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Laccase production by *Galerina* sp. and its application in dye decolorization

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The laccase producing fungus HC1 was isolated from a sample collected in the Bolivian Amazon region. Analysis of 5.8S and 28S rDNA, and internal transcribed spacer 1 and 2 sequences revealed that isolate HC1 belongs to genus *Galerina*. High production of laccase was achieved in basal salt medium supplemented with 30 g L^{-1} glucose, 10 g L^{-1} yeast extract and 0.01 g L^{-1} copper sulphate. The enzyme production was further improved by addition of 2,5-xylidine, α-benzoin oxime and *p*-coumaric acid, but strongly repressed by sinapyl alcohol, 3-(dimethylamino)benzoic acid, hydroquinone, 1-naphthaleneacetic acid, 2,6-dichlorophenol and 3-methyl-2-benzothiazolinone hydrazone. Cultivation under optimum conditions in the presence of 2 mmol L^{-1} 2,5-xylidine and 0.01 g L^{-1} copper sulphate, resulted in the enzyme yield of over 26 000 U L^{-1}. *Galerina* sp. HC1 could also produce laccase in media composed of orange peels both in submerged- and solid-state fermentations. The laccase (0.5 U ml^{-1}) from *Galerina* sp. was able to decolorize over 60% of 35 µmol L^{-1} Congo red in 2 h in the presence of 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt as mediator, and 65% of 30 µmol L^{-1} Coomassie Brilliant Blue G-250 in 5 min using syringaldehyde as mediator. The laccase was immobilized on cross-linked chitosan and used efficiently to decolorize the dyes.

Key words: Oxidase, inducer, immobilized enzyme, enzyme production, solid-state cultivation.

INTRODUCTION

Laccases (EC 1.10.3.2) are multicopper oxidases that are among the most abundant members of the oxidoreductase family, which catalyze oxidation of various substrates using molecular oxygen as the electron acceptor. These enzymes are attractive catalysts for oxidation under mild conditions and moreover do not require any expensive cofactor unlike several other oxidoreductases. Laccases display remarkably low substrate specificity catalyzing the oxidation of a fairly broad range of substrates such as phenolic compounds, diamines and aromatic amines with concomitant reduction of oxygen to water. The substrate range can be

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further extended in the presence of mediators-compounds that act as electron shuttles. So far, laccases have been used in delignification of pulp, detoxification of industrial effluents, bioremediation of soils contaminated by herbicides, pesticides and certain explosives, synthesis of drugs and cosmetic ingredients, medical diagnostics, etc. (Baldrian, 2006; Riva, 2006). They have even attracted attention for catalyzing decolorization of dyes that are used in large amounts (10^9 kg annually) in leather, textile and printing industries and have caused considerable environmental concern (Defrawy, 2002). Most of these dyes or their derivatives are mutagenic and carcinogenic (Weisburger, 2002), and about 10% of the total amount used in the industries is estimated to end up in effluents (Gopinath et al., 2009).

Laccases are widely distributed among plants, fungi, bacteria and insects. In plants, laccases are known to be involved in the synthesis of lignin by catalyzing the free radical polymerization of *p*-coumaryl-, coniferyl- and sinapyl alcohols (Morozova et al., 2007), while fungal laccases are involved in lignin degradation and pathogenesis (Baldrian, 2006), and are also considered to be necessary for fungal development and morphogenesis (Zhao and Kwan, 1999). In bacteria and fungi, laccases are useful in synthesis of pigments such as melanin (Henson et al. 1999) and detoxification of toxic compounds (Wu et al., 2008). In insects, the primary role of laccases is in cuticle sclerotization (Riva, 2006), a process where the cuticle stabilizes by cross-linking of reactive quinones. The widespread interest in laccases has led to efforts in search for organisms producing these enzymes, the great majority of which are fungi. Among the well-known laccase producers, *Trametes versicolor*, *Agaricus bisporus*, *Coriolus* spp., *Pleurotus ostreatus*, *Phlebia radiata*, *Pycnoporus cinnabarinus* and *Coprinus cinereus* belong to basidiomycetes.

Recently, we have purified and characterized a laccase from a *Galerina* species (Ibrahim et al., 2011), belonging to the group of litter degrading basidiomycetes growing invariably on dead bryophytes, on woody materials or on other plant debris. This paper reports studies on the production of laccase by this organism and its ability to decolorize a toxic azo dye and a triphenylmethane dye.

MATERIALS AND METHODS

Fungi, culture conditions and screening of laccase producers

Isolate HC1 was selected in a screening procedure involving 41 fungi collected in the upper Amazon River basin in Bolivia and has been deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen culture collection with an accession number of DSMZ 22662. The fungus was grown on modified malt extract agar containing: 45 g L^{-1} malt extract agar (HiMedia Laboratories, India) and 3 g L^{-1} peptone (Merck). The cultures were purified by repeated transfer to fresh plates, and grown at room temperature (around 22°C) for 12 days and then stored at 4°C, and sub-cultured on fresh agar plates every month. Screening for the presence of laccase activity was done by formation of green halos on solid ABTS medium containing: 1 g L^{-1} glucose, 15 g L^{-1} agar-agar (Riedel-de-Haën, Germany), 0.5 g L^{-1} yeast extract (Sigma-Aldrich), and 2.5 g L^{-1} ABTS (2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Sigma-Aldrich, Germany).

