{-1}$	206	1.000	100	18
<i>trans</i> -Resveratrol	$y = 1.03 \cdot 10^{-3}x - 6.05 \cdot 10^{-1}$	312	0.998	100	50
(+)-Catechin	$y = 1.13 \cdot 10^{-4}x + 2.21 \cdot 10^{-1}$	206	0.999	76	33
(-)-Epicatechin	$y = 7.31 \cdot 10^{-5}x + 5.01 \cdot 10^{-1}$	206	0.999	57	44
Hydroxytyrosol	$y = 1.39 \cdot 10^{-4}x - 3.46 \cdot 10^{-2}$	206	1.000	50	100
Sinapic acid	$y = 3.84 \cdot 10^{-4}x + 2.58 \cdot 10^{-1}$	217	0.998	67	149
Epicatechin gallate	$y = 1.61 \cdot 10^{-4}x + 2.34$	206	0.999	77	65
Syringic acid	$y = 2.47 \cdot 10^{-4}x - 6.80 \cdot 10^{-2}$	206	0.998	96	52
<i>o</i> -Coumaric acid	$y = 9.00 \cdot 10^{-5}x - 9.75 \cdot 10^{-2}$	217	1.000	100	25
<i>p</i> -Coumaric acid	$y = 1.04 \cdot 10^{-4}x - 1.26 \cdot 10^{-1}$	206	1.000	100	25
Vanillic acid	$y = 2.30 \cdot 10^{-4}x - 2.33 \cdot 10^{-1}$	206	0.999	83	30
Gentisic acid	$y = 1.01 \cdot 10^{-4}x + 2.39 \cdot 10^{-1}$	206	1.000	31	81
<i>p</i> -Hydroxybenzoic a.	$y = 1.02 \cdot 10^{-4}x + 4.11 \cdot 10^{-1}$	206	0.999	56	45
Salicylic acid	$y = 7.47 \cdot 10^{-5}x - 1.64 \cdot 10^{-1}$	206	0.999	84	30
Caffeic acid	$y = 3.29 \cdot 10^{-4}x + 4.89 \cdot 10^{-1}$	217	0.999	70	286
Gallic acid	$y = 7.43 \cdot 10^{-5}x + 3.27 \cdot 10^{-1}$	217	0.999	100	25
Protocatechuic acid	$y = 6.45 \cdot 10^{-5}x - 4.07 \cdot 10^{-3}$	206	0.999	22	114

^{a)} x is the peak area and y is the concentration in $\mu\text{g/ml}$

RESULTS

Total Phenolic Content and Total Antioxidant Potential

The wine samples were tested for total phenolic content and total antioxidant potential in two sets of analyses. In the first set of analysis, 5 wine samples (3 white and 2 red) from Brazil, 1 (red) from Chile, 1 (red) from Portugal and 1 (white) from Argentina were tested. The content of total phenols varied from 1615 to 2133 mg l^{-1} , averaging 1920 mg l^{-1} , for the red wines and from 216 to 353 mg l^{-1} , averaging 293 mg l^{-1} , for the white wines (Table 2). In the second set of analyses, 8 Italian wines samples were tested (4 red wines, 3 white wines, and 1 rosé wine). The content of total phenols varied from 3314 to 4177 mg l^{-1} , averaging 3760 mg l^{-1} , for the red wines and from 439 to 854 mg l^{-1} ,

averaging 634 mg l^{-1} , for the white wines. A value of 1304 mg l^{-1} for the rosé wine was found (Table 3).

The first set of analyses showed TAP values ranging from 556 to 859 mg l^{-1} , averaging 758 mg l^{-1} for red wines and from 103 to 172 mg l^{-1} , averaging 140 mg l^{-1} for white wines (Table 2). The second set of analyses showed TAP values ranging from 625 to 868 mg l^{-1} , averaging 766 mg l^{-1} for red wines and from 129 to 178 mg l^{-1} , averaging 153 mg l^{-1} for white wines. The value for the rosé wine was 284 mg l^{-1} (Table 3).

Assay of Polyphenols by Capillary Zone Electrophoresis

The concentrations of 18 polyphenols (listed in Table 4) were measured by capillary zone

Table 2. Contents of Total Phenolics and TAP Values of Wine Samples

Color, country	Vintage	Main variety	Total phenolics (mg l ⁻¹) ^a	TAP (mg l ⁻¹) ^a
white, Argentine	-	Chardonnay	216	103
white, Brazil	1998	blended	347	172
white, Brazil	1997	Riesling	353	165
white, Brazil	1998	Cabernet Blanc	256	121
red, Brazil	1996	blended	1947	808
red, Brazil	1996	Pinot	1984	807
red, Chile	1996	Cabernet Sauvignon	2133	859
red, Portugal	-	blended	1615	556
average white wines			293	140
average red wines			1920	758

^a Values are expressed as mg of gallic acid equivalents (GAE) l⁻¹.

