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Oxidative Transformation of Natural and Synthetic Phenolic Mixtures by *Trametes versicolor* Laccase

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The efficiency of *Trametes versicolor* laccase in the transformation of phenols (caffeic acid, catechol, hydroxytyrosol, methylcatechol, protocatechuic acid, syringic acid, *m*-tyrosol, 3-hydroxybenzoic acid, 3-hydroxyphenylacetic acid, 2,6-dihydroxybenzoic acid, 4-hydroxybenzaldehyde) usually present in waste water, such as that derived from an olive oil factory, was investigated. According to their response to 24 h laccase action the 11 phenolic compounds were classified in three groups: reactive (88−100% transformation), intermediate reactive (transformation lower than 50%), and recalcitrant (not transformed at all). The enzyme was able to transform the 11 substrates even when they were present in a mixture and also toward a phenolic extract from a Moroccan olive oil mill waste water (OMW) sample. The disappearance of protocatechuic, 3-hydroxyphenylacetic, and 2,6-dihydroxybenzoic acids, and 4-hydroxybenzaldehyde was enhanced whereas that of caffeic acid and *m*-tyrosol was depressed when the phenols were present in the mixture. A reduction of enzyme activity occurred in single and/or complex phenolic mixtures after enzymatic oxidation. No correspondence between phenol transformation and disappearance of enzymatic activity was, however, observed. The overall results suggest that laccases are effective in the transformation of simple and complex phenolic mixtures.

KEYWORDS: Laccase dephenolization; olive oil mill waste water; oxidative polymerization

INTRODUCTION

Phenols are very abundant environmental pollutants. They naturally occur in plant and soil and are the most widespread class of plant secondary metabolites (*1*). In addition to natural sources, phenolic compounds also enter the environment as wastes from several types of industrial and agricultural activities (*2, 3*). Phenolic compounds and their derivatives are considered priority pollutants because they are harmful toward living organisms, even at low concentrations (*4*).

One of the most abundant aqueous waste-containing phenols is that deriving from the olive oil industry [olive oil mill waste water (OMW)]. It is produced in large amounts in the main olive-producing countries of the Mediterranean region during oil extraction from olives. These effluents have a very high toxic activity due to their low pH, antimicrobial and herbicide activity, slow biodegradability, and inhibition of cellulolytic enzymes (*5–7*). As a consequence, they may affect the chemical and biological properties and the microbial and enzyme activity of both soil and water bodies. Moreover, they may interact with natural sources, such as plant materials, and be incorporated into humic materials. The phenolic compounds contained in OMW are considered major contributors to toxicity and antibacterial activity of this waste and limit their microbial degradability (*8*). Generally, OMW from different geographical origins are rich in a heterogeneous variety of phenols, and this may represent one the main problems for their detoxification (*9, 10*).

Oxidoreductive enzymes, such as laccases, peroxidases, and tyrosinases, are able to transform phenols through oxidative coupling reactions with production of polymeric products by self-coupling or cross-coupling with other molecules. The inclusion of phenolic pollutants in the humus as well as the formation of less soluble, high molecular mass compounds that may be easily removed from water may result (*2, 3, 11*). In previous work (*12*), we investigated the transformation of a mixture of four phenols, commonly present in OMW, by a plant laccase from *Rhus vernicifera*. The achieved results confirmed that laccase-mediated transformation of phenols depended on the nature and the initial concentration of the involved phenol, the time course of the reaction, and mainly the complexity of the phenolic incubation mixture. Indeed, different results were obtained when the four phenols were used as binary, ternary, and quaternary mixtures, showing that the simultaneous presence of more than one phenol in the reaction mixture differentially affected the transforming activity of the enzyme.

The purpose of the present study was to evaluate and quantify the performance of a laccase from the fungus *Trametes versicolor* in the presence of various phenolic compounds

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typically present in waste water with particular reference to OMW from different origins (*13*). Tests performed with *R. vernicifera*, *T. versicolor*, and *Coriolopsis polyzona* laccases indicated *T. versicolor* laccase the most efficient in the removal of either phenolic or nonphenolic substances and to decontaminate aqueous effluents. Phenols were tested as either single solutions or complex mixtures. A natural mixture of phenolic constituents extracted from an OMW sample was investigated, as well. Particular attention was devoted to the residual activity levels of the enzyme, after its initial catalytic action on added substrates, in order to evaluate the effectiveness of such a catalytic process for a cost-effective realistic application.

MATERIALS AND METHODS

Chemicals. Catechol, 3-hydroxyphenylacetic acid, protocatechuic acid, 3-hydroxybenzoic acid, caffeic acid, syringic acid, and 2,6-dihydroxybenzoic acid were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Methylcatechol and *m*-tyrosol were purchased from Fluka Chemie AG (Buchs, Switzerland), and 4-hydroxybenzaldehyde was from Merck-Schuchardt (Hohenbrunn, Germany). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Boehringer Biochemia (Mannheim, Germany). Highly purified hydroxytyrosol was a kind gift of Prof. R. Capasso of the Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, University of Naples Federico II, Italy (*9*). All other chemicals were from Sigma-Aldrich. All phenolic substrates were dissolved in buffer−dimethylformamide (1:5 v/v) and stored at 4 °C. The molecular structures and some characteristics of phenols are summarized in **Table 1**.

OMW Extract. An ethyl acetate extractable fraction (extract) was obtained from a Moroccan OMW sample by the Friederich method. According to El Hadrami et al. (*15*), the OMW sample had a pH of 4.9 (±0.2), a medium conductivity (11.6 ± 0.03 dS m^{-1}), relatively low amounts of Na, Ca, and Mg (<0.05 ± 0.01 g L^{-1}), and a moderate amount of K (3.5 ± 0.9 g L^{-1}). Total phenolics, sugar, and protein contents were 3.3 ± 0.2, 17.1 ± 0.2, and 2.0 ± 0.4 g L^{-1}, respectively. The OMW sample would contain simple phenols (protocatechuic acid, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, catechol, *m*-tyrosol, methylcatechol, and hydroxytyrosol), derivatives of hydroxycinnamic acid (caffeic acid, *p*-coumaric acid, and ferulic acid), flavonoids, anthocyanins, and polymeric phenols. Before phenol extraction, the OMW sample was centrifuged for 30 min at 10000 *g*, using a Sorvall SS34 rotor, and then filtered through Whatman No. 113 filters. After filtration, 50 mL of OMW was extracted with ethyl acetate (50 mL). After 8 h extraction, the organic extract was recovered and dried with a rotary evaporator at 70 Pa and 25 °C. The organic phenolic extract was then dissolved in 50 mL of deionized water and stored at 4 °C.