Identification of isolate HC1

Isolate HC1 was grown in Sabouraud broth (Sigma-Aldrich). After incubation for 7 days at 25°C, 1.5 ml of the culture was withdrawn, washed twice with sterile ultra-pure water before transferring the fungal mycelia to sterile Eppendorf tubes and vortexing with 0.3 g glass beads (425-600 µm; Sigma) to break the cells. Chromosomal DNA was purified from the lysate according to Sambrook et al. (1989) and used as template for the PCR reaction. Primers ITS1-F (5´-CTT GGT CAT TTA GAG GAA GTA A-3´) and ITS4-B (5´-CAG GAG ACT TGT ACA CGG TCC AG-3´) were used to amplify internal transcribed spacer regions 1 (ITS1) and 2 (ITS2), and the rDNA 5.8S and 28S as described by Gardes and Bruns (1993). The PCR reactions were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems) and products were purified after electrophoretic separation using a gel extraction kit (Qiagen). The purified product was sequenced in both directions with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) following the manufacturer's protocol. Sequence reactions were electrophoresed using ABI 3100 DNA sequencer.

Laccase production by isolate HC1

Thirty millilitres of basal medium (pH 6) composed of (g L^{-1}): NH_4NO_3, 0.04; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 0.1; KH_2PO_4, 0.2 and $CaCl_2 \cdot 2H_2O$, 0.01 were supplemented with varying concentrations of glucose, yeast extract and copper sulphate and autoclaved. After cooling, each 250 ml Erlenmeyer flaska containing the medium was inoculated with six agar discs (0.5 cm diameter) taken from 12 days old culture. The flasks were incubated at room temperature (22°C) with continuous agitation at 100 rpm. Samples were taken after 10, 15 and 20 days of cultivation for determination of laccase activity.

To 30 ml basal medium supplemented with 4 g L^{-1} glucose, 4 g L^{-1} yeast extract and 0.05 g L^{-1} $CuSO_4 \cdot 5H_2O$ were added different chemical additives (1 mol l^{-1}) or 1 g L^{-1} lignin (Eucalyptus Kraft lignin kindly provided by Innventia AB, Stockholm) and laccase production was followed over a period of 20 days. The additives used were 4-aminobenzoic acid, benzidine, benzoic acid, α-benzoin oxime, caffeic acid, coniferyl alcohol, *p*-coumaric acid, 2,6-dichlorophenol, 3-dimethylaminobenzoic acid (DMBA), 3,5-dinitrosalicylic acid (DNS), ferulic acid, hydroquinone, 3-methyl-2-benzothiazolinone hydrazine (MBTH), naphthalene, 1-napthol, 1-naphthaleneacetic acid, nicotinic acid, pyridoxine, pyrogallic acid, sinapyl alcohol, sinapic acid, 2,5-xylidine, veratryl alcohol (all from Sigma-Aldrich), methanol and ethanol, respectively. In another experiment, dried orange peels and bagasse fibers were used as inexpensive substrates for the production of laccase by isolate HC1. Thirty millilitres of the basal medium containing 0.05 g L^{-1} of $CuSO_4 \cdot 5H_2O$ and 20 g L^{-1} of orange peels or bagasse fibers cut up into small pieces were inoculated with the fungus and incubated as described above. Samples were withdrawn at different time intervals to determine the laccase activity.

Besides submerged fermentation, the enzyme production using orange peels was also studied by solid-state fermentation. The orange peels were soaked overnight in 0.2 mol l^{-1} KOH, washed extensively until neutralization and lyophilized. About 3 g of lyophilized peels were added to 100 ml flasks containing 25 ml of the basal medium and autoclaved. Some flasks were supplemented with copper sulphate or 2,5-xylidine or both.

Immobilization of the *Galerina* sp. laccase on cross-linked chitosan

The fungal culture was filtered using filter paper and the enzyme in the filtrate was partially purified using a combination of anion exchange and hydrophobic interaction chromatographies as described earlier (Ibrahim et al., 2011). A medium molecular mass chitosan (750 kDa) (Fluka) was cross-linked with glutaraldehyde (Sigma) according to Zhang et al. (2009) and extensively washed with water to remove the excess glutaraldehyde. Then, 20 ml of the partially purified laccase (6 U ml^{-l}, 75 U mg^{-l}) was added to 4 g of the cross-linked matrix and mixed overnight on a rocking table at 4°C. Subsequently, the suspension was centrifuged and thoroughly washed to remove the non-immobilized enzyme. The immobilized laccase preparation was stored at 4°C.

Dye decolorization using free or immobilized laccase

The efficiency of isolate HC1 laccase in degrading Congo red (azo dye, λ_{max} 500 nm) and Coomassie Brilliant Blue G-250 (triphenylmethane dye, λ_{max} 595 nm) was assessed using ABTS and syringaldehyde (Sigma-Aldrich), respectively, as mediators. The reaction mixture was composed of 25 mM sodium acetate buffer pH 5, 0.5 U ml^{-l} laccase, 35 µmol L^{-l} Congo red or 30 µmol L^{-l} Coomassie Brilliant Blue G-250 and 50 µmol l^{-l} mediator in a total volume of 800 µl. The change in the absorbance of the reaction mixture was followed for 3 h at the respective wavelengths. For comparison, the dyes were also treated with commercially available *Trametes versicolor* laccase under similar conditions.