electrophoresis in the Italian wines listed in Table 3. The results are shown in Table 4. A representative electropherogram is shown in Figure 2. In red wines, gallic acid was the highest of the polyphenols, ranging from 54.8 to 58.3 mg l⁻¹. The rosé wine presented a value of 14.3 mg l⁻¹. The highest level of this component in white wines was 3.5 mg l⁻¹. (+)-Catechin and (-)-epicatechin were the next most abundant phenolics, ranging from 13.8 to 15.2 mg l⁻¹ and 10.7 to 13.7 mg l⁻¹ in red wines, respectively. (-)-Epicatechin levels in all red wines were lower than the (+)-catechin levels. Tyrosol was around 6.0 mg l⁻¹ in red wines and varied from 1.1 to 3.0 mg l⁻¹ in white wines. The value in the rosé wine was 5.0 mg l⁻¹. The highest level of caffeic acid was found in the rosé wine (5.0 mg l⁻¹), followed by red wines (ranged from 2.7 to 3.6 mg l⁻¹). In one red Montepulciano wine, sample 6, the concentration of hydroxytyrosol was very low (0.5 mg l⁻¹). The level of this component in the other red wines ranged from 5.9 to 9.6 mg l⁻¹. The rosé wine presented a value of 6.1 mg l⁻¹ and in white wines ranged from 1.6 to 2.7 mg l⁻¹. Epicatechin gallate and protocatechuic acid presented values of 3.9-8.1

mg l⁻¹ and 2.6-7.2 mg l⁻¹ in red wines, respectively. Smaller amounts of the following compounds were found in the wines: *cis*-resveratrol (<3.5 mg l⁻¹), *trans*-resveratrol (<3.0 mg l⁻¹), sinapic acid (<2.4 mg l⁻¹), syringic acid (<3.9 mg l⁻¹), *o*-coumaric acid (<0.8 mg l⁻¹), *p*-coumaric acid (<2.9 mg l⁻¹), vanillic acid (<2.1 mg l⁻¹), gentisic acid (<1.0 mg l⁻¹), *p*-hydroxybenzoic acid (<1.3 mg l⁻¹), salicylic acid (<1.0 mg l⁻¹).

DISCUSSION

Recently much attention has focused on the protective biochemical function of naturally occurring antioxidants in biological systems and on the mechanism of their action (Kanner, Frankel, Granit, German & Kinsella, 1994). Albeit there is much interest in the antioxidant activity of red wine, it is uncertain which of the phenols exhibit the greatest antioxidant effect (Kerry & Abbey, 1997). Most aromatic plant acids exist as derivatives of benzoic acid or cinnamic acid. The existing methods used for the analysis of aromatic plant acids are generally high-performance liquid chromatography, thin-layer chromatography and

Table 3. Contents of Total Phenolics and TAP Values of Italian Wine Samples

Wines (samples)	Vintage	Main variety	Total phenolics (mg l ⁻¹) ^a	TAP (mg l ⁻¹) ^a
white (1)	1998	Greco di Tufo	854	178
white (2)	1997	Pinot Grigio	439	129
white (3)	1998	Verdicchio	610	152
rosé (4)	1998	blended	1304	284
red (5)	1998	Barbera	3314	625
red (6)	1998	Montepulciano	4177	868
red (7)	1996	blended	3791	805
average white wines			634	153
average red wines			3760	766

^a Values are expressed as mg of gallic acid equivalents (GAE) l⁻¹.

gas chromatography. In this work we have used capillary zone electrophoresis with diode array detection. This is a very efficient method because its high-resolution separation, simplicity of operation, versatility and sensitivity (Kulomaa, Siren & Riekkola, 1997; Hiermann & Hadl, 1998; Gu, Chub, O'Dwyer & Zeece, 2000), and the diode-array detector is a very powerful tool in qualitative and quantitative determination of natural substances (Da Costa, Horton & Margolis, 2000).

The amounts of phenolic materials vary considerably in different types of wine, depending on the grape variety, environmental factors in the vineyard, and the wine processing techniques (Frankel, Waterhouse & Teissedre, 1995). Our results confirm a variation in phenolic content among wine samples tested. These results are in agreement with the available literature (Kanner et al., 1994; Frankel et al., 1995; Sato, Ramarathnam, Suzuki, Ohkubo, Takeuchi & Ochi, 1996; Hurtado, Caldú, Gonzalo, Ramon Mínguez & Fiol, 1997; Simonetti, Pietra & Testolin, 1997; Campodonico et al., 1998). The presence of high concentrations of gallic acid in red wines would be expected since

this phenolic acid is principally formed by hydrolysis of flavonoid gallate esters, which are largely absent in white wines due to the lack of skin extraction (Frankel et al., 1995). Soleas et al. (1997a) presented data for concentrations of 15 polyphenolics in a range of white and red Canadian wines. They observed that in white wines, caffeic acid was the highest in Chardonnay and Vidal wines and p-coumaric acid in those from Seyval Blanc. The individual phenolic acids demonstrated a similar pattern among all of the red wines analysed, with gallic acid being the highest and the caffeic acid the second highest. Pinot Noir wines were highest in catechin, epicatechin and polydatin concentrations.

The total antioxidant potential (TAP) of wine samples was determined by bleaching of pre-formed ABTS radical cations. The addition of free radical scavengers to a solution containing ABTS-derived radical cations leads to a decrease in the absorbance of the sample at 734 nm that is proportional to the size of the wine aliquot (Campos, Escobar & Lissi, 1996). In general, the measurement of antioxidant ability uses standards

