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Full Length Research Paper

Decolorization of textile dyes by *Polyporus pseudobetulinus* and extracellular laccase

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The objective of this study is to obtain new laccase and enzyme source with remarkable dye removal potential. Thirty isolates of white rot fungi were screened for extracellular laccase-production using 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay as indicator. Among these, *Polyporus pseudobetulinus* strain WR77 exhibits the highest laccase activity and its suitable enzyme production medium contains; 1% (w/v) rice chaff, 0.5 g/L di-ammonium tartrate, and 0.01 g/L peptone as the carbon; inorganic and organic nitrogen sources; respectively. The laccase was 60-fold concentrated (by ammonium sulphate precipitation, Q-sepharose anion-exchange chromatography, and Superdex G-75 gel filtration chromatography) and gave the specific activity of 617.12 U/mg. The MW of prepared enzyme is 75.2 kDa under SDS-PAGE determination. Empirical analyzing results indicate that the optimum pH and temperature of the enzyme are around 40°C and pH 4, respectively. Furthermore, this enzyme can resist to wide pH range (4.0-11.0) with more than 95% maximum activity remained. The enzyme's K_m and V_{max}, with ABTS substrate, were 447.93 µM and 104.17 µmol/min/mg protein, respectively. The prepared enzyme was strongly inhibited by Hg^{2+} and Fe^{2+} but weakly (9.7%) stimulated by 10 mM Cu^{2+} ions. The strain WR77 shows good ability in decolorizing many synthetic dyes (200 mg/L initial concentration); Ambifix Blue H3R (98% in 8 days), Ambifix Yellow H3R (24% in 10 days) and Ambifix Red HE3B (50% in 18 days). The prepared laccase alone (5 U/ml) could decolorize Ambifix Blue H3R by 65% within 15 min and Malachite Green by 80% within 24 h. It can be concluded that new enzyme and source with satisfactory dye removal potential have been successfully achieved. Further studies should be attempted to evaluate their feasibility in industrial uses.

Key words: *Polyporus pseudobetulinus*, white-rot fungi, Laccase, dye decolorization, synthetic dyes.

INTRODUCTION

Lignin is a complex oxyphenyl propanoid polymer found in all vascular plants including herbaceous species where it provides rigidity to the wood part and takes responsibility in supporting and protecting the plants. Lignin polymer comprises a variety of monomers connected together by various C-C and C-O-C non-hydrolyzable bonds with an irregular arrangement of successive monomeric and inter-monomeric bonds (Dean and Eriksson, 1992), thus it can resist to hydrolytic attack. Nevertheless, it can still be degraded by various microorganisms, in particular, the white-rot fungi. These

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the fungi can produce several oxidative ligninolytic enzymes, in particular lignin peroxidase and laccase. In addition to lignin, it has been found that these enzymes have relatively low substrate specificity and can also degrade wide range of pollutants, such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbons, synthetic high polymers and various dyes (Arora and Sharma, 2010).

The dyes are extensively used in several industries including textile, paper, printing, leather-dyeing, cosmetic, pharmaceutical and food industries. Approximately 10-15% of used dyes are released into environment after manufacturing processes (Erkurt et al., 2007). These dyes are not only toxic to humans, animals and various aquatic life forms, but they also inhibit sunlight penetration and reduce photosynthetic activities of various ecosystems. Moreover, the exiting dyes usually come from synthetic origin and contribute to more complicate molecular structures making them difficult to biodegrade. Therefore, many attempts have been made to find out more dye removal tools in order to overcome this pollutant residual problem. Decolorization of these dyes by physical or chemical methods, including adsorption, coagulation/ flocculation, ion exchange, oxidation and electrochemical methods have been relatively successful but still have economic and methodological disadvantages (Erkurt et al., 2007). Alternatively, the dye decolorization using microbial enzymes has received a great attention in recent years due to its efficient applications. Certain families of fungi have typically proved to be suitable tools for textile effluent and dye removal treatments. Moreover, fungal mycelia can provide an additional advantage over single cell organisms in that they can solubilize their insoluble substrates by producing extracellular enzymes (Kaushik and Malik, 2009).