Dye decolorization by the immobilized laccase was studied by circulating 12 ml of the dye solutions in a column (5 x 1 cm internal diameter), packed with 500 mg of the preparation, at a flow rate of 1.6 ml min^{-l}. As a fraction of the dyes were adsorbed to the cross-linked chitosan (0.36 mg dye per g chitosan), the immobilized enzyme preparation was saturated with the respective dyes prior to studying the decolorization (the adsorbed dyes were not decolorized by laccase mediator system). When no further decolorization was achieved, a new dye solution (12 ml) containing the respective mediator (50 µmol L^{-l}) was pumped through the column.

One millilitre of the total dye solution was withdrawn at different time intervals to measure the residual absorbance at the specific wavelength, and returning the sample to the liquid stream. The decolorization yield was calculated as follows:

$$\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where, A_0 is the absorbance of the untreated dye and A_t is the absorbance of the treated dye after time t.

UV-visible scans (200 to 800 nm) were made for both the laccase treated and untreated dye solutions using PerkinElmer (Waltham, USA) spectrophotometer.

Assays for ligninases

The laccase activity was assayed following the method of Dong et al. (2005) using 1 mmol L^{-l} ABTS (ε_{420nm} = 36000 mol $l^{-l}.cm^{-l}$) as a substrate. Activity of the immobilized laccase was assayed in 20 ml reaction mixture with 5 mg immobilized enzyme particles. One unit of laccase activity was defined as the amount of enzyme that catalysed the oxidation of 1 µmol L^{-1} ABTS per min. The values reported are averages of at least two measurements.

The culture filtrate of isolate HC1 was also tested for other ligninolytic activities besides laccase. The activity of manganese peroxidase was determined according to the method of Castillo et al. (1994). One unit of manganese peroxidase activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol of azo dye resulting from the coupling of oxidized MBTH to DMAB per min (ε_{590nm} = 53000 mol $l^{-l}.cm^{-l}$). Lignin peroxidase (LiP) was assayed as described by Tien and Kirk (1988). One unit of LiP activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol veratryl alcohol per min (ε_{420nm} = 9300 mol $l^{-l}.cm^{-l}$). The versatile peroxidase activity was checked following the method described by Mohorcic et al. (2009) using 1-napthol as substrate (ε_{255nm} = 12800 mol $l^{-l}.cm^{-l}$).

RESULTS AND DISCUSSION

Isolate HC1 identification and its ligninase activities

Analysis of 5.8S and 28S rDNA, and the internal transcribed spacer 1 and 2 DNA sequences revealed that isolate HC1 belongs to genus *Galerina*. This fungus is considered as a polyphyletic organism, and more than 300 species have originated from the Northern Hemisphere (Gulden et al., 2005). The sequence of isolate HC1 showed 99% similarity to the corresponding sequence of *Galerina clavata* (sequence ID AY281021). However, as species delimitation in genus *Galerina* is mostly based on morphological and/or ecological characters, it needs further study to decide the taxonomic position of isolate HC1. Although, involvement of *Galerina* spp. in lignin decomposition has been described (Osono, 2007) and the presence of laccase activity in the culture of *Galerina patagonica* is reported (Tortella et al., 2008), there is no available report on production of laccase by any fungus that belongs to *Galerina*.

Galerina sp. HC1 was grown in liquid basal medium containing (g L^{-l}): glucose, 4; yeast extract, 4 and $CuSO_4 \cdot 5H_2O$, 0.05; and ligninase activities were followed for 20 days (Figure 1). Unlike most fungal cultures, which need more than five days of cultivation to produce detectable amount of laccase (Arora and Gill, 2000; Galhaup et al., 2002), laccase activity appeared on the 2^{nd} day and peaked to about 265 U L^{-l} on the 8^{th} day of cultivation. The fungus was able to produce at least four laccase isoforms and the major laccase was purified to homogeneity, characterized and used to modify lignin (Ibrahim et al., 2011). As shown in Figure 1, lower amounts of lignin peroxidase and manganese peroxidase were produced; however no versatile peroxidase activity was detected. The lignin peroxidase activity decreased drastically to a very low level after 16 days while Mn peroxidase activity remained stable with time. The presence of all the major lignin degrading enzymes, especially the lignin peroxidase that acts even on non-phenolic components of lignin, suggests that *Galerina* sp. HC1 could be an efficient lignin degrader (Cohen et al., 2001; Gold and Alic, 1993).