Table 4. Total Amount (mg l⁻¹)^a of Substances in the Analyzed Italian Wines

Wines (samples)	1	2	3	4	5	6	7
Substances							
Tyrosol (1)	1.1	2.3	3.0	5.0	5.9	5.9	6.0
cis-Resveratrol (2)	0.3	0.2	0.2	1.2	2.4	3.5	2.6
trans-Resveratrol (3)	0.3	0.2	0.1	0.8	2.3	3.0	2.2
(+)-Catechin (4)	4.9	0.6	0.1	3.5	13.8	14.0	15.2
(-)-Epicatechin (5)	2.8	0.3	0.6	3.3	11.0	13.7	10.7
Hydroxytyrosol (6)	2.7	1.9	1.6	6.1	9.6	0.5	5.9
Sinapic Acid (7)	0.1	0.2	0.4	0.5	0.9	1.7	2.4
Epicatechin Gallate (8)	1.3	ND	1.2	ND	3.9	8.1	4.8
Syringic Acid (9)	0.1	ND	0.2	1.5	2.2	3.0	3.9
o-Coumaric Acid (10)	ND	0.7	0.3	0.8	0.2	0.3	0.4
p-Coumaric Acid (11)	ND	1.0	0.8	2.7	2.3	2.9	1.0
Vanillic Acid (12)	0.1	0.1	0.6	0.3	2.0	2.1	2.1
Gentisic Acid (13)	ND	0.2	0.3	0.2	0.9	0.9	1.0
p-Hydroxybenzoic Acid (14)	0.2	ND	0.2	0.7	1.0	1.3	0.7
Salicylic Acid (15)	0.4	0.2	1.0	0.3	0.4	0.5	0.5
Caffeic Acid (16)	2.2	0.5	1.1	5.0	3.6	2.7	2.7
Gallic Acid (17)	2.2	3.5	0.6	14.3	54.8	58.3	55.2
Protocatechuic Acid (18)	1.1	0.1	0.4	2.4	2.6	7.2	5.1

^aany concentration value is the mean of three measures

ND = Not Detected

solutions of antioxidants compounds like Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or ascorbic acid. We have used gallic acid as standard considering the fact that this compound is generally present in high concentrations in red wines. In order to see the response of the Folin's and ABTS methods to different compounds, Campodonico et al. (1998) tested several mono and polyphenols. The data showed that both methods are able to titrate a variety of phenols and must be pointed out that the response of different phenolic groups may differ by a factor up to nearly 2. In the case of Trolox and propyl gallate (gallic acid propyl ester), the response was very similar considering that Trolox is a mono phenol and propyl gallate is a tri-phenol.

The data presented in Tables 2 and 3 indicated that red wines presented a substantial antioxidant

capacity. The red Chilean wine tested presented a very high charge of free radical scavengers, with is in agreement with Campos and Lissi (Campos & Lissi, 1996). A very close relationship between total phenolic content and total antioxidant potential for all wines was observed in both sets of results ($r = 0.9878$ (Figure 1A) and $r = 0.992$ (Figure 1B)). These results are in agreement with other reports in the literature (Sato et al., 1996; Simonetti et al., 1997; Campodonico et al., 1998; Henn & Stehle, 1998; Fogliano, Verde, Randazzo & Ritieni, 1999; Sánchez-Moreno, Larrauri & Saura-Calixto, 1999a). Total antioxidant potential also correlated with the concentrations of gallic acid ($r = 0.9572$, Figure 2A), (-)-epicatechin ($r = 0.9583$, Figure 2B) and (+)-catechin ($r = 0.9172$, Figure 2C). According to Frankel et al. (1995), the relative antioxidant activity of 20 selected California wines correlated

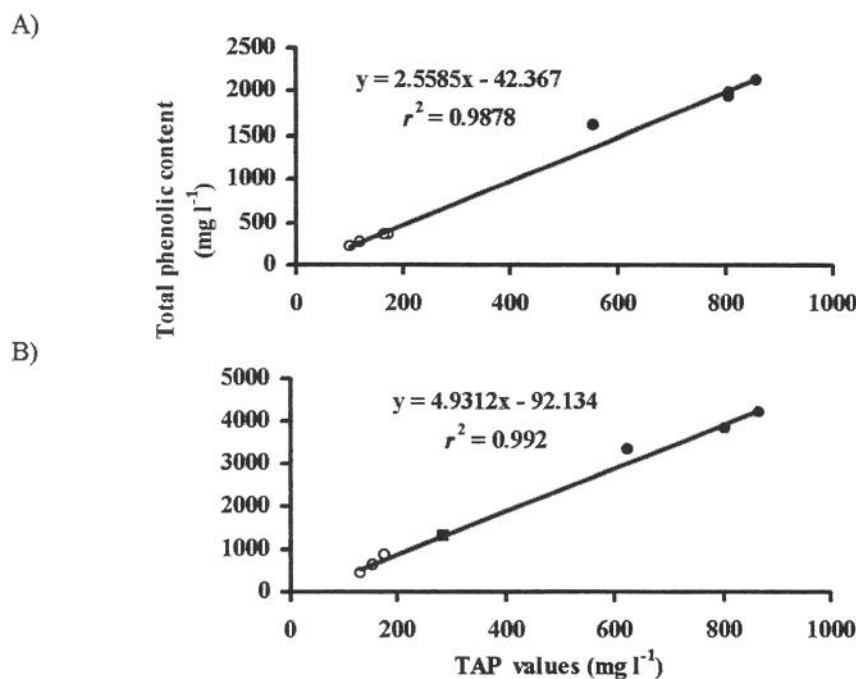


Figure 1. Relationship between total phenolic contents and TAP values of wines listed in Table 1 (A) and Table 2 (B). (○) white wine; (■) rosé wine; (●) red wine.

with total phenol contents of wines ($r = 0.94$) and with the concentrations of gallic acid ($r = 0.92$), catechin ($r = 0.75$), myricetin ($r = 0.70$), quercetin ($r = 0.68$), caffeic acid ($r = 0.63$), rutin ($r = 0.50$), epicatechin ($r = 0.45$), cyanidin ($r = 0.43$), and malvidin 3-glucoside ($r = 0.38$). Their work also showed that the antioxidant activity of different commercial wines toward low density lipoproteins oxidation is not a property of a single phenolic compound and that this activity is widely distributed among the phenolic phytochemical constituents. Sánchez-Moreno, Larrauri & Saura-Calixto I. (1999b) observed that the free radical scavenging activity of gallic acid was the highest, tannic acid, caffeic acid, quercetin, 3-tertiary-butyl-4-hydroxyanisole (BHA) and rutin activities were intermediate and that for ferulic acid, DL- α -tocopherol and resveratrol were the lowest.