White-rot fungi have been demonstrated to have the ability in decolorizing the synthetic dyes by their lignin peroxidase, manganese peroxidase and laccase (lignolytic enzymes). Generally, the fungi usually contain various combinations of the above three lignolytic enzyme types. Most of white rot fungal strains seem to produce laccase as main enzyme during the dye decolorization process. The laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) are multi-copper oxidases that catalyze oxidation reactions in many phenolic and organic substrates coupling with reduction reactions that transfer molecular oxygen to water (Murugesan et al., 2007; Thurston, 1994).

To date, numerous fungal strains have been tested for the ability to decolorize and mineralize various dyes. Unfortunately, the majority of dyes are chemically stable and still resistant to microbiological attack. Therefore, expanding the microorganism spectrums having alternative laccase abilities and novel laccase isoforms with different physicochemical and catalytic properties for the dye decolorization is still necessary. In this study, we screened 30 white rot fungi strains for their abilities to secrete laccase and selected one which contains the enzyme was purified, characterized, and evaluated for their decolorization capability against the synthetic dyes.

MATERIALS AND METHODS

Chemicals

2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), and Malachite green oxalate salt (MG) were purchased from Sigma-Aldrich (USA). Ambifix Blue H3R, Ambifix Yellow H3R, and Ambifix Red HE3B were purchased from Rachada Chemicals Co., Ltd. (Thailand). The reagents used in polyacrylamide gel electrophoresis (PAGE) were obtained from Plusone Pharmacia Biotech (Sweden), except that low molecular weight calibration kit was purchased from Amersham Pharmacia Biotech (UK). All other biochemical reagents and general chemicals used in the investigation were of analytical grade and used in received from.

Organisms

All 30 strains of white rot fungi used in this study were obtained from the culture collection of the Microbiology Department, Faculty of Science, Chulalongkorn University, Thailand. Each fungus was grown on 2% (w/v) malt extract agar (2% MEA) slants at 25°C for about seven days and maintained at 4°C until use. Each stocked fungus was sub-cultured every two or three months.

Enzyme assays

Laccase (EC 1.10.3.2) activity was measured at 30°C using 1 mM ABTS as a substrate. The assay mixture (1 ml) contained 880 µl of 100 mM sodium acetate buffer (pH 5.0), 100 µl of ABTS stock (final concentration 1 mM), and 20 µl of appropriately diluted crude enzyme. Then, the increased absorbance of the mixture was monitored at 420 nm (ε_{420} = 36.0 /mM cm) using a spectrophotometer (Murugesan et al., 2007). One changed unit was determined by 1 µmol of oxidized ABTS per minute.

Protein amount determination

Protein contents were determined by Bradford's procedure as described by Bollag et al. (1996). Briefly, Two-fold serial dilutions of the sample (50 µl) in deionized water were prepared in a 96 well flat-bottom plate along with the series of standard BSA solutions, and then 50 µl of Bradford's reagent was added to each analyzed well, the plate was shaken on a round orbit plate shaker (Biosan, OS-10, Latvia) and read at 595 nm using an ELISA plate reader (Biotek Synergy HT, Biotek instrument, USA). The OD was calculated for protein concentration via a standard curve derived equation. Besides, during column chromatographic separations, the protein amounts of eluent were determined by directly monitoring at 280 nm.

Selection of laccase-producing strains

All 30 fungal strains were activated on 2% MEA for one week. The ABTS agar medium (Pointing, 1999) was used for the selection of laccase-producing strains. Each plate was inoculated with one agar plug (8 mm diameter) from the edge of an actively growing test fungus and incubated for seven days at room temperature. Positive laccase producing strains were determined by appearance of green

zones around the colonies. Widths of the green zone directly relates to the amount of extracellular laccase produced. Thus the strain having the widest green zone diameter (cm) / colony diameter (cm) ratio was selected.

Identification of white rot fungi

After obtaining the fungal strains with highest laccase production, the species identification was conducted using systematic morphological and molecular marker approaches. Morphological determination bases on both macroscopic and microscopic characters whilst the molecular identification based on DNA sequence similarity of the internal transcribed spacer (ITS) regions within rDNA. These were achieved by data comparison between the attended isolates and those obtained from NCBI GenBank database. Genomic DNA was prepared from fresh mycelial culture of the selected fungal isolate and extracted with cetyltrimethylammonium bromide (CTAB), as described in Zhou et al. (1999). PCR amplification of the internal transcribed spacer (ITS) was carried out with a total volume of 35 µl comprised of approx. 100 ng genomic DNA, 1 × PCR master Mix (Fermentas, Califonia, USA), and 100 nM of ITS1F primer, and 500 nM ITS4 primer. The amplification was performed in a thermocycler with certain PCR program. The post PCR contents were purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and directly sequenced at both leading and lagging strands (using the ITSF1 and ITS4 primers, respectively) commercially available (Macrogen, Seoul, Korea). The complete consensus sequence was then used in NCBI GenBank BLASTn searching with default settings. Top 100 highest sequence similarity hits were recorded and compared. Species annotation of the deposited ITS sequences in the GenBank database were taken on trust and taken to convert into molecular operational taxonomic unit (MOTU) designated the fungal isolate to be the likely species in case the % sequence similarity was high enough (>97%).