Laccase production by submerged cultivation

Laccase production by different organisms is influenced

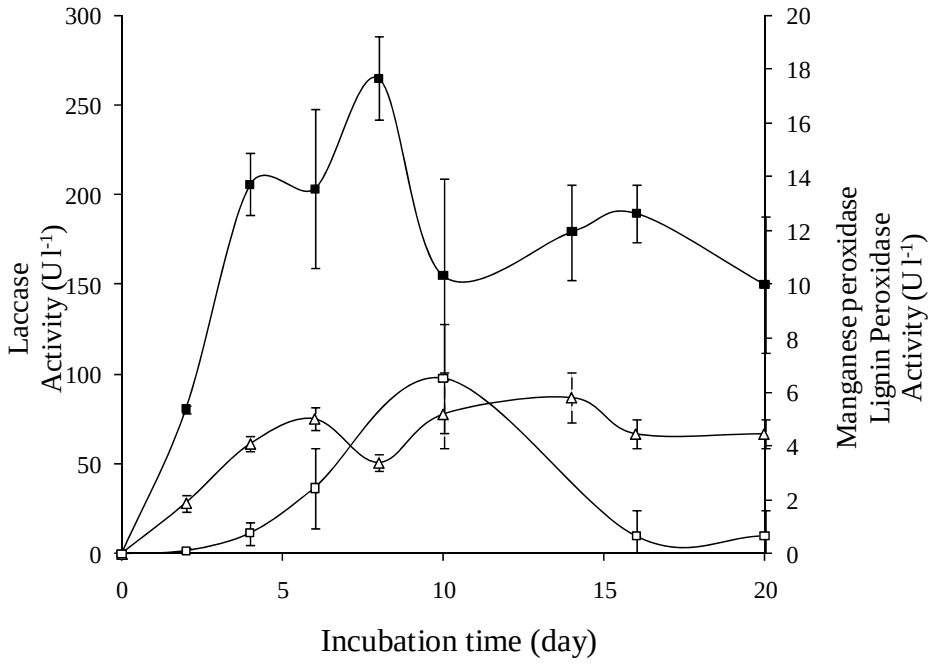


Figure 1. Production of laccase (■), manganese peroxidase (Δ) and lignin peroxidase (□) by *Galerina* sp. HC1 in basal medium supplemented with with 4 g L^{-1} glucose, 4 g L^{-1} yeast extract and 0.05 g L^{-1} $CuSO_4 \cdot 5H_2O$. Cultivation was done at room temperature with continuous agitation at 100 rpm.

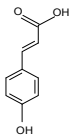
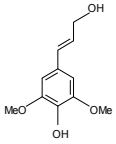
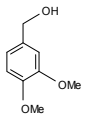
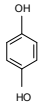
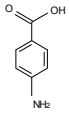
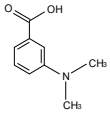
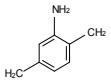
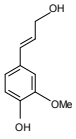
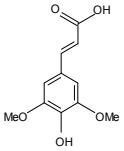
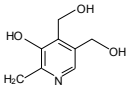
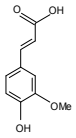
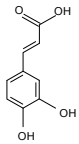
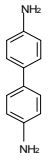
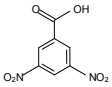
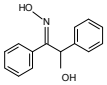
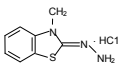
by glucose, yeast extract and copper concentrations in the culture medium, the effect of each nutrient being dependent on the concentrations of the others (Galhaup and Haltrich, 2001). Based on a preliminary study, three different concentrations of glucose, yeast extract and copper sulphate were chosen to study the effect on laccase production by *Galerina* sp. HC1 using a simple factorial design (data not shown). The highest laccase production was achieved at glucose-, yeast extract- and copper concentrations of 30, 10 and 0.01 g L^{-1}, respectively (data not shown). Increasing the glucose concentration to 60 g L^{-1} led to a decrease in the laccase activity in the medium. While supporting growth, glucose is known to repress expression of several genes encoding extracellular enzymes in fungi and yeast. However, the effect of glucose on laccase production varies for different organisms; reduced production with increasing glucose concentration has been reported for *Trametes versicolor* (Tavares et al., 2005) and *Phlebia* sp. (Arora and Rampal, 2002), whereas improved yields have been seen in *Trametes pubescens* (Galhaup et al., 2002).

Production of laccase by *Galerina* sp. HC1 decreased with increase in the concentration of yeast extract (data not shown), as reported earlier also for *Pycnoporus sanguineus* (Pandey et al., 2000), *Pleurotus ostreatus* (Prasad et al., 2005) and *Coriolus versicolor* (Revankar and Lel, 2006). In contrast, the production of laccase by *Trametes gallica* has been stimulated by organic nitrogen source (Dong et al., 2005).

Microbial requirements for copper are usually satisfied by very low concentrations of the metal (1-10 µmol L^{-1}), while high metal concentration exerts a toxic effect. Interestingly, *Galerina* sp. HC1 can grow at copper concentration as high as 1.2 g L^{-1}. Laccases are copper containing enzymes and often their production is stimulated by addition of copper salt to the culture medium (Soden and Dobson, 2001). The effect of copper on the production of laccase by *Galerina* sp. HC1 had a positive correlation with the concentration of yeast extract in the medium, implying that the laccase production was favoured with either increase or decrease in concentrations of both yeast extract and copper. Changes in their concentrations relatively inverse to each other led to reduction in the enzyme production. Yeasts are known to produce cysteine rich proteins-metallothioneins (MT) or other low molecular weight proteins that are capable of chelating relatively large quantities of copper ions (Brady et al., 1994). Hence, the observed effect may be due to the binding of copper to the yeast extract components that in turn may reduce the availability of copper for the fungus. On the other hand, at low yeast extract concentration, the copper included in the medium would satisfy the need for growth and laccase production.

Laccase production by *Galerina* sp. was further investigated in the presence of a number of additives that were selected either due to their existence in wood or

Table 1. Effect of additives on the production of laccase by *Galerina* sp. HC1. The 100% laccase activity corresponds to 508 U L^{-1}. The laccase production was followed for 20 days and the activity stated is the maximum production achieved during the cultivation period.