Enzymatic Activity Tests. A commercial laccase preparation (benzenediol:oxygen oxidoreductase, EC 1.10.3.2; 0.96 unit mg^{-1}) from the fungus *T. versicolor* was purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

Laccase activity was assayed at 25 °C using ABTS as the substrate. The standard conditions adopted were 10 mM ABTS in 0.1 M sodium acetate buffer, pH 3.65, and 25 °C (*12*). Oxidation of ABTS was followed by absorbance increases at 420 nm ($\epsilon_{420} = 36$ mM^{-1} cm^{-1}) (*16*). All activity tests were carried out directly in a Shimadzu UV/vis spectrophotometer by continuously monitoring the production of the chromogenic compound. Enzymatic activity was expressed in enzyme units (EU). One unit of enzymatic activity was defined as the amount of enzyme required to transform 1 μmol of ABTS/min at 25 °C and pH 3.65.

To determine kinetic parameters, activity assays were performed at 25 °C and pH 3.65 in 0.1 M sodium acetate buffer, by ranging the ABTS concentration from 0.025 to 30 mM. Because a substrate inhibition phenomenon was evident at ABTS concentrations higher than 10 mM, the experimental data were fitted to the modified Michaelis–Menten equation which takes into account substrate inhibition (*17*):

$$v = \frac{V_{max} S}{K_M + S + S^2/K_i}$$

where v is the specific enzymatic activity, V_{max} and K_M are the maximum velocity and the Michaelis constant in the absence of inhibition, and K_i is the substrate inhibition constant. The V_{max}, K_M, and K_i values were obtained by nonlinear regression analysis. Although the previous equation primarily applies to a single-substrate reaction, this kinetic treatment may be used because the concentration of oxygen, the other substrate participating to the reaction, may be considered in excess and constant over the time span of the reaction.

The inhibitory effect of 2,6-dihydroxybenzoic acid, 3-hydroxybenzoic acid, and 4-hydroxybenzaldehyde on laccase activity was monitored at 25 °C with ABTS concentrations increasing from 0.5 to 10 mM in 0.1 M sodium acetate buffer, pH 3.65, and in the presence of phenols at concentrations varying from 1 to 15 mM. The kinetic parameters were determined by a computed nonlinear and linear regression analysis according to the Michaelis–Menten (V vs S) and the modified Lineweaver−Burk ($1/V$ vs $1/S$) equations. To calculate the inhibition constant, the $1/V_{max\ apparent}$ values were plotted versus inhibitor concentrations, according to the secondary plot (*18*). The K_i values were obtained by linear regression analysis of the plotted data.

Transformation of Phenols. Caffeic acid, catechol, hydroxytyrosol, methylcatechol, protocatechuic acid, syringic acid, *m*-tyrosol, 3-hydroxybenzoic acid, 3-hydroxyphenylacetic acid, 2,6-dihydroxybenzoic acid, and 4-hydroxybenzaldehyde were incubated at 5 mM final concentration and 25 °C for 0.5, 1, 3, 5, and 24 h in 0.1 M sodium acetate, pH 5.0, in the presence of laccase (0.15 EU mL^{-1}) in 5 mL test tubes. Usually two control assays were performed: one without the enzyme to evaluate the volatilization and spontaneous transformation of phenol and the other lacking the phenol to determine the possible loss of the enzyme activity during the assay.

To evaluate the behavior of the enzyme in the presence of phenolic compounds in combination, experimental runs were also carried out with the 11 phenols in the mixture (each phenol at 3.3 mM final concentration) under the above specified conditions, if not stated otherwise.

In both experimental runs, samples were analyzed for both phenol concentration and residual enzymatic activity at each incubation time. Additionally, total organic C (TOC) was determined by the ash combustion procedure with a 1108 elemental analyzer (Fison Instruments, Milan, Italy). Calibration of the instrument with the appropriate standard (acetanilide) was carried out. Accuracy (<0.05%) and recovery of C (instrument detection limit 10 mg kg^{-1}) were checked by analyzing a sample of the standard material after each set of eight sample analyses.

For phenol determination, 1 mL of samples or controls was added to 0.125 mL of concentrated sulfuric acid to stop the enzymatic reaction. The samples were then centrifuged for 15 min at 10000*g* using a Sorvall RC-5 PLUS refrigerated superspeed centrifuge. The supernatants were filtered through 0.45 μm Acrodisc LC 13 PVDF filters (Pall Filtration & Separations SpA, Italy) and analyzed by high-performance liquid chromatography (HPLC). The transformation of each phenol (removal) was calculated by taking the difference between phenol levels in the experimental assay and the corresponding controls.

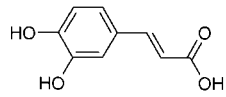
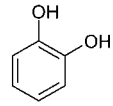
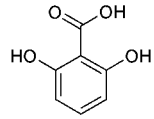
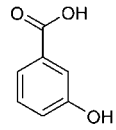
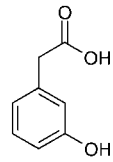
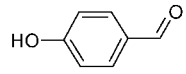
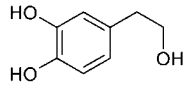
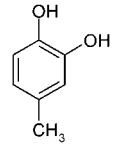
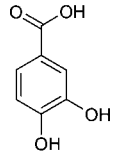
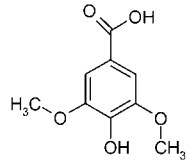
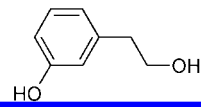
For residual enzymatic activity, usually aliquots (0.1 mL) of samples were withdrawn, placed in frozen tubes, and immediately assayed using ABTS, under standard conditions. The residual enzyme activities were expressed as percentages of the activity of the corresponding controls.

A different experimental scheme was followed for the determination of laccase activity toward the OMW extract. Separate specimens consisting of 2 mL of extract with 0.15 EU mL^{-1} of laccase were incubated at 25 °C under stirring for 0, 3, 6, 24, and 48 h. At the end of each incubation time, the samples and the corresponding controls (extract) without laccase were centrifuged and filtered, according to the conditions described for single phenols and analyzed for both phenol concentration and residual laccase activity.

All experimental tests were performed at least in triplicate, and SD was less than 5%.

HPLC Analyses. HPLC analyses were performed with an Agilent R 1100 instrument equipped with a pump and variable wavelength

Table 1. Chemical Structures and Redox Potentials of the Tested Phenols

Phenol	Structure	-(OH)$_x$	Substituent	Redox Potential (V)
Caffeic acid		2	-CH-CH-COOH (75)*	~ +0.53 (*27*)
Catechol		2	-	~ +0.53 (*27*)
2,6-dyhydroxybenzoic acid		2	-COOH (49)	~ +1.0[e] (*27*)
3-hydroxybenzoic acid		1	-COOH (49)	+1.02 (*28*)
3- hydroxyphenylacetic acid		1	-CH_2-COOH (63)	+1.06 (*26*)
4-hydroxybenzaldehyde		1	-COH (31)	--
Hydroxytyrosol		2	-CH_2-CH_2OH (47)	--
Methylcatechol		2	-CH_3 (15)	+0.77 (*26*)
Protocatechuic acid		2	-COOH (49)	~ +0.79 (*28*)
Syringic acid		1	-OCH_3 (32) -OCH_3 (32) -COOH (49)	+0.68 (*28*)
m-Tyrosol		1	-CH_2CH_2OH (47)	--

*Values in parentheses indicate the molecular masses of the substituents.

absorbance detector set at 280 nm and at 254 nm. A 25 × 4.6 mm i.d., 5 μm, Zorbax ODS (dimethyloctadecylsilane) column (Agilent Technologies, Italy) and a 30 × 4.6 mm i.d., 7 μm, Brown Lee Spheri-5 RP300 guard column (PerkinElmer) were used.