Soleas, Tomlinson Diamandis & Goldberg (1997b) analysed the concentrations of 17 phenolic constituents in a red wine by a number of multiple regression models for their contribution to total

antioxidant status. On the basis of single analysis of each phenolic in wine matrix, only seven were significantly correlated with total antioxidant status of the wine sample; the highest values for r were observed for vanillic and gallic acids. With statistical modelling utilizing both linear and non-linear approaches to predict the total antioxidant status of wine samples from their polyphenol content, the best results were obtained with vanillic acid, trans-polydatin, catechin, m-coumaric acid, epicatechin, quercetin, cis-polydatin and trans-resveratrol. Although syringic and gallic acids were significantly correlated with total antioxidant status in a univariate analysis, they do not contribute to a statistical description of this parameter to the eight constituents already identified. Ghiselli, Nardini, Baldi & Scaccini (1998) showed that the protective effect of wine is mainly due to the anthocyanic fraction (quantitatively the more abundant phenolic subclass in red wine), although their results do not exclude the possibility of a synergistic action

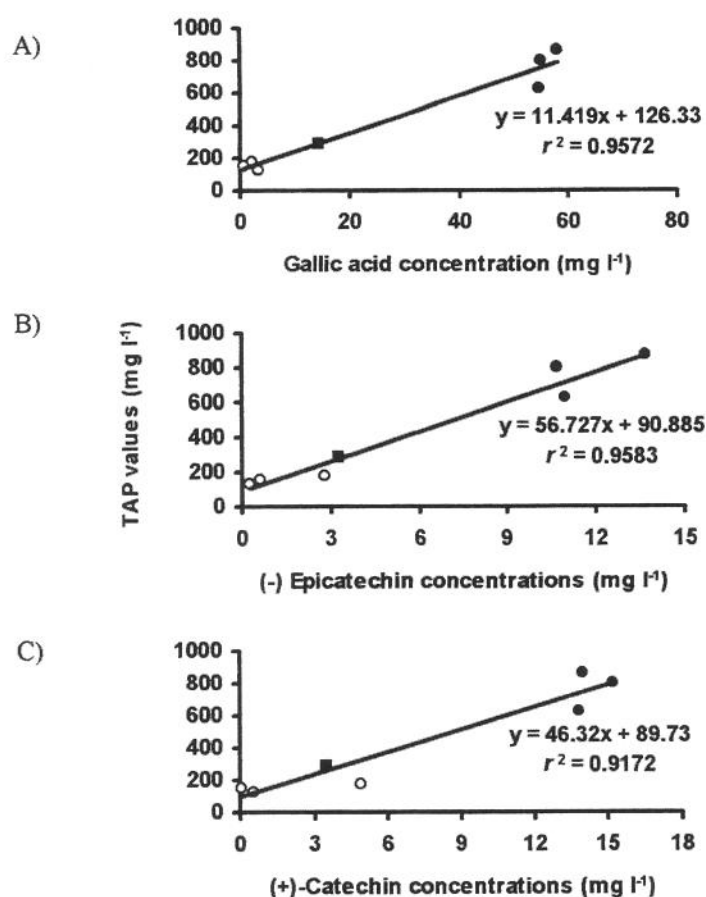


Figure 2. Relationship between gallic acid (A), (-)-epicatechin (B) and (+)-catechin (C) concentrations and TAP values of wines.

among the different classes of polyphenols. Also the anthocyanic fraction showed a high free radical scavenging power in relation to the other tannic fractions by De Gaulejac et al. (1999). Kerry and Abbey (1997) observed that the antioxidant property of red wine is due predominantly to monomeric catechins, procyanidins, monomeric anthocyanidins and phenolic acids. Simonetti et al. (1997) suggested that absorption and metabolism studies should be preferably focused upon gallic acid derivatives and flavonols, since they are the most significant phenols in red wines. Kondo, Kurihara, Miyata, Suzuki & Toyoda (1999) investigated the antioxidative effects and mechanisms of catechins

using liquid chromatography/mass spectrometry, spectrophotometric analyses, and semiempirical molecular orbital calculations. The authors observed that (-)-epicatechin would be gently converted to an anthocyanin-like compound. According to the mechanisms, the compound produced from (-)-epicatechin by radical oxidation can also function as an antioxidant.

Capillary zone electrophoresis coupled with diode array detector has shown to be a powerful tool able to resolve a complex matrix as wine; furthermore sample pre-concentration step improves method sensitivity allowing determining concentrations at sub-ppm level.

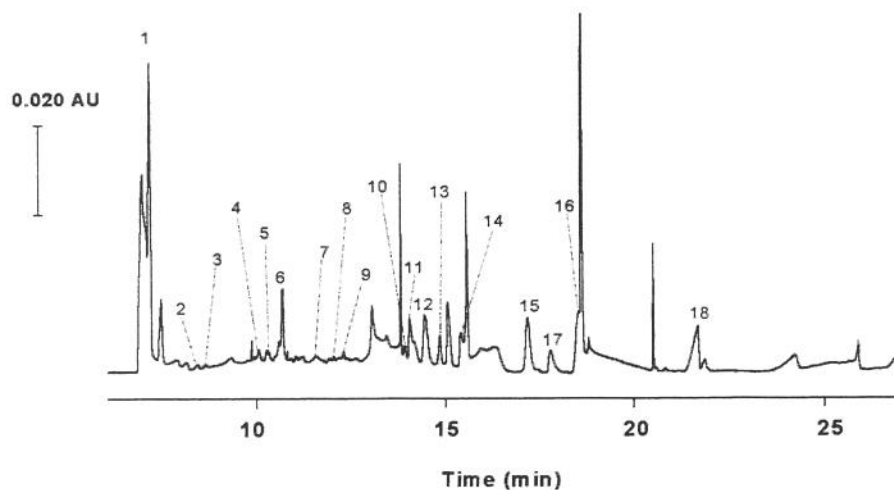


Figure 3. Electropherogram of a white wine extract (Verdicchio, 1998). For peak identification, see Table 4.

