African Journal of Biotechnology Vol. 6 (10), pp. 1255-1259, 16 May 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Fungal laccase: copper induction, semi-purification, immobilization, phenolic effluent treatment and electrochemical measurement

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Accepted 2 March, 2007

The laccase activity induced by copper ions in *Trametes versicolor* by two inducers was studied and the aims were to show that it is possible to obtain a high laccase activity in almost 12 of the enzymes product using a basic culture medium but different copper concentration. Laccase was inducted by 2,5-xylidine and copper sulfate. Semi-purification of the crude laccase was carried out through precipitation and the column separation with NaCl gradient. In order to apply in an effluent treatment, laccase was immobilized on different vitroceramics supports, pyrolytic graphite and also on a carbon fiber electrode as biosensor. The maximum laccase activity was 40,774.0 U L^{-1} at the 12th day. The best support for immobilization was pyrolytic graphite (glutaraldehyde treated-94% efficiency). Total phenol removal in Kraft E1 effluent was 19% for immobilized pyrolytic graphite with the mediator present. The biosensor prepared with this material showed a good linear response to catechol. The optimization of laccase activity induction through the combination of 2,5-xylidine and cooper sulfate was obtained and led to its use in environmental remediation.

Key words: *Trametes versicolor*, laccase induction, semi-purification, immobilization, effluent treatment.

INTRODUCTION

Laccases are produced from different fungi and their capabilities are markedly different depending on the source, number of isoforms, molar mass, optimum pH, and specificity for the substrate (Bollag and Leonowicz, 1984; Durán et al., 2002; Gianfreda and Rao, 2004; Gianfreda et al., 1999). Studies of the favorable conditions for laccase production have been carried out (Ikehata et al., 2004; Tavares et al., 2005).

The addition of an inducer such as xylidine in *Trametes versicolor* (Bollag and Leonowicz, 1984; Couto et al., 2002) and in *Trametes villosa* (Yaver et al., 1996) has been studied. Induction by copper for the white rot fungi *Pleurotus ostreatus* (Palmieri et al., 2005; Baldrian et al., 2005), *Trametes trogii* (Levin et al., 2005), *Trametes hirsuta* (Rodríguez Couto et al., 2006), *Myceliophthora thermophila* (Alcalde et al., 2005), *T. versicolor* (Minussi et al., 1999ab; Tavares et al., 2005; Kajita et al., 2004) have been found to increase laccase production. Few studies reported the laccase induction activity conjugating both inducers, 2,5-xylidine and copper ions (Gaulhaup et al., 2002). Minussi et al. (1999b) reported a laccase preliminary induction with variable 2,5-xylidine concentrations and a fixed copper salt concentration (0.02 mmolL^{-1}) on *Lentinus edodes, T. versicolor, T. villosa* and *Brotritis cinerea* in liquid medium. Tavares et al. (2005) found that at 0.075 mmol L^{-1} copper sulfate and 0.030 mmol L^{-1} 2.5-xylidine, an laccase induction from 190 to 360 U L^{-1} occurred. In some species, the addition of inducer compounds to the culture initiates a new extracellular biosynthesis form of laccases; hence, the constitutive forms are continuously produced (Bollag and Leonowicz, 1984).

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There are studies on the efficiency of laccases and peroxidases, such as horseradish peroxidase (HRP), in the phenolic and pulp and paper industry effluent treatment (Peralta-Zamora et al., 1998; Freire et al. 2000). Laccase has been used efficiently in its immobilized form, in order to be economically feasible (Durán et al., 1995, 2002; Taprab et al., 2005). Good perspectives are been published concerning the use of *in situ* remediation of contaminated regions (Baldrian, 2004) in the food Industry, olive oil production (D'Annibale et al., 1999), beverage industry such as wine and fruit juices (Minussi et al., 2002), and in textile dyes decolouration (Champagne et al., 2005).

The use of immobilized enzymes in several supports by chemical and physical processes can resolve the limitations of enzymatic instability (Gianfreda et al., 1999; Durán and Esposito, 2000; Durán et al., 2002). Carbonaceous materials such as activate carbon, vitroceramics and pyrolytic graphite are supports largely employed in analytical methodology, mainly due to the possibility to use hydroxyl and carboxylic groups on the surface to form covalent bound with enzymes. The immobilization via covalent binding is important when bifunctional reagents, such as carbodiimide and glutaraldehyde, are used (Durán et al., 2002). Leonowicz (1988) demonstrated that laccase from *Rhizoctonia praticola* and *T. versicolor* immobilized by covalent binding on celite, glass porous beads and activated carbon exhibited high activity and was efficient in the removal of phenolic compounds in industrial effluents.