For analysis of single phenols an isocratic elution was performed at a flow rate of 1 mL min^{-1} with a mobile phase composed of acidified water and acetonitrile (70:30 v/v). The acidified water was prepared with 1 mL of concentrated acetic acid. All phenols were analyzed at

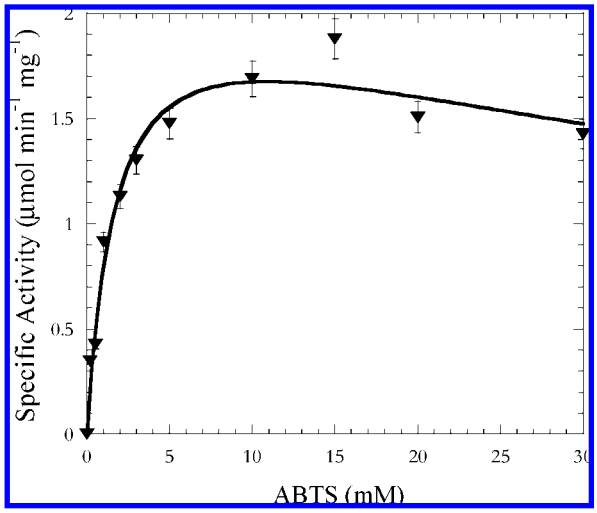


Figure 1. Specific activity versus ABTS concentration. Error bars indicate standard deviations.

280 nm, except for protocatechuic acid and 2,6-dihydroxybenzoic acid, which were analyzed at 254 nm.

In the case of experiments with the synthetic phenolic mixture and the OMW extract, elution gradients were used at a flow of 0.5 mL min^{-1} with a mobile phase composed of water acidified with 1 mL L^{-1} of orthophosphoric acid (solvent A) and acetonitrile−water (70:30 v/v) (solvent B); the analysis of the synthetic phenolic mixture was made according to following elution program: gradient to 75% A in 18 min; to 50% A in 30 min; to 100% B in 10 min; to 97% A in 5 min; isocratic elution 97% A for 5 min. The elution program for the OMW extract was as follows: isocratic elution with 85% A for 5 min; gradient to 50% A in 35 min; to 100% B in 10 min; to 85% A in 10 min; isocratic elution 85% A for 5 min. The phenolic compounds were identified on the basis of their retention times and their spectra in comparison with standards. Coinjection and elution with different concentrations of the related standards were used to ensure the identity and quantity of the compounds.

RESULTS AND DISCUSSION

Preliminary Investigations. To choose the standard activity assay conditions, laccase activity was preliminarily tested at increasing ABTS concentrations (**Figure 1**). Results indicated that at ABTS concentrations higher than 15 mM a substrate-inhibition phenomenon appeared. The kinetic parameters calculated by the modified Michaelis–Menten equation were V_{max} = 1.82 μmol min^{-1} mg^{-1}, K_M = 1.75 mM, and K_i = 20 mM. Bourbonnais and Paice (*19*) reported that an inhibition at high ABTS concentrations appeared evident for laccase from another strain of *T. versicolor*, but no numerical evaluation was made. Laccases from both plant (*R. vernicifera*) (unpublished results) and fungal (*Cerrena unicolor*, *Pleorotus ostreatus*) (*20, 21*) origins showed similar substrate inhibition phenomena.

The behavior shown in **Figure 1** could be explained by the reaction between the substrate and the enzyme–substrate complex (ES) to form a nonproductive ternary complex ES_2, not capable of splitting into enzyme and product. The high value of K_i, however, suggests that the phenomenon occurs only at high substrate concentrations. Therefore, in the following the activity of the enzyme was measured at ABTS concentrations lower than 15 mM to avoid any possible inhibition by the substrate.

Transformation of Phenols in Single Solution. Preliminary tests were performed to select the experimental conditions in terms of pH and amount of enzyme to be used in the presence of phenols. Assays were performed by incubation of 5 mM catechol at 25 °C for 24 h in a pH range between 3 and 7.5 and with different amounts of laccase. Results demonstrated that the enzyme showed the higher activity at pH 5.0 and with 0.15 EU mL^{-1} laccase concentration. pH 5.0 was also chosen because it is very close to that of the OMW sample used in this study (*15*).

Eleven phenols chosen among the most abundant components of OMW were tested as potential substrates for the enzyme. Typically, the total phenol content in olive oil has been reported to vary with olive oil quality (*22*). The concentration of a single phenol ranges from 0.196 mM (syringic acid) to 2.47 mM (*m*-tyrosol) (*13*). According to literature reports about the specific activity and substrate affinity of *T. versicolor* laccase (*23*), investigations were performed at 5 mM phenol concentration to assess the ability of the enzyme to transform large quantities of phenolic compounds. Moreover, to evaluate the degree of phenol oxidation as a function of the enzyme stability, tests were performed at five different incubation times, and at each incubation time the residual laccase activity was measured following the standard conditions.

According to their response to laccase action under the experimental conditions adopted, the 11 phenolic compounds were classified in three groups: reactive, intermediate reactive, and recalcitrant. The first group included catechol, caffeic acid, hydroxytyrosol, methylcatechol, and syringic acid whose transformation ranged from 88% to 100% after 24 h incubation time. Intermediate reactive were considered those phenols, 3-hydroxybenzoic acid, 3-hydroxyphenylacetic acid, *m*-tyrosol, and protocatechuic acid, that reached a transformation lower than 50% in the same time span, and the last group, recalcitrant, including 2,6-dihydroxybenzoic acid and 4-hydroxybenzaldehyde, was not transformed at all.

The time course of transformation (%) of reactive and intermediate reactive phenols by laccase was also monitored (**Figures 2** and **3**). A sudden removal of hydroxytyrosol occurred in the initial phase of the reaction (**Figure 2**). After 1 h incubation, about 95% of the hydroxytyrosol concentration was transformed. With increasing the incubation time, a slower, quite constant transformation took place. The transformation of the other reactive phenols progressively increased with time. Indeed, after 1 h incubation 18%, 21%, 37%, and 15% transformation on average occurred for catechol, methylcatechol, caffeic acid, and syringic acid, respectively. At 24 h incubation, quite complete oxidation was reached for all phenols (**Figure 2**).