The effect of different carbon and nitrogen sources on laccase production

The optimal carbon and nitrogen (organic and inorganic) sources for maximal laccase production were preliminarily evaluated using a sequential univariate approach as follows.

Different carbon sources on laccase production

The selected fungal strain was precultured on 2% MEA for one week and then laccase production was carried out at 25°C for 20 days on a rotary shaker at 120 rpm in 250 ml flasks containing 100 ml of basal medium (LBM) supplemented with 1% (w/v) of one of investigated carbon sources (rice chaff, biogases, rice bran, rice straw and sawdust). The initial pH of the medium was adjusted to 5.5. Every two days of cultivation, the mycelium was harvested from certain amount of culture aliquot by filtrating through Whatman no. 1 filter paper and the filtrate was further used to determine the laccase activity. The carbon source giving maximal laccase production was selected and its efficacy was estimated under 0.5, 1.0 and 2.0% (w/v) concentrations using the same condition as described above. All experiments were done in triplicate and the results are expressed as mean ± standard deviation (SD).

Different nitrogen sources on the laccase production

In order to optimize the inorganic nitrogen source, the $C_4H_{l2}N_2O_6$ in ammonium hydrogen phosphate ($(NH_4)_2PO_4$) or ammonium the LBM formula was replaced by one of these inorganic nitrogen sources [ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), persulfate ($(NH_4)_2S_2O_8$] at nitrogen concentration of 0.25, 0.5 and 1.0 g/L. All remaining procedures were conducted as method previously described except that the inorganic nitrogen in LBM medium was modified by supplementing with optimal carbon source type and concentration.

In organic nitrogen sources optimization, the yeast extract ingredient in the LBM medium (which now was adjusted to be optimal carbon and inorganic nitrogen sources) was replaced by 0.005, 0.01, and 0.02 g/L of one of these ingredients (peptone, urea, soy bean, and corn steep). The optimal organic nitrogen type and concentration were selected for further experiment use. All experiments were done in triplicate and the results were expressed as mean ± SD.

Laccase purification

Laccase was purified from the selected white rot fungal strain (WR77) grown in the LBM culture medium with optimal carbon and nitrogen sources for 12 days. The culture medium was separated from mycelia by centrifugation at 15,000 × g for 30 min (Beckman Coulter, USA). The supernatant was then fractionated with 80% saturated ammonium sulphate. The precipitant was harvested by centrifugation and taken to dialyze (membrane molecular weight cut-off 3,500 Da) in 4°C deionized water. The crude protein was loaded (10 ml at 50 mg protein/ml) onto a Q-sepharose fast flow column (1.6 cm × 15 cm, previously equilibrated with 50 mM sodium acetate, pH 5.0). After that, the column was washed with 50 mM sodium phosphate buffer (pH 5.0) until the A_{280} reading was less than 0.02. Then, bound protein was eluted with a linear salt gradient (0-1.0 M NaCl) at 2.0 ml/min, and collected in each 10 ml fraction.

The eluted fractions were assayed for laccase activity and the absorbance at A_{280} was recorded. Peak corresponding fractions were pooled together and dialyzed against running buffer before being loaded (5 ml at 10 mg protein/ml) onto a Superdex G-75 column (1.6 cm × 60 cm) which was equilibrated and consequently eluted with 0.1 M NaCl / 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 1 ml/min. Each 5 ml fraction was collected and assayed for laccase activity. Finally, the peak corresponding fractions were pooled together and some may need to be concentrated by lyophilization before used.