In recent years, carbon fibers have been widely exploited in electrochemical studies (Kubota et al., 2002). Biosensors can make ideal sensing systems to monitor the effects of environmental pollution due to their biological base, ability to operate in complex matrices, short response time and small size. In this study, laccase in crude extracts from *T. versicolor* immobilized on carbon-fiber electrode using a carbodiimide-glutaraldehyde system showed good response and retained its activity (Kubota et al., 2003).

MATERIALS AND METHODS

Laccase production

Laccase was obtained from *T. versicolor* (CCT 4521) cultivated for 20 days at 30°C and 240 rpm in a liquid medium containing (g L^{-1}) following Von Hunolstein et al. (1986). The laccase production was induced by 2,5-xylidine 1 mmol L^{-1} with 96 h of fungal culture.

Laccase semi-purification

The culture filtrate from the procedure described above in the presence of 0.1 mmol L^{-1} of copper sulfate (through 380 meshes) was frozen, unfrozen, filtrated through a Millipore 0.45 µm membrane and lyophilized. A solution containing 2.0 g of lyophilized crude extract, 30 mL citrate-phosphate and buffer 75 mmol L^{-1} (pH 5.0) was precipitated with ammonium sulfate at 90%. The precipitate was eluted in a Sephacryl S-200 (Sigma) column using the same buffer as mobile phase. The fractions containing laccase activity were collected. Another precipitation and elution was done and eluted in order to eliminate the dark pigments from the filtrate. The fractions with laccase activity were collected and lyophilized. The lyophilized was resuspended in citrate-phosphate buffer 10 mmol L^{-1} (pH 5.0) and applied to a column containing DEAE cellulose (Leonowicz and Bollag, 1988). The laccase was eluted with citrate-phosphate buffer 10 mmol L^{-1} and a NaCl gradient of zero to 1 mol L^{-1} was applied. The fractions obtained were lyophilized and stored in a freezer.

Enzyme activity assay

The reagent syringaldazine was used as substrate for spectrophotometric determination of laccase activity. One laccase unit was defined as the enzyme quantity needed to oxidize 1 µmol of substrate min^{-1} and per liter of broth culture (Szklarz et al., 1989).

Laccase immobilization

The semi-purified laccase immobilization was studied in three porous vitroceramic materials containing 0.5 g of support in 5 mL of enzymatic solution and in pyrolytic graphite containing 0.1 g of support per 100 µL of semi-purified laccase. Laccase solution containing around 1000.0 U L^{-1} was immobilized in the support previously treated with carbodiimide for 90 min and immobilized after 24 h (carbodiimide solution: 14 mg of carbodiimide in 1 mL of acetate buffer 0.1 mol L^{-1}, pH = 4.8), and glutaraldehyde (1 mL of glutaraldehyde 5% solution). The immobilization content was measured at 0, 90 and 180 min.

Carbon fiber biosensor immobilization

The carbon fibers microelectrodes were pretreated at a potential of +0.8 V for 180 s. The electrodes were allowed to react for 2 h with 7 mg of dicyclohexylcarbodiimide dissolved in 500 µL of 0.05 mol L^{-1} acetate buffer at pH 4.8 and then dipped into a glutaraldehyde aqueous solution (10% m/v) with laccase (40 U mL^{-1}) for 30 min.

Electrochemical measurement

To investigate the carbon fiber microelectrodes, a saturated calomel electrode was used as the reference, a platinum wire as the counter and about 10 - 20 carbon fibers (5 mm in length) as the working electrodes. A potentiostat model PGSTAT 10 from AUTOLAB (Netherland) connected to a PC was used for data acquisition and potential control.

Kraft E1 effluent treatment

The effluent treatment efficiency was evaluated through the phenol reduction (APHA, 1995). In the case of pyrolytic graphite, a mediator 1-hydroxybenzotriazole (HBT) (0.34 mol L^{-1}) was added in the effluent treatment.

Total phenol determination

A reaction mixture contained 1000 µL of sample, 250 µL sodium carbonate-tartarate and 25 µL of Folin reagent, after 30 min at

Table 1. Laccase activity of *T. versicolor* (U L^{-1}) in function of the production days and different copper sulfate concentration.