Correspondingly, the reaction mixtures achieved different colors, which intensified with time. At 24 h incubation mixture colors ranged from bright yellow and light brown to intense yellow and dark brown for caffeic and syringic acids and hydroxytyrosol, respectively. Light amber to dark brown color was observed for catechol solutions, whereas an intense brown color constant with incubation time was achieved by methylcatechol solution. The formation of brown solid material visible to the naked eye was observed after catechol transformation; by contrast, no visible precipitate formation was observed for the other phenols. Moreover, when the data of **Figure 2** were elaborated as $\log(C/C_0)$ vs time (where C and C_0 stand for phenol concentration at t and zero time), a straight line was obtained to indicate that the reaction followed first-order kinetics (data not shown).

A completely different behavior was observed for intermediate reactive phenols (**Figure 3**). After 24 h incubation, a low transformation (%) occurred for *m*-tyrosol and protocatechuic acid reaching at maximum 39% and 45%, respectively. During the entire incubation time (1–24 h) the transformation order of

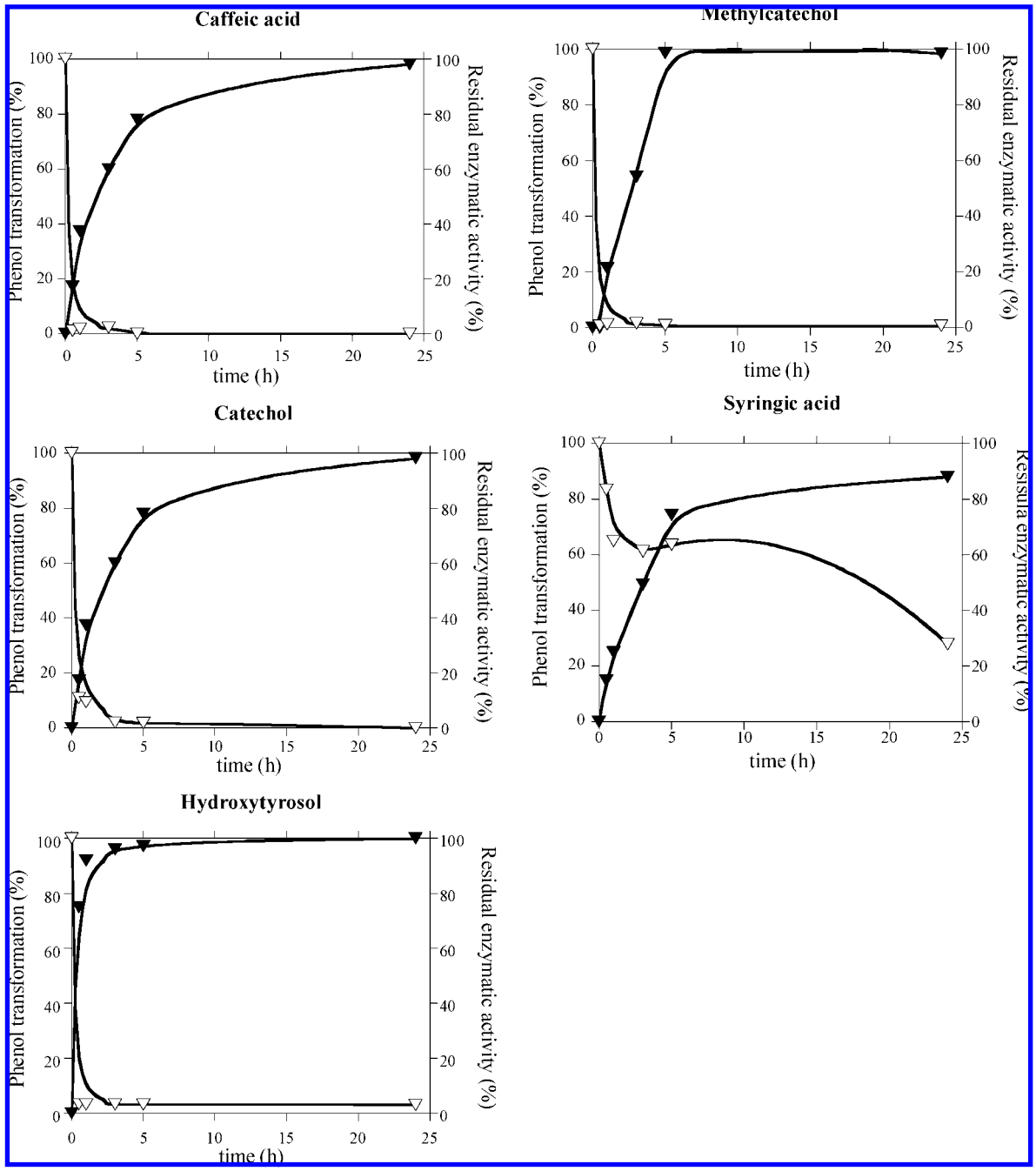


Figure 2. Transformation (%) of 5 mM reactive phenols (▼) at 25 °C and pH 5.0 with 0.15 EU of laccase and residual activities (%) of laccase (▽) in the phenolic solutions. All SDs were smaller than the symbol sizes.

phenols was *m*-tyrosol ≥ protocatechuic acid ≥ 3-hydroxybenzoic acid > 3-hydroxyphenylacetic acid. As shown in **Figure 3**, a measurable removal of *m*-tyrosol and 3-hydroxybenzoic and 3-hydroxyphenylacetic acids (23%, 15%, and 20%, respectively) occurred within the first 0.5–1 h of incubation. Afterward, phenol removal remained relatively constant within 5–24 h of incubation. A progressive removal of protocatechuic acid occurred with time, and 45% transformation was measured by the end of the investigation. A light yellow or yellow color was achieved by the reaction mixtures of *m*-tyrosol and 3-hydroxybenzoic acid, which did not intensify as incubation time increased. Neither coloration of the mixture nor the formation of solid material was observed for the other phenols.

Very low or no measurable transformation occurred for 2,6-dihydroxybenzoic acid and 4-hydroxybenzaldehyde even after 24 h incubation. The reaction mixtures remained uncolored throughout the incubation period. Therefore, the two phenols were classified recalcitrant.

Analysis of HPLC chromatograms indicated that diverse peaks, corresponding to various byproducts, appeared concomitantly with substrate removal, in particular after reactive phenol transformation. The removal of catechol, hydroxytyrosol, methylcatechol, caffeic acid, and syringic acid, and also of protocatechuic acid and *m*-tyrosol, gave rise to the appearance of a common peak, at shorter elution time (~2.5 min), indicative of a compound with an increased polarity (*24*). By contrast, no additional peaks were observed after removal of the other phenols. Moreover, no variations of TOC levels before and after laccase action were measured, thus confirming that transformation and no mineralization of phenols occurred.

