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# Oxidation of phenyl compounds using strongly stable immobilized-stabilized laccase from *Trametes versicolor*

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ABSTRACT

The hydrolysis of phenolic compounds using an immobilized and highly active and stable derivative of laccase from *Trametes versicolor* is presented. The enzyme was immobilized on aldehyde supports. For this, the enzyme was enriched in amino groups by chemical modification of its carboxyl groups. The aminated enzyme was immobilized with a high recovered activity (over 60%). Aldehyde derivatives were more stable than soluble or aminated-soluble enzyme and the reference derivatives after incubation in different inactivating conditions (high temperatures, different pH values or presence of organic cosolvents). The most stable derivative was obtained immobilizing the chemically aminated enzyme at pH 10 on aldehyde supports with a stabilization factor approximately 280 fold after incubation at pH 7 and 55 °C. In addition, it was possible to prepare immobilized derivatives with a maximal enzyme loading of 60 mg $g^{-1}$ of support. This derivative could be reused for 10 reaction cycles with negligible lost of activity.



## 1. Introduction

Several types of industrial and agricultural wastes contain phenol compounds. For example, chlorophenols, largely used as wide-spectrum biocides and nitrophenols, widely employed in the chemical industry, accumulate in soils, sediments, surface waters and animals because of their continuous usage and recalcitrant nature [1]. Moreover, a lot of phenol compounds are recognized as endocrine disruptors since they possess estrogenic or antiestrogenic activities so interfering with the endocrine system [2]. Generally, these recalcitrant compounds produced in industries as paper, oil or different ink industries cannot be degraded by the active sludge in the treatment plants. Because of that the general trend is treating these compounds with different enzymes in order to transform them into degradable compounds or other non-toxic ones [3]. The use of enzymes in these processes has a lot of advantages because of their high activity in environmental conditions such as room temperature, neutral pH or atmospheric pressure, their high selectivity and specificity. One of the enzymes are being used extensively is laccase.

Laccases are produced from various sources [4–7]. This enzyme is able to oxidize different aromatic compounds such as phenol or dye compounds into other more easily degradable by the active sludge in the treatment plants [8]. Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) catalyze the oxidation of a variety of organic compounds including methoxyphenols, phenol, *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, and lignin-related molecules to *o*-, *m*-, *p*-quinones or radical species [9] not requiring hydrogen peroxide as co-substrate or any cofactors for its catalytic reaction. This makes this enzyme of great importance in different processes such as lignin modification [10], paper strengthening [11,12], juice manufacture [13], hair coloring [14] or in other different environmental processes [15,16]. Laccase from *Trametes versicolor* is one of the most widely used in various processes such as described in the literature [17–20]. It is a monomeric enzyme of approximately 70 KDa of molecular weight [21].

However, the use of enzymes is not always easy on an industrial scale because they have a number of limitations due to their biological origin. Thus enzymes are soluble and they cannot easily be used for many reaction cycles; they are quite unstable under conditions of high temperatures, extreme pH values or use of organic co-solvents or toxic products. The availability of a strongly active and stable biocatalyst is key for the application of these processes at industrial scale. One way to avoid these problems consists in the immobilization of enzymes. Immobilization promotes the reusability of the biocatalyst during different reaction cycles. This enzyme was previously immobilized using different methodologies. Some of them immobilized this enzyme through different systems such as entrapment in different polymers as alginate [22,23], dendrimers

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using poly(amido amine) (PAMAM) [24] or gelatin [25], preparation of different systems as liposomes [26], microcapsules [27] or cross-linked enzyme aggregates (CLEAs) [28]. However, the most used system consisted in using different supports to immobilize the enzyme. There are a great variety of supports used for the immobilization as Amberlite IR-120H beads [29], membranes [30], oxidized carbon nanotubes and graphene oxides [31,32], sulfo polyester resins [33], exchange resins [34], magnetic nanoparticles [35–37]. Among all these methods, the recovered activity is different depending on the immobilization protocol, but the stabilization generally is moderated and lowly studied. Best reported examples give stabilization around 10–15 fold better than soluble enzyme [19,38].