Determination of enzyme purity by native-PAGE, SDS-PAGE and laccase enzyme staining

The enzyme from each purification step was analyzed for its native protein pattern and purity according to the method described by Bollag et al. (1996). The native PAGE was carried out at 10 and 5% (w/v) acrylamide for separating and stacking gels, respectively, using 100 mM Tris-glycine (pH 8.3) as the electrode buffer. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK). After electrophoresis, protein bands in the gel were visualized after Coomassie blue R-250 (Sigma) staining procedure and after native-PAGE resolution finished, the laccase zymograms were visualized by immersing the gel into sodium acetate buffer (pH 5.0) contained 5 mM ABTS at room temperature.

Discontinuous reducing 0.1% (w/v) SDS-PAGE was performed according to the procedure described by Laemmli (1970) using 15% and 5% (w/v) acrylamide for resolving and stacking gels, respectively. Electrophoresis was run at a constant current of 20

mA per slab in a Mini-Gel Electrophoresis unit. Low molecular weight standards (5 μg each) were co-resolved on each gel and used to determine the (subunit) molecular weight of the prepared laccase enzyme.

Laccase characterization

Temperature optimum and temperature stability

The effect of temperature on the laccase activity of the enriched laccase fraction (post-superdex-75) was determined by incubating the enriched laccase fraction (1 mg/ml) in 100 mM sodium acetate buffer (pH 5.0) at various temperatures (4-90°C at 10°C intervals) for 30 min. The thermostability of the laccase was investigated by preincubating the enriched laccase fraction at various temperatures (40-70°C in 10°C intervals) in the same buffer for the indicated fixed time intervals (10-120 min), cooling to 4°C and then assaying the residual laccase activity as described in above. The control incubation was set at 100% activity and the activities of the samples from the different temperatures were expressed relative to that of the control (100% activity).

pH optimum and pH stability

Incubating the enriched laccase fraction in buffers of broadly similar salinity levels, but varying in pH from 2-12, was used to assess the pretreatment pH stability and the pH optimum of the laccase. The buffers used were (all 20 mM) glycine-HCl (pH 2-4), sodium acetate (pH 4-6), potassium phosphate (pH 6-8), Tris-HCl (pH 8-10) and glycine-NaOH (pH 10-12). The enriched laccase fraction was mixed (1 mg/ml final concentration) in each of the different pH-buffer compositions, plus the control (50 mM Tris-HCl (pH 7.0). For pH optimuma, the above laccase-buffer mixtures were left for 30 min at room temperature and then adjusted back to 50 mM Tris-HCl (pH 7.0) and assayed for laccase activity as described in above. The control incubation was set at 100% activity and the activity of the samples from the different pH buffers were expressed relative to that of the control (100% activity). For the pH stability, the different pH buffer-enzyme mixtures were adjusted in substrate concentration as described in above for laccase activity assay, and performed over 30 min. The activity of the enzyme in each pH was then related to that of the control, set to 100%.

Various metal ions and reagents

The effects of various metal ions on prepared laccase activity was studied by incubating the enzyme in presence of each divalent metal ion (Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Hg^{2+}) or EDTA at the concentration of 0.5, 1.0, 5.0 and 10.0 mM, respectively, for 30 min (the corresponding salts are shown in Table 3). Then the enzyme activity was tested as described in above and expressed as relative laccase activity (%) corresponding to the reaction mixture without additional metal ions (set as 100% activity).

Enzyme kinetics

The enzyme kinetic constants, for ABTS substrate, were assayed at concentrations between 0.025 and 2.0 mM in 100 mM sodium acetate buffer (pH 5.0). The substrate oxidation rates were determined by measuring the absorbance increase at the respective wavelengths and Michaelis constants (K_m and V_{max}) were calculated from Lineweaver-Burk plots.

Dye decolorization

The selected fungal strain was precultured on 2% MEA for one week before decolorization of each Ambifix Blue H3R, Ambifix Yellow H3R, Ambifix Red HE3B and malachite green (MG) (added in liquid culture medium to make 200 mg/L of initial concentration) was evaluated in 250-ml Erlenmeyer flasks containing 100 ml medium. Flasks were inoculated with three plugs (6 mm diameter) of pregrown mycelium and were incubated at 25°C for twenty days on rotary shaker (120 rpm). Each sample was separately taken and the absorbance of the residual dye was followed by measuring the absorbance at 585 nm for Ambifix Blue H3R, 415 nm for Ambifix Yellow H3R, 535 nm for Ambifix Red HE3B and 620 nm for MG. The dye removal efficiency was expressed as the percentage of remained dye per initial dye.