The findings are in agreement with that widely demonstrated with other laccases; i.e., the efficiency of the enzymatic

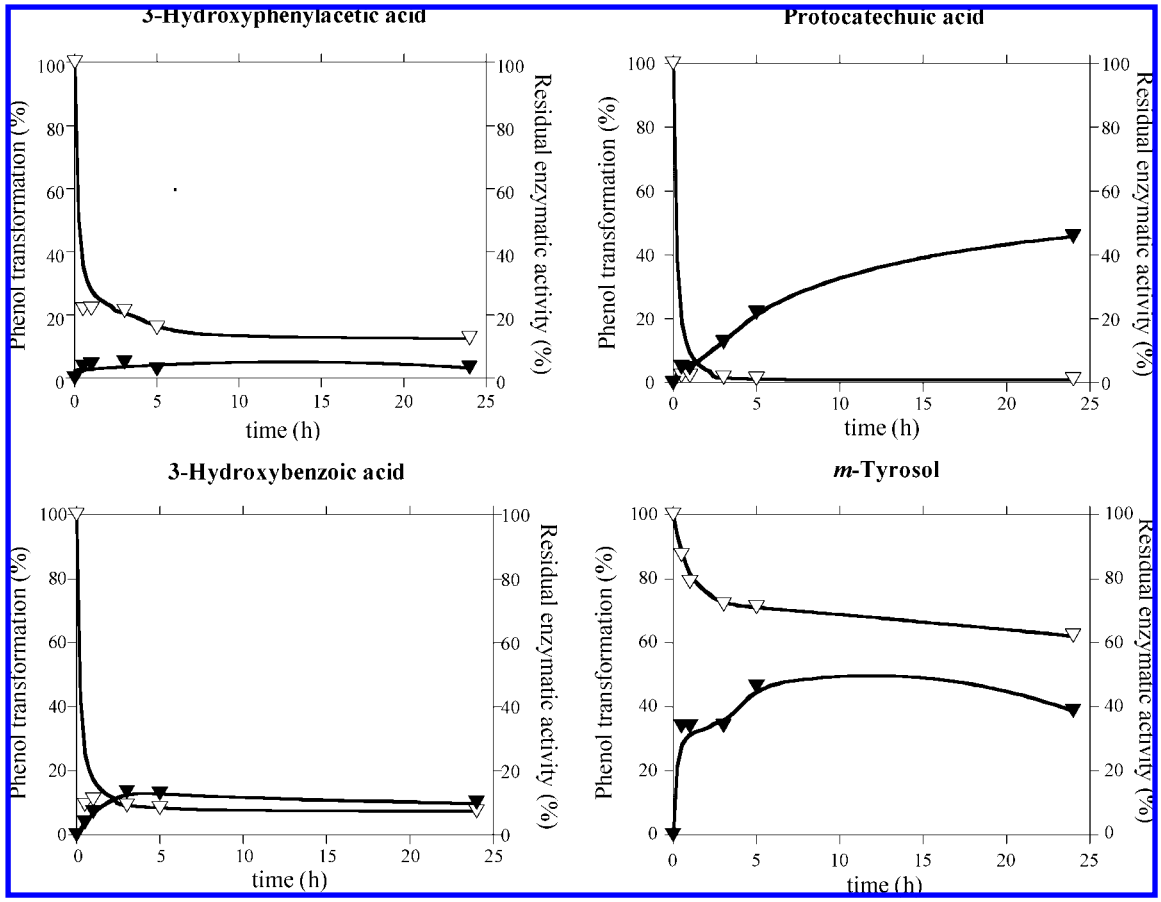


Figure 3. Transformation (%) of 5 mM intermediate reactive phenols (▼) at 25 °C and pH 5.0 with 0.15 EU of laccase and residual activities (%) of laccase (▽) in the phenolic solutions. All SDs were smaller than the symbol sizes.

transformation is strictly dependent on the chemical features of the phenolic substrate such as the number of −OH groups present, the nature and molecular mass of the substituents, and their position on the aromatic ring (*25*). As expected, the intensity and degree of mixture colors and the formation of solid material after transformation were a function of phenol oxidation; i.e., a notable qualitative correspondence between phenol removal and mixture colors occurred.

The lower or absent reactivity of the intermediate reactive and recalcitrant phenols might be explainable by their molecular structure and their higher redox potential (*26, 27*). In particular, the substituents that behave as electron-donating (e.g., $-CH_3$ or $-CH_2CH_3$) are those easier to give up an electron, to decrease oxidation potential, and to increase oxidation rate. By contrast, substituents that are electron-withdrawing (e.g., $-NO_2$, −Cl, −COOH) are harder to give up an electron, and usually they increase oxidation potential and decrease oxidation rate (*3*). Both the intermediate reactive and recalcitrant phenols have substituents that are electron-withdrawing (**Table 1**).

The different initial lower transformation rate exhibited by methylcatechol and catechol with respect to that of hydroxytyrosol is less explicable. Indeed, the three phenols all have two OH groups and differ from each other by having methylcatechol and hydroxytyrosol as an electron-donating substituent ($-CH_3$ and $-CH_2CH_2OH$, respectively) in the *para* position on the aromatic ring, whereas catechol does not (**Table 1**). Therefore, a similarity of behavior of methylcatechol with hydroxytyrosol rather than of methylcatechol with catechol would have been expected (*12*). However, the molecular structure of the substrate, the size of the substituent, and its *ortho* or *para* position may strongly influence the redox potential of the substrate, thus determining the final response to laccase oxidation (*29*). The different behavior shown in **Figure 2** for the three phenols at lower incubation times seems to be also in contrast with that reported in the literature (*24, 25*), i.e., that the higher the molecular mass of the substituent (as in the case of hydroxytyrosol and methylcatechol, in the order listed), the less efficient the laccase transformation of phenols. By contrast, the results observed at higher incubation times are in agreement with this trend.

During the incubation period, the residual laccase activities of supernatants were measured under standard conditions. No corresponding measurements of protein concentration were possible because of the strong interference of phenolic compounds with many routine protein assays (*30*).

The results of activity assays carried out at each data point of **Figures 2** and **3** demonstrated that after 1 h incubation the residual enzymatic activity suddenly decreased by more 90% for catechol and it fell to near zero for hydroxytyrosol. The reduction of laccase activity was complete also for caffeic acid, whereas 35% and 60% of the initial enzymatic activity was still detected for methylcatechol and syringic acid, respectively (**Figure 2**). A similar though less pronounced decrease of laccase activity was also observed for both the intermediate reactive and recalcitrant phenols (**Figure 3**). An exception to these findings was observed when *m*-tyrosol was used as the substrate. After *m*-tyrosol oxidation the residual enzymatic activity reduced to only 60–70%, and it remained high by the end of the experiment.