CONCLUSION

A positive correlation between the total antioxidant potential of wine and the gallic acid, (-)-epicatechin, (+)-catechin concentrations and total phenol content has been demonstrated in the present study. Total antioxidant potential, by bleaching of ABTS radical cations and using gallic acid as standard, could be a practical and easy measurement to evaluate the characteristics of different wines. Capillary zone electrophoresis could be a powerful tool to analyse polyphenols content of white and red wines, with an opportune sample pre-concentration step.

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PHENOLIC REMOVAL IN MUSTS BY LACCASE FROM *Trametes versicolor*

Rosana C. Minussi^{1,2}, Massimo Rossi³, Luciano Bologna³, Domenico Rotilio³, Gláucia M. Pastore¹ and Nelson Durán^{2*}

¹Food Biochemistry Laboratory, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (UNICAMP); ²Biological Chemistry Laboratory, Instituto de Química, Universidade Estadual de Campinas (UNICAMP), C.P. 6154, CEP 13083-970, Campinas – SP and Núcleo de Ciências Ambientais, Universidade de Mogi das Cruzes, Mogi das Cruzes – SP, Brazil; ³Consorzio Mario Negri Sud, “G. Paone” Environmental Health Center, 66030 Santa Maria Imbaro, Italy.

Abstract

The potential of laccase from *Trametes versicolor* for phenolic removal in must for wine stabilization was evaluated. Total phenolic content, total antioxidant potential, and polyphenols were monitored from 0 to 3 hr 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 total antioxidant potential. Phenol degradation was very rapid for catechins, and less rapid for stilbenes (*cis* and *trans* resveratrol) and derivatives of cinnamic (ferulic and caffeic) and benzoic (syringic, vanillic, and gallic) acids. We can specifically conclude 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 electrophoresis, CZE

Introduction

Within the European Union, wine is defined as “the fermented juice of fresh grapes or the liquid, known as grape must, which is derived from grapes by pressing”. At crushing and pressing, the wine maker has to make choices that will affect the style of wine. Both colour and astringency derive from polyphenols concentrated particularly in the stems, seeds, and skins (Gibson, 1997). Wine phenolic composition depends on the grapes used to make the wine and on the vinification conditions (Cheynier *et al.*, 1997). Polyphenolic components of wine fall into one of two major classes. Nonflavonoids 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 (Soleas & Goldberg, 1999).

A basic requisite of wines is that their optimal organoleptic properties should remain unchanged until consumption (Zamorani, 1989). Due to a complex sequence of events, where the polyphenols (coumaric acid derivatives, flavans, and anthocyanins) play an important role, oxidative reactions catalysed by iron, copper, and enzymes and that also involve aldehydes, amino acids and proteins, can occur in musts and wines causing turbidity, colour intensification and flavour alterations. This phenomenon of oxidation is known as madeirization (Zamorani *et al.*, 1993).

In traditional wine technology, the madeirization prevention can avail itself of stabilizing procedures that either act on catalytic factors, block oxidizers, or remove 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. These polyphenolic substances would be hydrolysed and oxidized by such enzymes, being previously destabilized by polymerization, flocculation, and easily eliminated by clarification (Zamorani, 1989).

Enzymatic preparations have been studied in the wine industries for about 60 years, beginning in the decade of the 30's with preparations for juice clarification. Today, the wine industries have available technology for the application of several industrial enzymes. Pectinases, β -glucosidases, β -glucanases, oxidases, ureases, proteases and lysozyme are important examples of enzymatic preparations that can be used in wine production (Bisson & Butzke, 1996). Although the use of enzyme preparations in the food industry is well established and expanding rapidly, enzyme processing in enology is less common (Zamorani, 1989). 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 (Cantarelli, 1986). This treatment is interesting for its specific action and is a mild technology with less drastic effects 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, specifically phenols and anilines, while concomitantly reducing molecular oxygen to water (Thurston, 1994; Gianfreda *et al.*, 1999). Laccase exhibits some important requirements to be used for polyphenols elimination in wines, such as optimum pH around 2.5-4.0, stability in acid medium and reversible inhibition with sulphur dioxide, among

others. The literature reports many studies on the use of laccase for fruit juice and wine stabilization (Cantarelli, 1986; Cantarelli *et al.*, 1989; Zamorani, 1989; Maier *et al.*, 1990; Cantarelli & Giovanelli, 1991; Giovanelli & Ravasini, 1993; Stutz, 1993; Zamorani *et al.*, 1993; Brenna & Bianchi, 1994; Lante *et al.*, 1992, 1996; Minussi *et al.*, 1998a,b; Minussi *et al.*, 1999). Maier *et al.* (1990) evaluated the polyphenols percentage, coloration, 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 any addition of SO₂. Cantarelli & Giovanelli (1991) 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) in place of the hyperoxidation. The enzyme laccase was added with concentrations of 5-20 units/mL to the must, which was then filtered (with and without PVPP) or clarified with bentonite or gel + silica solution, or sulphited (5 g SO₂/mL). The results demonstrated that the enzymatic treatment coupled to filtration with PVPP reduced the quantity of oxidized polyphenols. A comparative analysis among the hyperoxidation and enzymatic treatments showed compatibility in terms of coloration but sensitive differences in the organoleptic characteristics related to the grape variety used.