Additive	Structure	Activity (%)	Additive	Structure	Activity (%)	Additive	Structure	Activity (%)
None	---	100	*p*C		200	S Al		5
Lignin	Complex	194	VA		14	HQ		0
4AB		14	DMBA		0	Phenol		117
N Ac		109	B Ac		122	X		374
C Al		110	S Ac		49	P		152
F Ac		36	C Ac		70	BZD		72
P Ac		91	DNS		108	DP		0
N		108	B		233	EtOH		91
MBTH		1	1-Nac		<1	MeOH		135

4AB: 4 Aminobenzoic acid; N Ac: nicotinic acid; C Al: coniferyl alcohol; F Ac: ferulic acid; P Ac: pyrogallic acid; N: naphthalene; MBTH: 3-methyl-2-benzothiazolinone hydrazone; *p*C: p-coumaric acid; VA: veratryl alcohol; DMBA: 3-dimethylaminobenzoic acid; B Ac: benzoic acid; S Ac: sinapic acid; C Ac: caffeic acid; DNS: 3,5-dinitrosalisylic acid; B: α-benzoin oxime; 1-Nac: 1-naphthaleneacetic acid; S Al: sinapyl alcohol; HQ: hydroquinone; X: 2,5-xylidine; P: pyridoxine; BZD: benzidine; DP: 2,6-dichlorophenol; EtOH: ethanol; MeOH: methanol.

structural similarity to the natural substrate of laccase-lignin or its precursors. The effect of ethanol and methanol was also tested because their addition has resulted in increased laccase production by other fungal strains (Meza et al., 2005). As summarized in Table 1, the best inducer was 2,5-xylidine followed by α-benzoin oxime, *p*-coumaric acid, lignin, pyridoxine and methanol. However, the toxic nature of most aromatics restricts their use in industrial applications. The induction achieved by lignin is economically attractive because of its availability in large amounts as a by-product of paper pulp. Additives like 3-(dimethylamino) benzoic acid, sinapyl alcohol, 1-naphthaleneacetic acid, 2,6-dichlorophenol and 3-methyl-2-benzothiazolinone hydrazone hydrochloride strongly repressed laccase production by *Galerina* sp. HC1. The fungus did not grow in the presence of hydroquinone, which is a known antioxidant that efficiently inhibits the growth of several fungi (Elwakil and El-Metwally, 2000).

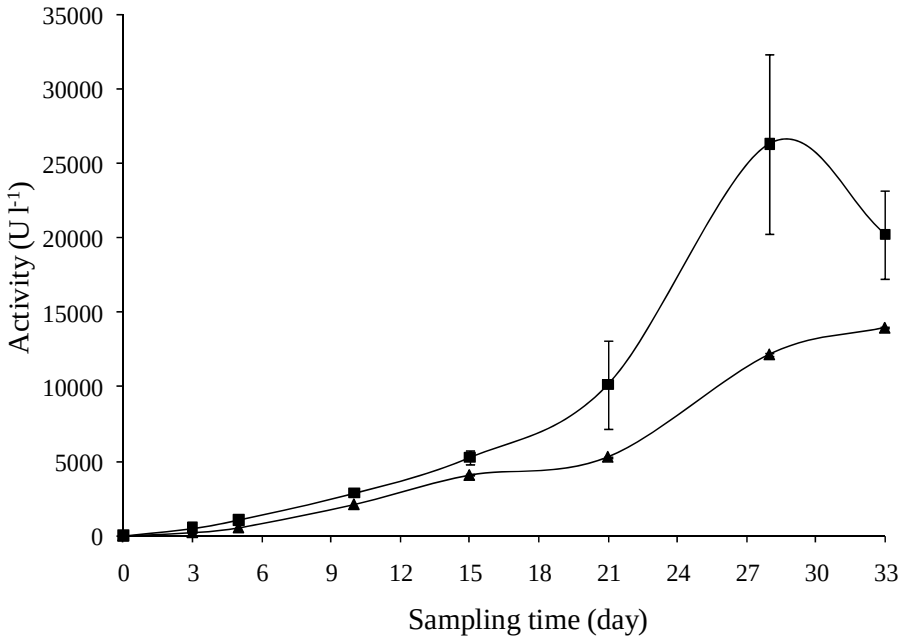


Figure 2. Time course of laccase production by *Galerina* sp. HC1 during cultivation under optimized conditions at 22°C. The medium contained 30 g L^{-1} glucose, 10 g L^{-1} yeast extract, 0.01 g L^{-1} copper sulphate, and 2 mmol L^{-1} 2,5-xylidine (■) or 2 mmol L^{-1} α-benzoin oxime (▲).

The effect of different concentrations of the three best inducers, 2,5-xylidine, α-benzoin oxime and *p*-coumaric acid, respectively, on laccase production was further tested (data not shown). Laccase production was highest at the inducer concentration of 2 mmol L^{-1} after 15 days of cultivation, amounting to over 5800 U L^{-1} in the presence of xylidine, 3800 U L^{-1} with α-benzoin oxime, and 3400 U L^{-1} with *p*-coumaric acid. Higher concentrations of xylidine (>2 mmol L^{-1}) resulted in lower growth and enzyme production.

Laccase production was then studied in the medium under optimal nutrient and inducer conditions determined above, that is, 30 g L^{-1} glucose, 10 g L^{-1} yeast extract and 0.01 g L^{-1} copper sulphate and 2 mmol L^{-1} xylidine or α-benzoin oxime. As high as 26 000 U L^{-1} laccase activity was produced in the presence of 2 mmol L^{-1} xylidine after 28 days of cultivation (Figure 2), which exceeded that reported for other fungi including several strains of *Trametes* (Font et al., 2003; Rodríguez et al., 2004). The exception is *Trametes pubescens* that produced 740 000 U L^{-1} in a fed-batch cultivation at low level of glucose which avoids repression of the enzyme synthesis (Galhaup and Haltrich, 2001; Galhaup et al., 2002).