Activity tests were also performed on samples filtered with filters ((Minisart cellulose acetate filters, $<0.2\ \mu m$) that specifically adsorb phenols and phenol derivatives. Activity values

similar to those obtained without sample filtration were obtained, thus ruling out a direct inhibitory effect on laccase activity by polymeric products derived from phenol oxidation and/or by unreacted phenolic substrates. A reduction of laccase activity has usually been detected in treatments showing a measurable removal of phenolic substrates (*12, 21, 24*). An inverse relationship between residual laccase activity and phenol removal was observed, and the greater the phenol transformation, the higher the laccase activity reduction (*21, 24*). This phenomenon was explained by the disappearance of laccase active molecules complementary to the phenol oxidation. Active enzymatic molecules may be removed from the solution and incorporated (entrapped, adsorbed, and/or bound) into the newly formed products, probably of soluble polymeric nature. The precipitation of proteic molecules could also take place when solid material is detected. A partial and/or complete loss of enzymatic activity may result from the immobilizing process of the laccase molecules. An exception to these findings, but in the opposite direction of the results found, was the high levels (76–89%) of laccase residual activity measured with respect to the great oxidation (>90%) of phenolic, humic precursors such as resorcinol, gallic acid, and pyrogallol (*24*). Therefore, while the decrease of laccase activity observed with reactive phenols was predictable, that measured with intermediate and, especially, with recalcitrant phenols was less expected, thus suggesting that different mechanisms were probably involved in the interaction between them and laccase molecules.

Inhibition of laccase activity was then hypothesized when very low residual enzymatic activity was associated with the low level of phenol transformation. ABTS activity tests were performed with the less transformed substrates, 2,6-dihydroxybenzoic acid, 3-hydroxybenzoic acid, and 4-hydroxybenzaldehyde.

Figure 4 shows the Michaelis–Menten plots obtained for 3-hydroxybenzoic acid and 4-hydroxybenzaldehyde. The kinetic parameters V_{max} and K_M and the corresponding V_{max}/K_M values, as determined by the Michaelis–Menten and Lineweaver−Burk equations, were quite comparable to each other, indicating that the experimental data were good and properly weighted (data not shown).

The results in **Figure 4** do confirm an inhibitory effect of the two phenols on laccase activity, but with different mechanisms. By increasing 3-hydroxybenzoic acid concentrations, the V_{max} values reduced from 3.29 to 2.97 μmol min^{-1} mg^{-1} and the K_M values increased from 0.11 to 0.49 mM. In contrast, both of the kinetic parameters decreased for 4-hydroxybenzaldehyde. The V_{max} decreased from 2.64 to 1.08 μmol min^{-1} mg^{-1} and the K_M from 0.25 to 0.05 mM. In the case of 3-hydroxybenzoic acid the simultaneous decrease of V_{max} and increase of K_M are indicative of a noncompetitive inhibition mechanism, where the enzyme shows an apparent lower efficiency for the substrate and a reduced reaction velocity. A smaller catalytic efficiency (V_{max}/K_M) may result. By contrast, the trend of the two kinetic parameters at increasing 4-hydroxybenzaldehyde concentrations suggests that an uncompetitive inhibition mechanism takes place.

To calculate the inhibition constants K_i, the data were plotted according with the secondary plot (*18*). The two constants calculated were 0.11 and 0.015 M for 3-hydroxybenzoic acid and 4-hydroxybenzaldehyde, respectively. The significant lower value of K_i for 4-hydroxybenzaldehyde indicates that the aldehyde behaves as a considerably stronger inhibitor than 3-hydroxybenzoic acid.

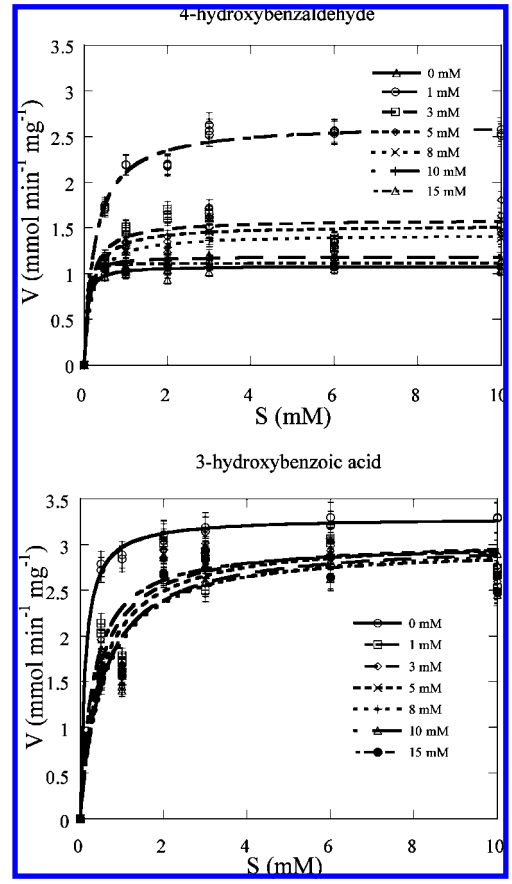


Figure 4. Michaelis–Menten plots of inhibition tests with 4-hydroxybenzaldehyde and 3-hydroxybenzoic acid. Error bars indicate standard deviations.

An unclear kinetic trend was observed for 2,6-hydroxybenzoic acid (data not shown). Activity values fluctuated with changing both ABTS and phenol concentrations. It was not possible to elaborate the data with any of the kinetic models following Michaelis−Menten kinetics.

The results achieved in the inhibition tests still confirm the complexity of relationships existing between laccase and its substrates when phenolic compounds are involved.

Transformation of Phenols in Synthetic and Natural Mixtures. Investigations were performed to evaluate the efficiency of the enzyme toward the 11 phenols when present in combination to better simulate an aqueous waste. A natural mixture consisting of the phenolic extract from the Moroccan OMW sample was tested, as well.

Table 2 shows the removal of the 11 phenols after 24 h incubation of their mixture with laccase. The corresponding removal of each phenol when incubated in a mixture lacking hydroxytyrosol (i.e., a mixture made by only 10 phenols) is also reported. For comparison purposes, the transformations of each phenol in single solution are also shown. It is noteworthy that the enzyme was capable of transforming the 11 substrates even when present in the mixture. Moreover, the simultaneous presence of such a high number of phenolic substrates determined some enhancing or depressing effects toward the transformation of some of them.

From the results of the mixture with or without hydroxytyrosol (**Table 2**), it appears evident that the composition of the mixture may influence the removal of some phenols. This effect is much more evident when comparing the removal of phenols in the mixtures or alone. Generally, an enhanced phenol

Table 2. Transformation (%) of Phenols in Different Mixtures at 25 °C and pH = 5.0[a]

phenol	transformation (%) mixture with hydroxytyrosol	mixture without hydroxytyrosol	alone
catechol	100	100	100
caffeic acid	38	86	99
2,6-dihydroxybenzoic acid	7	11	0
methylcatechol	100	100	98
3-hydroxyphenylacetic acid	13	12	3
hydroxytyrosol	100		
4-hydroxybenzaldehyde	19	19	0
syringic acid	100	85	87
m-tyrosol	10	22	39
protocatechuic acid	99	97	46

[a] SDs were less than 5%.

removal was observed in the complex mixtures. For instance, the disappearance of protocatechuic and 3-hydroxyphenylacetic acids increased from 46% and 3% to 99–97% and 13–12%, respectively, when the phenols were present in the 11 or 10 phenolic mixture. Even the two recalcitrant phenols, 2,6-dihydroxybenzoic acid and 4-hydroxybenzaldehyde, were removed though to a small extent. By contrast, a detectable depressing effect was observed on the transformation of *m*-tyrosol, which decreased from 39% to 10% and 22% in the mixture with or without hydroxytyrosol (**Table 2**). After laccase action, the complex mixtures achieved an intense rust-brown color, and the presence of a small amount of solid material, indicative of products of size and molecular mass such as to induce their precipitation, was observed.