The aim of this study was to evaluate the potential of laccase from *Trametes versicolor* in must phenolics removal for wine stabilization. Total phenolic content, total antioxidant potential and polyphenols were monitored from 0 to 3 hr of must treatment.

Materials and Methods

Enzyme Production: Laccase was obtained by *Trametes versicolor* CCT 4521 grown for 20 days at 30°C and 240 rpm in a liquid medium containing (g/L): peptone, 10; malt extract, 5; CuSO₄ · 5H₂O, 0.005 and glucose, 20; at pH 5.4. Laccase induction consisted in the addition of 0.5 mM 2,5-xyldine at 96 hours 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% ammonium sulphate. The enzyme was eluted in a gel chromatography column (Sephacryl S-200, Sigma) and lyophilised. The laccase activity in a liquid stock solution was around 100 U/mL.

Enzyme assay: Laccase activity was assayed by measuring oxidation of syringaldazine. 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. Measuring the increase in A₅₂₅ for 5 minutes monitored syringaldazine oxidation. Enzyme activity was expressed in units; 1 U was defined as 1 µmol of syringaldazine oxidized per min.

Must Treatments: Montepulciano d'Abruzzo (red), Montonico (white) and Moscato (white) musts were tested. Laccase activities of 1 and 5 U/mL were added to the Montepulciano must. The 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 hr of must treatment with laccase. The treatment was conducted in the dark with slow agitation.

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 Na₂CO₃ and 12 g of Na₂C₄H₄O₆ · 2H₂O in 1 L of distilled water) and 25 µL of Folin-Ciocalteu reagent were added. The absorbance of the sample was measured at 700 nm after 30 minutes of reaction. The results were expressed as mg of gallic acid equivalents (GAE)/L.

Total Antioxidant Potential (Bleaching of 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 hours at 50°C in 0.02 M phosphate buffer pH 7.0 (Campodonico *et al.*, 1998). To 996 µL of the ABTS radical cation, 4 µL of the must sample adequately diluted were added (Minussi *et al.*, 2002). The absorbance of the sample was measured after 15 minutes at 734 nm. Gallic acid was used as standard and the results were expressed as mg of gallic acid equivalents (GAE)/L.

Assay of Polyphenols by Capillary Zone Electrophoresis (CZE). CZE was employed to quantify polyphenols in musts according to Rossi *et al.* (1998). 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. Capillary electrophoresis analyses were performed using a P/ACE 5500 (Beckman Instruments Inc., Fullerton, CA, USA), equipped with a diode-array detector (DAD), and elaborated 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 ID (Beckman) with

effective and total lengths of 50 cm 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 $3.45 \cdot 10^3$ Pa pressure for 7 seconds. Electrophoretic buffer composition was 25 mM phosphate, 10 mM borate, at pH 8.8. 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 seconds, at a pressure of $3.45 \cdot 10^3$ Pa (data not shown).

Results and Discussion

Phenolic removal from musts by laccase from *Trametes versicolor* for wine stabilization was studied. Total phenolic content, total antioxidant potential and polyphenols reductions were monitored from 0 to 3 hr of must treatment with laccase.

Investigators have certified the antioxidant properties of polyphenols that are present in red wines; however, the specific role of individual compounds remains elusive (Brouillard *et al.*, 1997; Kaur & Kapoor, 2001; Landbo & Meyer, 2001). 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) (Figure 1). The total phenol reductions after 3 hours 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 (1993) 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 selectivity's, one can select those that have the least on affect 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 detailed studies in this area should be made. The treatment of white musts with 1 U/L laccase showed higher reduction in total phenol than in total antioxidant potential. The white must Moscato treatment resulted in 9.2 and 32.1% of total antioxidant and total phenols reductions, respectively (Figure 2A). The results with white must Montonico treatment indicated reductions of 13.9 and 33.4% in total antioxidant potential and total phenol, respectively (Figure 2B). In this way, the treatment of these must types with laccase is indicated.

The polyphenol removal in musts should be selective, since if it is indiscriminate, the wine 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

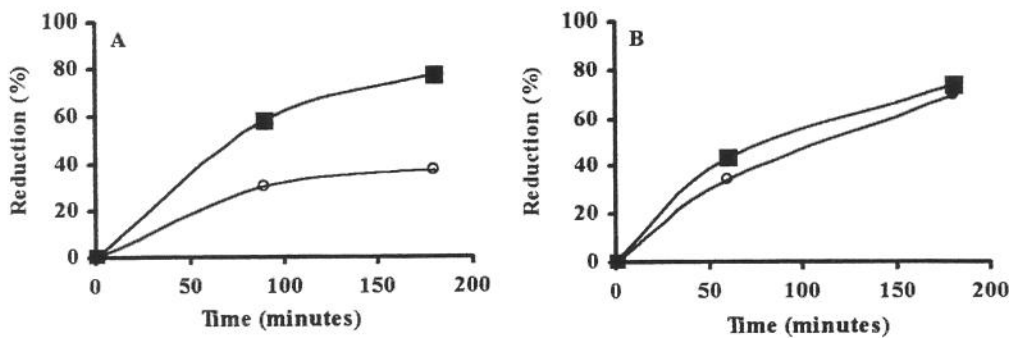


Fig. 1. Total phenols (○) and total reactive antioxidant potential (■) reduction (%) of red must (Montepulciano d'Abruzzo) treated for 180 minutes with 1 U/mL (A) and 5 U/mL (B) of laccase by *Trametes versicolor*.