Agricultural residues as substrates for laccase production

Orange peels and sugarcane bagasse, the two agricultural residues available in abundance and also rich in sugars, vitamins and minerals (Pandey et al., 2000; Mamma et al., 2008), were tested as substrates for cultivation of *Galerina* sp. HC1 and production of laccase. Use of such renewable materials that may otherwise have nil value would potentially reduce the enzyme production costs. At the residues concentration of 20 g L^{-1} that is determined to be equal to the chemical oxygen demand of 4 g L^{-1} glucose, the laccase production was 4 times higher than the maximum production achieved using glucose as carbon source (Figure 3). Orange peel contains many aromatics especially *p*-coumaric acid, and soluble and insoluble carbohydrate compounds (Ersus and Cam, 2007) that may stimulate laccase production. Bagasse, on the other hand, contains high amount of lignin (25%) (Prasad, 2005) which makes accessibility of polysaccharides difficult without pretreatment, and provided only slightly higher laccase activity than glucose.

The orange peels were further considered as substrate for laccase production using solid-state fermentation that has many advantages over submerged fermentation in terms of low liquid volumes and related processing costs (Lonsane and Ramesh, 1990). The peels were soaked in the basal medium prior to inoculation with the fungus, and absorbed most of the liquid when suspended at a concentration of about 12% (w/v), resulting in a wet solid mass. Laccase production was found to be about 0.5 U g^{-1} of the dried orange peels, and supplementation of the peels with copper, 2,5-xylidine, and combination of the two, increased the activity 5, 6 and 10 fold, respectively

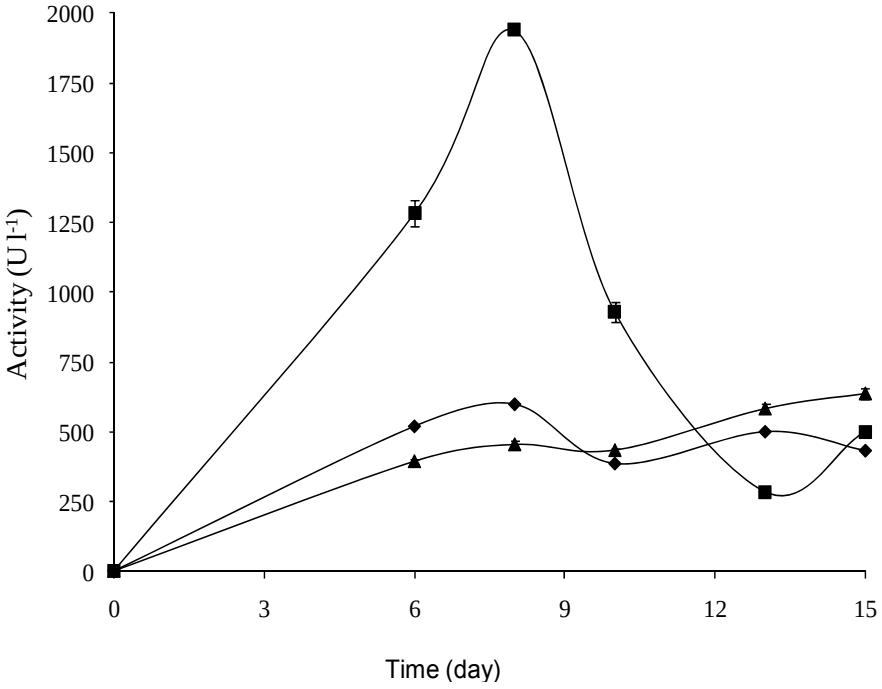


Figure 3. Production of laccase by *Galerina* sp. HC1 using 20 g L^{-1} sugarcane bagasse (♦), 20 g L^{-1} orange peels (■) or 4 g L^{-1} glucose (▲), respectively as substrates during submerged cultivation at 22°C. The experimental details are given in the text.

(data not shown). When compared with the production level achieved in submerged fermentation using orange peels, the laccase production using orange peels in solid state fermentation resulted in very low enzyme activity.

Decolorization of dyes using *Galerina* sp. HC1 laccase

The dye decolorization activity of the partially purified laccase from submerged cultivation of *Galerina* sp. HC1 was evaluated using Congo red and Coomassie Brilliant Blue G-250 as model dyes. Decolorization of both dyes by the laccase in the presence of mediators was comparable to that achieved with *T. versicolor* laccase which has been reported to be the most efficient enzyme in decolorization of dyes (Figure 4) (Liu et al., 2004). The initial decolorization rate for Coomassie Brilliant Blue was faster, reaching more than 50% decrease in absorbance in less than 5 min, while similar degree of decolorization of Congo red took up to 30 min (Figure 4). This could be due to the presence of electron donating methyl and methoxy groups on the triphenylmethane dye that have earlier been shown to enhance laccase activity (Abadulla et al., 2000). Furthermore, decolorization was relatively faster with syringaldehyde as the mediator (Figure 4), which is attractive due to its availability from lignin that is among the primary renewable resources (Villar et al., 2001). In this study, maximum decolorization of Coomassie blue was obtained in the presence of syringaldehyde (Figure 4b). On the other hand, the highest decolorization of Congo red was achieved using ABTS but not syringaldehyde. Moreover, it has been observed that Congo red decolorizes (Figure 4a) at a slower rate than that of Coomassie blue (Figure 4b). Resistance of Congo red to decolorization by laccase from *Trametes* sp. SQ01, an enzyme that efficiently decolorizes various azo-, triphenylmethane-, and anthraquinone dyes has been known (Yang et al., 2009). After 24 h treatment, only 47% of the Congo red was decolorized by the pure laccase of *Trametes* sp. SQ01. Thus, the decolorization achieved by the *Galerina* sp. HC1 laccase relatively in short time is encouraging. This shows that screening of different organisms and their enzymes for dye decolorization is beneficial, as it may result in more efficient dye decolorizers than the commonly studied organisms (Chander et al., 2004).