Taking into account the low stability of the soluble enzyme and necessity of obtaining more stable catalysts, in the present paper the immobilization of a commercial laccase from *T. versicolor* using aldehyde supports is proposed. The optimized catalyst in terms of activity and stability will be used in the oxidation of some phenolic compounds.

## 2. Materials and methods

### 2.1. Materials

Laccase from *T. versicolor* (2.2 IU $mg^{-1}$), 2,2′-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid) (ABTS), ethylene diamine (EDA), sodium borohydride, sodium metaperiodate, ethanolamine, glycidol (2,3-epoxypropanol), 2,6-dichloro-indophenol, pyrogallol and catechol and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were supplied by Sigma Chem. Co. (St. Louis, MO, USA). Sepharose 10BCL supports and cyanogen bromide (CNBr) activated Sepharose were supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK). Cellulose dialysis tubes (cut-off 12–14 KDa) were from Spectrum Labs (Breda, The Netherlands). Other reagents were always of analytical degree.

### 2.2. Methods

In all cases the experiments were performed in triplicate and the maximal error was never higher than 5%.

#### 2.2.1. Enzyme assay

Standard laccase activity was determined by oxidation of ABTS at 25 °C [39]. The substrate solution was composed by 2 mL of ABTS (3 mM) in sodium acetate buffer (10 mM at pH 4.5). Then a suitable amount of soluble enzyme or immobilized preparation was added and the oxidation of ABTS was followed by measuring the increase of the absorbance at 418 nm in a 1 cm path length spectrophotometric cell ($\varepsilon_{ABTS}$ = 36,000 $M^{-1}$ $cm^{-1}$). One international unit (IU) of laccase activity corresponds to the oxidation of 1 μmol ABTS per minute under these conditions.

#### 2.2.2. Chemical amination of laccase

A mixture composed by a *T. versicolor* laccase solution (1 mL with 1 IU $mL^{-1}$), 5 mL of 1 M ethylene diamine (EDA) at pH 4.75 and 0.012 g of solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was performed. After 90 min of gentle stirring at 4 °C, the solution was dialyzed 5 times against 50 volumes of distilled water and stored at 4 °C [40].

#### 2.2.3. Preparation of the supports

Cyanogen bromide Sepharose was hydrated as suggested by the supplier (1 g dry support was suspended in 10 mL of water at pH 2–3 and stirred for 30 min. Then, support was dried under vacuum before being used for the immobilization). Sepharose aldehyde supports were activated and oxidized as was previously described [41].

#### 2.2.4. Immobilization of the enzyme

A laccase solution (9 mL with 1 IU $mL^{-1}$ or the correspondent amount in the maximal load of enzyme experiments) in sodium bicarbonate (0.1 M) at the indicated pH (9 or 10) was added to 1 g of aldehyde-Sepharose. At different times, samples of the supernatant and suspensions were taken and the activity was assayed. When the immobilization process was completed, the different preparations were incubated at the indicated pH (9 or 10) during 3 h. Then, 10 mg of solid $NaBH_4$ were added in a single addition and left under stirring during 30 min. Finally the support was washed with water.

Immobilization yield was referred to the percentage of enzyme activity that disappeared from the enzyme solution compared with the activity of a blank solution without support.

Expressed activity was calculated as the ratio between the final activity after incubation at different pHs of the immobilized enzyme and the immobilized enzyme activity measured at the optimal pH. All experiments were performed using a low enzyme activity in order to avoid diffusion problems that could alter the apparent activity and/or enzyme stability.

The immobilization on cyanogen bromide Sepharose was performed as in the case of aldehyde supports. In this case immobilization was carried out at 4 °C and pH 7 during short times. In the indicated cases this derivative was aminated by adding to 1 g of laccase derivative 10 mL of EDA (1 M) and of EDAC (10 mM) at pH 4.75 during 90 min. Finally, this preparation was washed with water.

#### 2.2.5. Stability of biocatalysts

Various preparations were incubated in different indicated conditions. Periodically, residual activity was determined as described above.