Measurements made at different incubation times (0.5, 1, 3, 5, and 24 h) indicated that a progressive increase of each phenol removal occurred (data not shown). For example, the transformation of catechol, methylcatechol, and syringic acid increased from 32%, 61%, and 35% at 0.5 h incubation to 100%, 100%, and 85% at the end of the incubation, respectively. No further increased transformation of any of the 11 phenols was observed at 48 h incubation.

The reciprocal influence of different phenols in mixture on their transformation by laccase was also evaluated with a natural phenolic mixture, the phenolic extract from the Moroccan OMW sample. Preliminarily, attempts were made to confirm and identify the phenols present in the OMW extract and to determine their concentrations (*15*). HPLC analysis of the Moroccan OMW extract revealed the presence of several aromatic components that affected the complexity and the nature of the mixture. On the basis of comparison of retention times and spectra with standards, only six phenols usually recognized in OMW extracts (*13*) were identified (i.e., 30 mM hydroxytyrosol, the most abundant, 9.4 mM *m*-tyrosol, 3.7 mM methylcatechol, and 0.19 mM syringic, 5.4 mM caffeic, and 0.08 mM protocatechuic acids). Other phenols were likely present in the OMW sample and not identifiable, at least with the HPLC conditions used.

Table 3 reports the transformation at three different incubation times of the six phenols identified in the natural mixture and, for comparison, that at 24 h incubation of a synthetic mixture made by the six phenols identified in the natural OMW extract and at equal concentration levels. The data of **Tables 2** and **3** clearly indicate that the transformation of hydroxytyrosol was quite independent of the complexity of the reaction mixture whereas its presence influenced the transformation of the other phenols. By contrast, the removal of methylcatechol or *m*-tyrosol

Table 3. Transformation (%) of Phenols in the OMW Extract and in the Six-Phenol Synthetic Mixture[a]

phenol	transformation (%) OMW extract 3 h	OMW extract 6 h	OMW extract 24 h	synthetic mixture[b] 24 h
caffeic acid	31	28	84	95
hydroxytyrosol	23	25	90	72
methylcatechol	33	33	32	100
protocatechuic acid	0	0	45	48
syringic acid	40	54	65	59
m-tyrosol	48	77	78	5

[a] SDs were less than 5%. [b] The mixture contained 30 mM hydroxytyrosol, 9.4 mM *m*-tyrosol, 3.7 mM methylcatechol, 0.19 mM syringic acid, 5.4 mM caffeic acid, and 0.08 mM protocatechuic acid in 0.1 M sodium acetate, pH 5.0.

was strongly affected by the simultaneously presence of other phenols in the reaction mixture. Indeed, the transformation of methylcatechol was strongly depressed in the OMW extract as compared to the synthetic mixtures. By contrast, *m*-tyrosol was transformed at very high percentage in the natural mixture whereas it ranged from 5% (mixture of six phenols) (**Table 3**) to 10% or 22% in the mixture with 11 or 10 phenols in combination, respectively (**Table 2**).

Analysis of HPLC chromatograms indicated that diverse peaks at high retention times (30–50 min), and corresponding to byproduct with lower polarity, appeared concomitantly with the substrate removal from synthetic and natural mixtures after laccase action. The most intense peak was observed for the mixture made by the six phenols (**Table 3**), where the concentration of some of them reached values up to 30 mM (hydroxytyrosol). Less intense peaks were observed with the mixture made by 10 or 11 phenols (**Table 2**) where the concentration of each phenol was lower. These results were totally opposite to those observed with phenols incubated in single solution (i.e., appearance of peaks with higher polarity), thus confirming that products with different properties and features are likely produced not only when different phenols are treated in mixture but also when their concentrations are different.

For both the synthetic and natural mixtures, a sudden decrease of laccase activity to near zero, which remained constant with time, occurred after 0.5 h incubation (data not shown). These results, along with those reported in **Figures 2** and **3**, confirm not only the fate of laccase molecules after their action on phenolic substrates but also the mechanisms hypothesized for the transformation of phenols by the enzyme. As demonstrated and discussed by Bollag and Liu (*31*) and Gianfreda et al. (*25*), the main action of the enzyme is primarily devoted to produce free radicals from the substrate. Once these are formed, they can further undergo a laccase-catalyzed oxidation or the reaction may proceed also spontaneously, i.e., in the absence of active enzymatic molecules, to form quinones, dimers, trimers, and polymeric species from phenols.

The response of phenols to laccase action may be strongly influenced by the presence of other substances that may behave as mediators and/or competitive inhibitors. Indeed, it has been well recognized that certain compounds of either phenolic or nonphenolic nature may behave as active mediators of laccase action. Specific molecules such as ABTS, 1-hydroxybenzotriazole (HBT), and phenolic humus precursors are able to induce the transformation of even recalcitrant molecules. They are, usually, highly reactive substrates in the presence of laccase,

which produces the free radicals that cause the transformation and subsequent polymerization of less or even nonreactive substances.

In light of these considerations the results of **Tables 2** and **3** are easily explainable. Evidently, highly reactive substrates such as hydroxytyrosol, methylcatechol, or catechol acted as mediators for those less reactive substrates and promoted their transformation. Also, the response of *m*-tyrosol in the OMW extract (**Table 3**) could be explained by assuming that other phenolic substances acting as active cosubstrates and not identified by the adopted HPLC conditions were present in the extract and enhanced the transformation and removal of the phenol. By contrast, the reduced transformation of caffeic acid could be due to competitive inhibitory effects by the presence of several enzyme susbtrates.

In conclusion, *T. versicolor* laccase efficiently promoted the transformation of a large number of different phenolic compounds when tested alone or in complex mixtures. Its efficiency was demonstrated also toward a natural mixture such as the organic extract from a sample of olive oil mill waste water. The tested phenols showed a different reactivity to laccase action, depending on their molecular structure and features. When used in combination, enhancing or depressing counteracting effects were observed. The behavior of some of them as active mediators or inhibitors of laccase action was hypothesized.

In each of the situations investigated, laccase generally lost its catalytic activity to a great extent, whether a corresponding significant removal of phenol was or not measured.

These results confirm that laccases are effective in the transformation of simple and complex phenolic mixtures and can be regarded as an efficient tool for the detoxification of phenolic wastes. The loss of enzyme activity, however, still constitutes a possible drawback for an effective use of the catalyst at large scales. The improvement of the process by laccase immobilization on solid supports and its use in batch or continuous reactors as well as the use of alternative, nonenzymatic oxidative catalysts is under investigation.

ABBREVIATIONS USED

OMW, olive mill waste water; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); EU, enzyme unit.