Decolorization of the dyes was further investigated using *Galerina* sp. laccase immobilized on cross-linked chitosan. Chitosan is a deacetylated chitin, and is cheap and abundant, having use in various applications. About 12 U of the *Galerina* sp. HC1 laccase was immobilized per gram of cross-linked chitosan, which was equivalent to 76% of the initial activity used in the immobilization process. Since chitosan has high dye binding capacity (Chiou and Li, 2003; Wang and Hu, 2007), the cross-linked chitosan-enzyme preparation was first saturated with respective dyes without adding any mediator in the

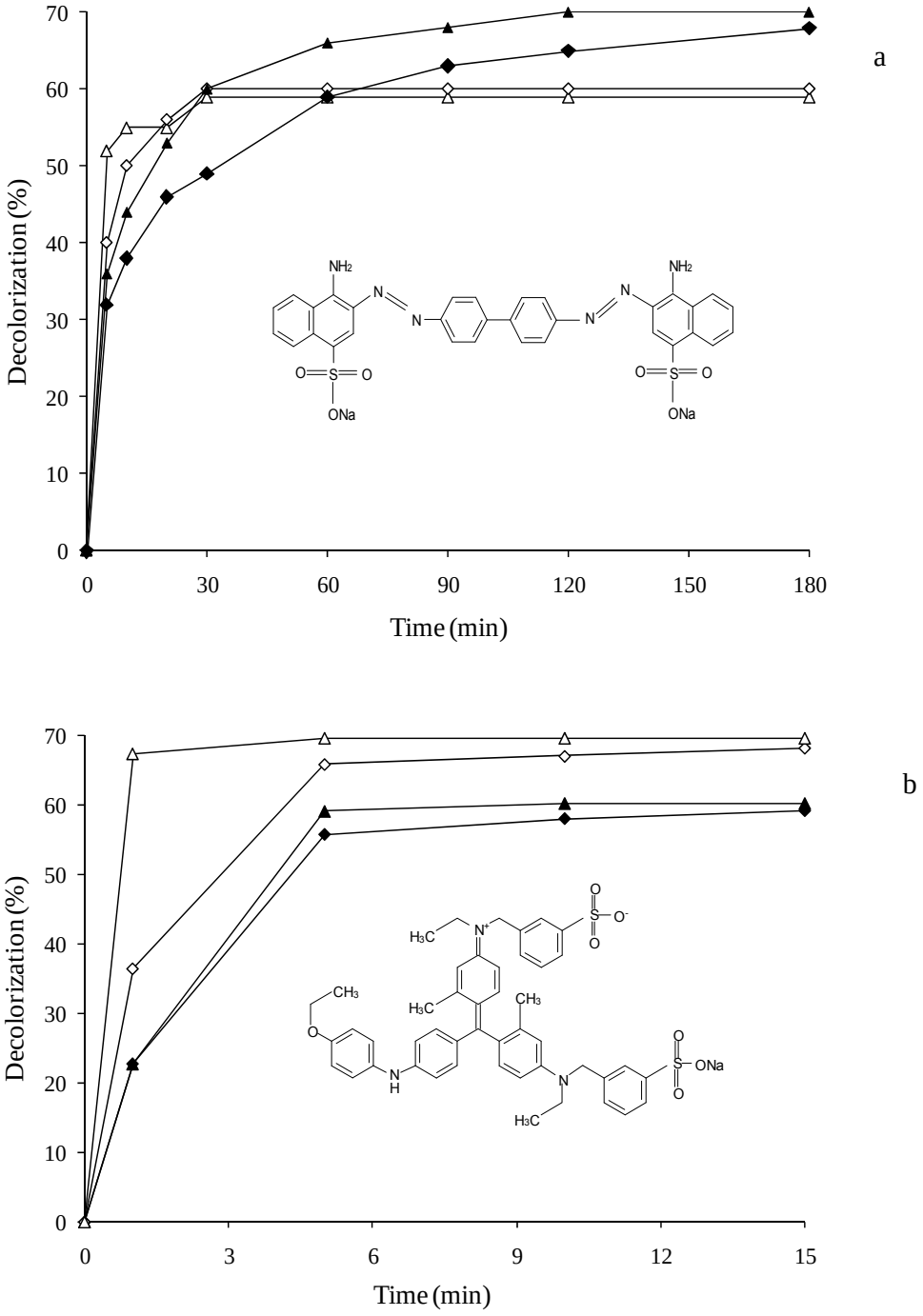


Figure 4. Decolorization of (a) Congo red and (b) Coomassie Brilliant Blue G-250 using *Galerina* sp. HCl (diamonds, ◊ & ♦) and *T. versicolor* (triangles, Δ & ▲) laccases in the presence of ABTS (filled symbols) and syringaldehyde (open symbols) as mediators.