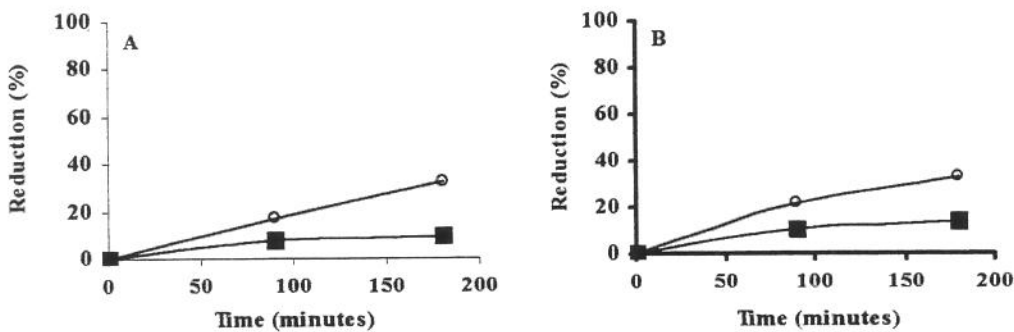


Fig. 2. Total phenols (○) and total reactive antioxidant potential (■) reduction (%) of white musts (Moscato (A) and Montonico (B)) treated for 180 minutes with 1 U/mL of laccase by *Trametes versicolor*.

Table 1. Must components determined by Capillary Zone Electrophoresis.

PEAK N°	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)

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 possibility to study the enzymatic mechanism of 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 Figures 3 and 4.

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).

According to Cantarelli (1986), mutant laccase from *Polyporus versicolor* (optimum pH 2.7)

eliminated around 70% catechin and 90% of anthocyanidines of model solutions in 3 hours of treatment. The laccase action on grape juice showed a removal of 50% of total polyphenols and the analysis for HPLC of a must treated with the active and inactive enzyme extract demonstrated the efficacy of this laccase, superior in action specificity and stabilization when compared to the physical-chemical treatment. The use of the laccase in a must resulted in a stable wine with good flavour. The results indicate the potential of laccase as a clarifying agent, followed by the addition of silica solution or a thermal treatment, and an ultrafiltration for the removal of the oxidized products and enzymatic protein. Lante *et al.* (1996) observed decreased of phenolics present in musts and wines treated with the immobilized laccase by covalent binding in polysaccharides supports.

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'	60'	180'	0'	45'	90'	180'
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 (1U/L) action in Montonico and Moscato (white musts)

Substances	Montonico				Moscato			
	0'	45'	90'	180'	0'	45'	90'	180'
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

Seivili *et al.* (2000) showed high removal of (-)-epicatechin, ferulic and *o*-coumaric acids by immobilized laccase in white grape must.

Preliminary studies with laccase from *Trametes 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 (Minussi *et al.*, 1998a) and 34% of phenolic compounds in wines were obtained (Minussi *et al.*, 1998b). Siringic acid is quickly oxidized to 2,6-dimethoxy-1,4-benzoquinone by laccase from *Azospirillum lipoferum* (Faure *et al.*, 1996). The gallic acid was oxidized to quinone by the laccase from *Botrytis cinerea* (Viterbo *et al.*, 1993). Resveratrol was oxidized to epsilon-viniferin by two isoenzymes of the laccase of *Botrytis cinerea* (pI of 4.35 and 4.30) (Pezet, 1998). Vanillic acid reacts slowly with laccase forming dimers (Tatsumi *et al.*, 1994). According to Espin and Witchers (2000), 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 (Takahama, 1995).

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, facilitating their elimination of the must and with reutilization possibility.

Conclusions

We can conclude that the use of laccase in white wines is perfectly feasible. This would allow a more soft and ecologically correct treatment, which could diminish processing costs and avoid deterioration of wines for long storage times. In the case of the red wines, the use of the laccases is not indicated because the phenols with large antioxidant properties should be preserved.

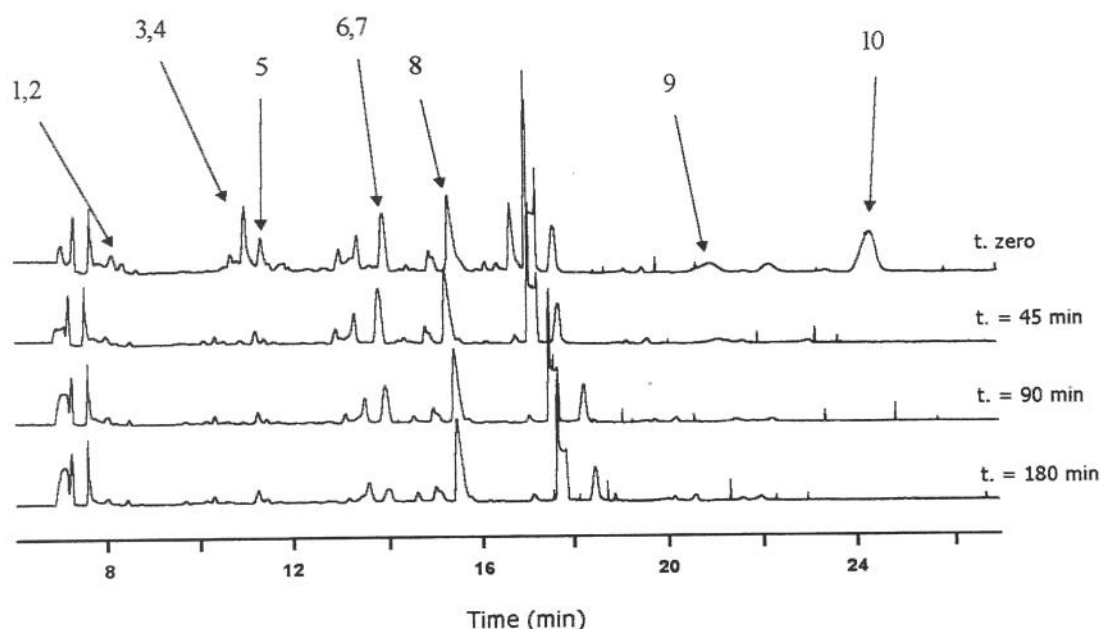


Fig. 3. Electropherograms of liquid-liquid extracted samples of red must (Montepulciano d'Abruzzo) treated with 1U/L of laccase by *Trametes versicolor*.