To evaluate enzyme decolorization, the reaction mixtures (200 μL) containing the crude enzyme (final laccase activity 5 U/ml) and each dye (100 mg/L Ambifix Blue H3R, Ambifix Yellow H3R, Ambifix Red HE3B or MG) was tested in 50 mM sodium acetate buffer, pH 5.0. Changes in absorbance were detected by the spectrophotometer (Synergy HT Biotek, USA).

RESULTS AND DISCUSSION

Selection of laccase-producing strains and fungus identification

To select the best laccase-producing strain from total 30 strain white rot fungi, each strain was cultured on ABTS-agar medium seven days before the green zone around the colony were recognized and measured. Presence of laccase activity causes the clear medium turn green due to the ABTS is oxidized to be ABTS-azine (Niku-Paavola et al., 1990). The green zone width to colony diameter ratio was used in laccase activity comparison. Although all 30 isolates were found to secrete laccase, the isolate WR77 showed the highest ratio (Table 1), and therefore it was selected for further study.

For morphological identification, the basidocarp of the isolate WR77 is 7-9 cm broad and 0.5-1.0 cm thick, pileate to substipitate contain a thin cuticle on the pileus which is shell-shaped and bulgy. There is no stipe observed from fruiting bodies which are shelf-like, firmly attach to the bark, upper surface is cream-white color. Dimitic hyphal system with arboriform vegetative hyphae, no reaction to Melzer's Reagent (nonamyloid) is observed in cell content or wall. For genotypic identification, the ITS region of isolate WR77 was amplified, directly sequenced and compared to those in the NCBI GenBank database using the BLASTn algorithm. The PCR amplicon containing the its sequence of the isolate WR77 is 532 bp long and exhibited highest sequence identity (86%) to *Polyporus pseudobetulinus*. Thus, both the morphological, cultural and molecular characteristics are consistent with isolate WR77 belonging to *Polyporus* and being related to *P. pseudobetulinus*. Some members of the genus *Polyporus* have previously been reported to produce extracellular laccase production, such as *P. anceps* (Petroski et al., 1980), *P. anisoporus* (Vaitkyavichyus et al., 1984) and *P. pinsitus* (Heinzkill et

Table 1. Selection of the white rot isolate by laccase secretion as determined by the clear green to colony diameter ratio.

Isolate	Green zone diameter (mm)	Colony diameter (mm)	Green zone / colony diameters
WR5	4.53 ± 0.03	1.43 ± 0.03	3.16
WR6	6.07 ± 0.05	2.67 ± 0.04	2.28
WR9	7.23 ± 0.12	5.45 ± 0.07	1.32
WR12	6.07 ± 0.05	4.03 ± 0.01	1.50
WR17	8.07 ± 0.01	8.50 ± 0.01	0.95
WR19	8.50 ± 0.03	8.50 ± 0.03	1.00
WR24	5.57 ± 0.03	3.83 ± 0.03	1.45
WR25	6.17 ± 0.03	4.20 ± 0.03	1.47
WR26	5.13 ± 0.01	8.03 ± 0.08	0.64
WR28	6.47 ± 0.03	3.07 ± 0.02	2.11
WR33	8.50 ± 0.02	8.50 ± 0.01	1.00
WR35	8.50 ± 0.01	8.50 ± 0.05	1.00
WR43	8.50 ± 0.01	4.50 ± 0.10	1.89
WR48	6.85 ± 0.03	2.53 ± 0.02	2.70
WR50	8.50 ± 0.03	8.50 ± 0.03	1.00
WR51	5.03 ± 0.03	2.57 ± 0.06	1.96
WR54	7.23 ± 0.03	5.83 ± 0.07	1.24
WR57	8.50 ± 0.02	6.37 ± 0.05	1.34
WR58	8.50 ± 0.01	3.43 ± 0.03	2.48
WR60	2.07 ± 0.05	2.77 ± 0.03	0.75
WR61	6.20 ± 0.01	7.03 ± 0.02	0.88
WR63	6.10 ± 0.02	3.83 ± 0.01	1.55
WR66	5.43 ± 0.05	8.50 ± 0.02	0.64
WR67	6.63 ± 0.03	6.60 ± 0.07	1.00
WR73	7.00 ± 0.05	4.27 ± 0.05	1.64
WR75	6.47 ± 0.03	4.73 ± 0.05	1.37
WR76	5.53 ± 0.03	1.73 ± 0.03	3.19
WR77	6.27 ± 0.03	1.87 ± 0.03	3.36
WR80	3.60 ± 0.05	5.85 ± 0.10	0.62
WR81	5.77 ± 0.03	1.95 ± 0.03	2.96

Data are shown as the mean ± 1 SD diameter and are derived from 3 replicates.

al., 1998).