Copper sulfate (mmol L^{-1})	Days				
	4	8	12	16	20
0.000	10.8	15.6	39.8	50.3	64.0
0.005	72.7	4,617.0	7,447.0	10,373.0	10,091.0
0.020	69.0	2,860.0	7,654.0	7,849.0	10,580.0
0.040	75.3	3,917.0	34,769.0	13,449.0	15,233.0
0.070	73.5	2,626.0	39,550.0	10,534.0	11,073.0
0.100	38.8	3,963.0	40,774.0	11,230.4	12,915.0

Table 2. Extracellular laccase semi-purification from *T. versicolor*.

Purification (Steps)	Volume (mL)	Total activity (U L^{-1})	Total protein content (µg/mL)	Specific activity (U mg^{-1})	Purification (folds)
Crude extract	900.0	2,643.1	2,517.1	1.05	1.0
$(NH4)_2SO_4$ Precip.					
Sephacryl S-200 (1)	60.1	8,557.8	414.8	20.6	9.6
$(NH4)_2SO_4$ Precip.					
Sephacryl S-200 (2)	44.0	10,254.9	276.3	37.1	35.4
DEAE Cellulose					
(Fraction 1)	83.0	3,656.7	84.4	43.3	41.3
(Fraction 2)	97.0	49.7	11.4	4.3	4.1

20°C, the absorbance was measured at 700 nm (APHA, 1995).

RESULTS

Copper sulfate influence on fungal laccase production

Table 1 presents the laccase activity values in a production period (days 4, 8, 12, 16 and 20) for the fungus *T. versicolor* at different copper sulfate concentration (0, 0.005, 0.02, 0.04, 0.07 and 0.1 mmol L^{-1}) and 1 mmol L^{-1} of 2,5-xylidine 1.

Laccase semi-purification

A large part of the pigments were removed in the first two steps of purification by gel filtration (Sephacryl S-200). In the first gel filtration, the 18 - 43rd fractions (2.4 mL each) were collected. The second fraction contained the 29 - 62nd fractions. In these two steps, the purification factor (intercalated with ammonium sulfate precipitations) increased 35.4 folds. The laccase semi-purification results are exhibited in Table 3.

The enzyme activity obtained from the gel filtration was lyophilized and applied to DEAE cellulose column. In this type of column, it was observed that a small amount of the protein was bound to the column (fraction 1), when directly eluted without NaCl addition. The pigment was retained on the column with a purification factor around of 41.3 folds (Table 2). In order to be sure that all the laccase was eluted from the column, a NaCl solution 0.1 mol L^{-1} in the same buffer was added. Fractions with low laccase activities were found. Then, 1.0 mol L^{-1} of NaCl in the same buffer was applied and a peak of very low laccase activity was found.

Laccase immobilization

Table 3 shows the immobilization of laccase in vitroceramic 1 that immobilized over 37% laccase after carbodiimide treatment prior to immobilization. Table 4 shows the data for laccase immobilization in untreated vitroceramic 2. This material exhibited low porosity. After 90 min the immobilization yield was 9%. For vitroceramic 2 treated with carbodiimide, a yield of 11% was achieved after 180 min.

The immobilization was also attempted with a vitroceramic 3, which was prepared under strict conditions to generate a very low porosity material. In this experiment, no observed immobilization was found in the immobilized laccase in the untreated support and one treated with carbodiimide and glurataldehyde simultaneously.

Table 3. Immobilization of *T. versicolor* laccase in vitroceramic 1.

Time (min)	Control	Vitroceramic Immobilization	%
0	1091.9 $U.L^{-1}$ (± 3)	1091.9 $U.L^{-1}$ (± 3)	0%
90	1091.9 $U.L^{-1}$ (± 3)	984.2 $U.L^{-1}$ (± 9)	37.0%
180	1115.2 $U.L^{-1}$ (± 2)	1025.5 $U.L^{-1}$ (± 4)	28.5%

Table 4. Immobilization of *T. versicolor* laccase in vitroceramic 2.