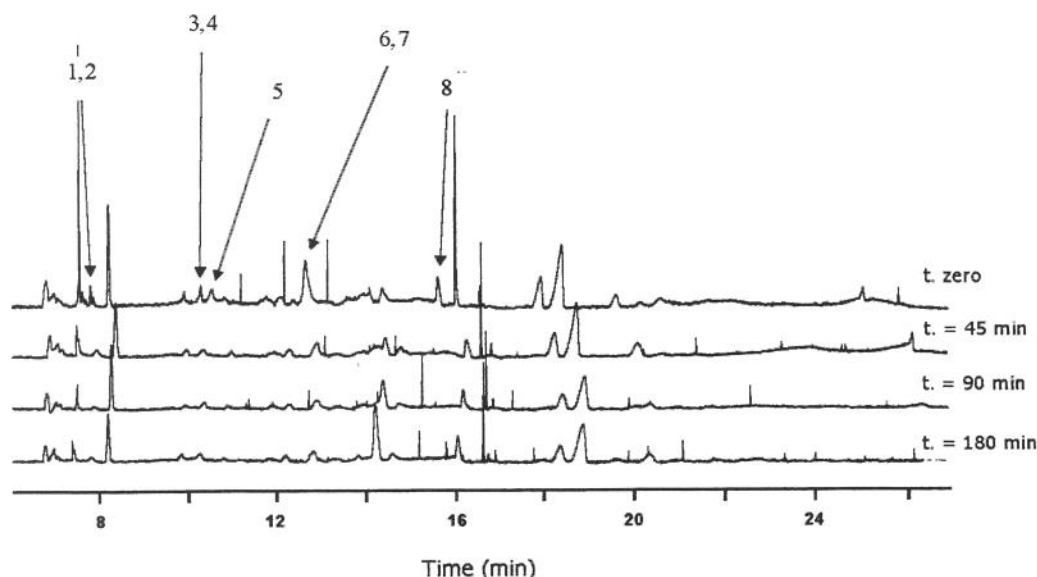


Fig. 4. Electropherograms of liquid-liquid extracted samples of white must (Montonico) treated with 1U/L of laccase by *Trametes versicolor*.

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CONCLUSÕES GERAIS

1. Todos os fungos estudados produziram lacase nas condições estudadas. A maior atividade de lacase foi obtida com o fungo *Trametes versicolor* CCT 4521 cultivado em meio líquido na presença de 2,5-xilidina e cobre como indutores. Foram observadas duas formas de lacase (L1 e L2) no meio de cultivo. O rendimento geral da purificação (duas etapas cromatográficas) foi em torno de 38% com fatores de purificação de 8 e 35 vezes para L1 e L2, respectivamente.
2. As duas formas apresentaram a mesma massa molecular de 66 kDa, temperatura ótima a 40°C e teor de carboidratos em torno de 7%. Os valores de pH ótimo (4,0 e 5,0) e Km (28 e 5 µM) foram obtidos para L1 e L2, respectivamente, usando siringaldazina como substrato. L1 se mostrou relativamente estável a 60°C após 20 minutos, enquanto L2 apresentou inativação em torno de 70% nas mesmas condições.
3. As lacases purificadas foram capazes de oxidar uma grande variedade de compostos e foram fortemente inibidas por azida sódica, L-cisteína e ditioneitol.
4. As seqüências de N-terminal mostraram aproximadamente 75% de resíduos idênticos entre as duas formas e similaridades em torno de 40-60% com outras lacases produzidas por fungos de degradação de madeira.
5. Lacase-ácido hidroxâmico mostrou-se o melhor sistema enzima-mediador estudado na redução de fenóis totais e carbono orgânico total em efluente papaleiro (E₁). O sistema lacase-1-hidroxibenzotriazol demonstrou eficiência na redução de coloração e fenóis totais em efluente de óleo de oliva.
6. *Lentinus edodes* demonstrou a maior habilidade na descoloração de corantes têxteis em meio sólido. Os resultados obtidos indicaram uma possível relação entre a produção de sideróforos e a descoloração dos corantes.
7. Alta correlação foi obtida entre o teor total de fenóis e o potencial antioxidante em vinhos. Ácido gálico, catequinas e epicatequinas, os compostos fenólicos mais abundantes observados nos vinhos tintos, também mostraram alta correlação com o potencial antioxidante.
8. O método de determinação do potencial antioxidante total utilizando a descoloração dos radicais cátions de ABTS e usando ácido gálico como padrão mostrou-se prático e rápido na avaliação de diferentes vinhos. A utilização de eletroforese capilar se mostrou bastante eficiente na determinação de compostos fenólicos em vinhos.
9. Lacase semipurificada de *T. versicolor* CCT 4521 demonstrou potencial na redução de fenóis em mostos visando estabilização de vinhos. Os resultados do tratamento de mosto tinto com lacase indicaram alta redução no potencial antioxidante, embora isto não tenha sido observado no tratamento de mosto branco. Os resultados indicam que estudos mais detalhados devem ser realizados nesta área.