solution. The dye solution was re-circulated over a bed of 500 mg immobilized enzyme preparation until no more dye was observed to bind to the matrix. Then fresh dye solutions containing mediators were used for laccase mediated decolorization in consecutive batches. It was possible to decolorize more than 97% of the 35 µmol l^{-1} Congo red solution (7 batches of 12 ml each), however less volume (3 batches each with 12 ml) of 30 µmol l^{-1} Coomassie Brillant Blue G-250 solution was decolorized and thereafter the decolorization efficiency of the immobilized enzyme declined (Figure 5). This is in agreement with the earlier report of Katuri et al. (2009) on

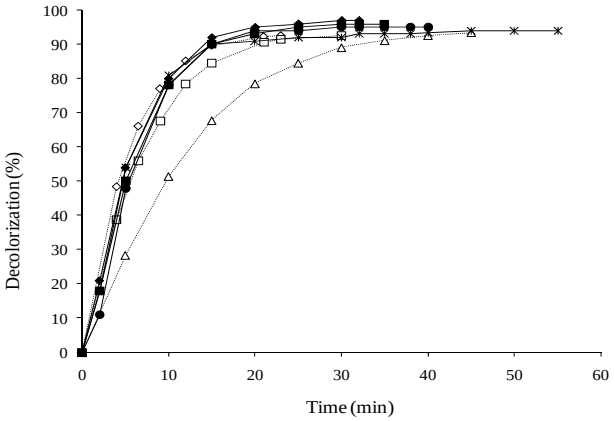


Figure 5. Re-use of immobilized laccase for decolorization of Congo Red (filled symbols and continuous line) and Coomassie Brillant Blue G-250 (open symbols and dashed lines). The used mediators are ABTS and syringaldehyde, respectively. Symbols represent: (diamonds) first batch, (square) second batch, (triangle) third batch, (circle) fourth to sixth batch, and (cross) seventh batch.

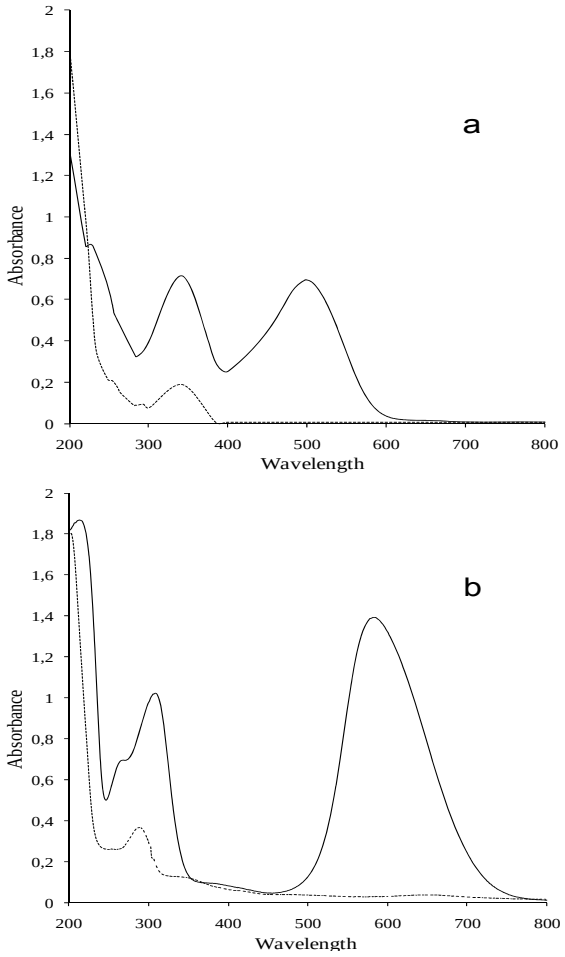


Figure 6. UV-visible scan of (a) Congo red and (b) Coomassie Brilliant Blue G-250 before (continuous line) and after (dashed line) treatment with laccase and the respective mediator.

decolorization of azo dyes using chitosan bound laccase.

UV-visible scans of the treated dyes showed disappearance of the band in the visible region and substantial decrease in absorbance in the UV region (Figure 6), which indicates significant modification of the dye compounds by the laccase-mediator system.

The results of this study demonstrate that *Galerina* sp. is a potential new source of laccase and even other oxidizing enzymes. Agricultural residues could serve as useful substrates for the fungus, instead of glucose and yeast extract, indicating potential for reducing enzyme production costs and also providing added value to the wastes. Further increase in the enzyme yield should be possible e.g. by using fed-batch mode of cultivation.

The laccase produced by *Galerina* sp. HC1 showed the ability to extensively decolorize a toxic azo dye and a triphenylmethane dye, which represent groups making up the bulk of the commercial dyes. The availability of such enzymes make enzymatic dye decolorization more preferable to other relatively expensive methods such as radiation, fenton oxidation, ozonation, photochemical oxidation, etc. Further studies on the mechanism of decolorization and determining the reaction end product(s) would be of interest in order to ensure that the effluent obtained is non-toxic or show if the enzymatic method is to be supplemented with another degradation technique to achieve the desired results.

Conflict of interests

The author(s) have not declared any conflict of interests.

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