Time (min)	Control	Vitroceramic 2 with carbodiimide (%)	Vitroceramic 2 (%)
0	1,060.8 $U.L^{-1}$	1,060.8 $U.L^{-1}$	1,060.8 $U.L^{-1}$
90	1,035.3 $U.L^{-1}$ (± 38)	984.0 $U.L^{-1}$ (± 10) / (5 %)	939.7 $U.L^{-1}$ (± 9) / (9 %)
180	1,094.0 $U.L^{-1}$ (± 3)	970.3 $U.L^{-1}$ (± 4) / (11 %)	1,013.6 $U.L^{-1}$ (± 16) / (7 %)

Laccase immobilization using pyrolytic graphite treated with carbodiimide and glutaraldehyde gave 93.5% yield in 180 min. Using only carbodiimide or glutaraldehyde treatment a 90.3 and 94% yields were found, respectively.

Kraft E1 effluent treatment

In the kraft E1 effluent treatment with laccase immobilized on vitroceramic 1 treated with carbodiimide for 90 min and immobilized after 24 h, a 10% phenol reduction was found after 180 min of treatment. In the case of laccase immobilized on untreated vitroceramic 2 and laccase immobilized in vitroceramic 2 with carbodiimide treatment (after 90 min of carbodiimide treatment the laccase was immediately immobilized), the first exhibited better results than the latter material (90 min of treatment a 14% phenol reduction was observed). In the case of the carbodiimide treatment, only 7% after 180 min of treatment was obtained. Pyrolytic graphite treated with glutaraldehyde in the presence of HBT as mediator showed to be the most efficient. A value of 19% phenol reduction was found compared with 16% for the laccase in its free form.

Biosensor application

The biosensor prepared in this study exhibited a sensitivity value of 16.0 ± 0.3 nA μmol L^{-1} (r^2 of 0.999 and coefficient of variation of less than 2%) at an applied potential of -100 mV with standard solutions of catechol (100 μmol L^{-1}, pH = 5.0). The calibration curve obtained for catechol, using the developed biosensor at an applied potential of -100 mV vs SCE (Saturated Calomelane Electrode) in 0.1 mol L^{-1} phosphate buffer (pH 5.0), showed a good linear response range between 1 - 80 μmol L^{-1} of catechol and was adjusted to the equation Δi (nA) = 0.296 (± 0.223) + 16.10 (± 0.005) (correlation coefficient of 0.9993 for n = 9).

DISCUSSION

Laccase activities were negligible in the absence of copper sulfate in culture with *T. versicolor*. The highest laccase activity was between 0.070 and 0.100 mmol L^{-1} of copper sulfate concentration and the value was 40,774.0 U L^{-1} at the 12th day.

The laccase semi-purification show that most of the pigments present in the crude extract was removed by the Sephacryl S-200 step. Using the DEAE cellulose, around 41.3 folds purification was obtained and a laccase isoenzyme was detected. The different fractions obtained with laccase activities could be due to the presence of isoenzymes.

One possible explanation for the immobilization efficiency results is the different vitroceramic porosity. The vitroceramic was characterized by X-rays diffratometry and Raman spectroscopy (Gimenez et al., 2001) and shows different porosities. Vitroceramic 1 (lixiviation for long period), exhibited no β-$Ca_3(PO_4)_2$ residues. This indicates a material with large porosity and better chances to bind the enzyme. This characteristic was diminished in vitroceramic 2, where β-$Ca_3(PO_4)_2$ residues were found causing a porous reduction available for the enzyme immobilization. The vitroceramic 3 has very low porosity since no lixiviation was carried out to eliminate β-$Ca_3(PO_4)_2$ residues and presents large quantities of TiO_2 and $LiTi_2(PO_4)_3$ residues, resulting in a bad support. In summary, the vitroceramic 1, which has a larger porosity than vitroceramic 2 and 3, was one of the most effective materials for the laccase immobilization, resulting in 37% efficiency. The pyrolytic graphite (94%efficiency) immobilized laccase efficiently with glutaraldehyde treatment alone instead of carbodiimide presence.

Total phenol removal in Kraft E1 effluent was 19 and 14% by using laccase immobilized in pyrolytic graphite in the presence of mediator and vitroceramic 2 without mediator. The most efficiency phenol removal using laccase immobilization was obtain with pyrolytic graphite treated with glutaraldehyde in the presence of HBT presenting 19% of phenol reduction compared to 16% with the laccase in its free form. The mediator presence increases the effluent treatment efficiency. The carbon fiber biosensor exhibited a good operational range and sensitivity, and an excellent recovery values for catechol determinations for 4 replicates, indicating that the biosensor can be applied in the analysis of environmental monitoring with no significant influence from the matrix.

ACKNOWLEDGMENTS

Financial support from FAPESP and CNPq is acknowledged.

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