LITERATURE CITED

(1) Hattenschwiler, S.; Vitousek, P. M. The role of polyphenols in terrestrial ecosystem nutrient cycling. *Trends Ecol. Evol.* **2000**, *15*, 238–243.

(2) Gianfreda, L.; Bollag, J.-M. Isolated enzymes for the transformation and detoxification of organic pollutants. In *Enzymes in the Environment: Activity, Ecology, and Applications*; Burns, R. G., Dick, R. P., Eds.; Marcel Dekker: New York, 2002; pp 495–538.

(3) Gianfreda, L.; Iamarino, G.; Scelza, R.; Rao, M. A. Oxidative catalysts for the transformation of phenolic pollutants: a brief review. *Biocatal. Biotrans.* **2006**, *24*, 177–187.

(4) Mills, W. B.; Procella, D. B.; Ungs, M. S.; Gherins, S. A.; Summers, K. V.; Mok, L.; Rupp, G. L.; Bowie, G. L. *Water Quality Assessment: A Screening Procedure for Toxic and Conventional Pollutants in Surface and Groundwater*; EPA/600/6-85/002a; USEPA: Washington, DC, 1985.

(5) Krogmeir, M. G.; Bremner, J. M. Effects of phenolic acids on seed germination and seedling growth in soil. *Biol. Fertil. Soils* **1989**, *8*, 116–122.

(6) Gonzalez, M. D.; Moreno, E.; Quevedo Sarmiento, J.; Ramos Cormenzana, A. Studies on antibacterial activity of waste waters from olive oil mills (alpechin): Inhibitory activity of phenolic and fatty acids. *Chemosphere* **1990**, *20*, 423–432.

(7) Perez, J.; De la Rubia, T.; Moreno, J.; Martinez, J. Phenolic content and antibacterial activity of olive oil waste waters. *Environ. Toxicol. Chem.* **1992**, *11*, 489–495.

(8) Capasso, R. The chemistry, biotechnology and ecotoxicology of the polyphenols naturally occurring in vegetable wastes. *Curr. Top. Phytochem.* **1998**, *1*, 145–156.

(9) Capasso, R.; Cristizio, G.; Evidente, A.; Scognamiglio, F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. *Phytochemistry* **1992**, *31*, 4125–4128.

(10) Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M. A.; Crisitnzio, G. Antibacterial polyphenols from olive oil mill waste water. *J. Appl. Bacteriol.* **1995**, *79*, 393–398.

(11) Sannino, F.; Gianfreda, L. An oxidative enzyme for the detoxification of polluted systems. *Res. Adv. Water Res.* **2000**, *1*, 101–114.

(12) Gianfreda, L.; Sannino, F.; Rao, M. A.; Bollag, J.-M. Oxidative transformation of phenols in aqueous mixtures. *Water Res.* **2003**, *37*, 3205–3215.

(13) Casa, R.; D'Annibale, A.; Pieruccetti, F.; Stazi, S. R.; Giovannozzi Sermanni, G.; Lo Cascio, B. Reduction of the phenolic components in olive-mill wastewater by an enzymatic treatment and its impact on durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere* **2003**, *50*, 959–966.

(14) Iamarino, G. Trattamento e destino delle acque di vegetazione. Ph.D. Thesis (in Italian), Università degli Studi di Napoli Federico II, Naples, Italy, 2007.

(15) El Hadrami, A.; Belaqziz, M.; El Hassni, M.; Hamifi, S.; Abbad, A.; Capasso, R.; Gianfreda, L.; El Hadrami, I. Physico-chemical characterization and effects of olive oil mill wastewater in fertirrigation on the growth of some Mediterranea crops. *J. Agron.* **2004**, *3*, 247–254.

(16) Wolfenden, B. S.; Willson, R. L. Radical cations as reference chromogens in kinetic studies of one-electron transfer reactions: pulse readiolysis studies of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate). *J. Chem. Soc., Perkin Trans.* **1982**, *2*, 805–812.

(17) Laidler, K. J.; Bunting, P. S. *The Chemical Kinetics of Enzyme Action*, 2nd ed.; Clarendon: Oxford, 1973; p 84.

(18) Birch, P. An Introduction to Enzyme Kinetics, **1996** (http://www-biol.paisley.ac.uk/Kinetics/).

(19) Bourbonnais, R.; Paice, M. G. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett.* **1990**, *267*, 99–102.

(20) Palmieri, G.; Giardina, P.; Marzullo, L.; Desiderio, B.; Nitti, G.; Cannio, R.; Sannia, G. Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 632–636.

(21) Gianfreda, L.; Sannino, F.; Filazzola, M. T.; Leonowicz, A. Catalytic behavior and detoxifying ability of a laccase from the fungal strain *Cerrena unicolor*. *J. Mol. Catal. B: Enzym.* **1998**, *14*, 13–23.

(22) Tuck, K. L.; Hayball, P. J. Major phenolic compounds in olive oil: Metabolism and health effects. *J. Nutr. Biochem.* **2002**, *13*, 636–644.

(23) Madzak, C.; Mimmi, M. C.; Caminade, E.; Brault, A.; Baumberger, S.; Briozzo, P.; Mougin, C.; Jolivat, C. Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. *Protein Eng., Des. Sel.* **2006**, *19*, 77–84.

(24) Filazzola, M. T.; Sannino, F.; Rao, M. A.; Gianfreda, L. Effect of various pollulants and soil-like constituents on laccase from *Cerrena unicolor*. *J. Environ. Qual.* **1999**, *28*, 1929–1938.

(25) Gianfreda, L.; Xu, F.; Bollag, J.-M. Laccase a useful group of oxireductive enzymes. *Bioremed. J.* **1999**, *3*, 1–25.

(26) Monzani, E.; Gatti, A. L.; Profumo, A.; Casella, L.; Gullotti, M. Oxidation of phenolic compounds by lactoperoxidase. Evidence for the presence of a low-potential compound II during catalytic turnover. *Biochemistry* **1997**, *36*, 1918–1926.

(27) Mai, C.; Schorman, W.; Huttermann, A. Chemo-enzymatically induced copolymerization of phenolics with acrylate compounds. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 177–186.

(28) Mirando, M. A.; Galindo, F.; Amat, A. M.; Arques, A. Pyrylium salt-photosensitised degradation of phenolic contaminants present in olive oil wastewaters with solar light. Part II. Benzoic acid derivatives. *Appl. Catal. B: Environ.* **2001**, *30*, 437–444.
(29) Xu, F. Oxidation of phenols, anilines and benthenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry* **1996**, *35*, 7608–7614.
(30) Winters, A. L.; Manchin, F. R. Modification of the Lowry assay to measure proteins and phenols in covalently bound complexes. *Anal. Biochem.* **2005**, *346*, 43–48.
(31) Bollag, J.-M.; Liu, S.-Y. Biological transformation processes of pesticides. In *Pesticides in the Soil Environment: Processes, Impacts, and Modeling*; Cheng, H. H., Ed.; Soil Science Society of America Inc.: Madison, WI, 1990; pp 169–211.

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