Inactivation was modeled based on the deactivation theory proposed by Henley and Sadana [42]. Inactivation parameters were determined from the best-fit model of the experimental data which was the one based on two-stage series inactivation mechanism with no residual activity. According to it, biocatalyst inactivation proceeds through two sequential steps of progressively less active enzyme species until a final completely inactive species is obtained, as represented in the following scheme:

$$E \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2$$

where $k_1$ and $k_2$ are first-order transition rates constants, $E$, $E_1$ and $E_2$ are the corresponding enzyme species. The mathematical model representing this mechanism is

$$a = \left(1 + \alpha\left(\frac{k_1}{k_2 - k_1}\right)\right)\exp^{(-k_1 \cdot t)} - \left(\alpha\left(\frac{k_1}{k_2 - k_1}\right)\right)\exp^{(-k_2 \cdot t)} \quad (1)$$

where $\alpha$ represents the residual activity at time $t$ and $\alpha$ is the ratio of specific activity of enzyme species $E_1$ with respect of that of the native enzyme species $E$.

Considering only one step mechanism of enzyme inactivation ($k_2 = 0$), and residual activity ($\alpha \neq 0$), the model is first order inactivation with residual activity, represented by

$$a = (1 - \alpha)\exp^{(-k_1 \cdot t)} + \alpha \quad (2)$$

Inactivation parameters were determined from the best-fit model of the experimental data. Half-life (time at which the residual enzyme activity is half of its initial value; $t_{1/2}$) was used to compare the stability of the different biocatalysts, being determined by interpolation from the respective models described by Eq. (1) or Eq. (2).

#### 2.2.6. Oxidation of different compounds

Commercial enzyme (0.02 mg) or 0.04 g of the most stable immobilized derivative (5 mg of enzyme per gram of support) was added to 1 mL of a solution at pH 7 or 4.5 of different compounds (2,6-dichloro-indophenol, pyrogallol and catechol).

In order to measure the activity with the different substrates, derivatives with low load of enzyme were used again. In all cases activity was expressed as μmol of product $min^{-1}$ $mg^{-1}$ of immobilized enzyme. At different times, aliquots of solution were taken and the absorbance measured. Supports were previously saturated with different substrates to avoid possible absorptions. Molar extinction coefficients ($\varepsilon$) were: 12,097 $M^{-1}$ $cm^{-1}$ at pH 7 measured at 600 nm and 12,109 $M^{-1}$ $cm^{-1}$ at pH 4.5 at 520 nm for 2,6-dichloro-indophenol; 928 $M^{-1}$ $cm^{-1}$ (at pH 7 and 4.5) measured at 450 nm for *o*-benzoquinone, oxidation product from catechol; and finally 4283 and 3724 $M^{-1}$ $cm^{-1}$ at pH 7 and 4.5, respectively, and measured at 420 nm for purpurogallin that is the oxidation product of pyrogallol as previously described [43,44].

#### 2.2.7. Reuse of the derivatives

In order to assess the operational stability of the most stable preparations, several consecutive cycles were performed measuring the increase in the absorbance using ABTS (3 mM) as substrate in sodium acetate buffer (10 mM at pH 4.5) at 418 nm and 25 ° C. After each cycle, the immobilized preparation (20 IU/mL) was washed three times with 5 volumes of acetate buffer (10 mM at pH 4.5).

#### 2.2.8. Determination of the enzyme structure

Enzyme structure was extracted from Protein Data Bank (PDB file: 1gyc). Modeling was performed using the program Pymol Molecular Graphics System. Amination of the structure was simulated using this program considering a total amination of carboxylic moieties.

## 3. Results and discussion

### 3.1. Immobilization of the enzyme on aldehyde supports

As it was commented above, the availability of a heterogeneous and strongly stable biocatalyst is key for use at industrial scale. Thus, laccase was offered to an aldehyde support for its immobilization. The immobilization on aldehyde supports is performed by a multipoint mechanism [41]. Therefore, incubation at alkaline pH

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