Carbon and nitrogen sources determination and optimization

Carbon source

Evaluation for the different carbon source effects in the LBM, in term of extracellular laccase production, revealed that the maximum laccase activity (approx. 0.40 U/ml) was obtained after a 16-day cultivation period with rice bran as the carbon source (Figure 1A). However, only a slightly lower laccase level (approx. 0.37 U/ml) was obtained after a 12-day cultivation period with rice chaff as the carbon source. The laccase production with rice straw as the carbon source was much slower than previous two sources and only reached 0.22 U/ml after 20 days cultivation. In contrast, sugarcane bagasse and sawdust gave the lowest laccase levels (Figure1A).

The laccase production rate in rice bran carbon source treatment was slower than rice chaff treatment. This could be due to presence of other enzyme secreting contributes to hydrolyze the rice bran components (e.g. cellulases, β-glucosidases and xylanases) at the early stages of growth. Since, rice bran contains high total carbohydrate level (~82% (w/w)) of which the main composition (31%) is hemicellulose (Claye et al., 1996), the arabinoxylan mainly composes of xylose and arabinose (Mod et al., 1978). Although rice chaff main components are hemicelluloses, but its lignin proportion (21-26%) is still high (Yang et al., 2004) thus it could induce laccase synthesis. This observation agrees to a report in grape seeds which have relatively high lignin

content and become the best solid carbon source for laccase production by *Trametes hirsuta* (Moldes et al., 2003). From this result, the rice chaff was selected as carbon source since the laccase activity was comparable to that from the rice bran, but the rice chaff is cheaper and generally available.

It was found that increasing of the rice chaff concentration from 0.5 to 1 or 2% (w/v) significantly increased the laccase production level, for example after 12 days culture from 0.21 U/ml to 0.37 and 0.32 U/ml, respectively (Figure 1B). Thus, the optimum concentration of the rice chaff was 1% (w/v). Increasing the rice chaff to 2% (w/v) might cause carbon and nitrogen excess, which directly affect (reduce) the ligninolytic enzyme production. Many agricultural wastes have been investigated for use as substrates in laccase production through white rot fungi. Stajic et al. (2006) reported that the highest level of laccase activity in *Pleurotus eryngii* was found in submerged culture with dry ground mandarine peels as the carbon source after seven days cultivation (999.5 U/L), but the highest level of laccase activity in *Pleurotus ostreatus* strain No. 493 was observed under solid state fermentation (SSF) conditions of grapevine sawdust after 10 days cultivation (2144.6 ± 57.8 U/L).

Nitrogen source

It was found from the investigation of different inorganic nitrogen source and concentrations (0.25, 0.5 and 1.0 g/L) that the 0.5 g/L di-ammonium tartrate gave maximum laccase production (0.37 U/ml), and thus was the best concentration (Figure 1C). Indeed, most of given inorganic nitrogen sources gave respective optimal laccase productions at 0.5 g/L and the laccase production decreased at thereafter range.

Likewise, the different organic nitrogen sources typically gave their optimal laccase production at 0.01g/L and the production became diminished at higher (0.02 g/L) or lower (0.005 g/L) concentrations. The overall maximum laccase production (approx. 0.39 U/ml) was obtained with 0.01 g/L peptone as the organic nitrogen source (Figure 1D). It was reported that, the peptone could stimulate laccase production in *Lentinus edodes* (Buswell et al., 1995), and *Phanerochaete chrysosporium* (Srinivasan et al., 1995). However, Stajic et al. (2006) found that both *P. eryngii* and *P. ostreatus* strain no. 493,gave the highest laccase production at 20 and 30 mM $(NH_4)_2SO_4$, respectively.

Laccase enrichment capability

The condition consisted of all optimized parameters were used for laccase enrichment. After an initial 80% saturation ammonium sulphate cut, with 96% protein removed and giving a 6.5-fold laccase activity enhancement (Table 2), the culture supernatant was collected, dialyzed, and subjected to Q-sepharose anion-exchange chromatography (Figure 2A). The laccase eluted at very low NaCl gradient (60-200 mM) appeared as a well separated sharp peak. A 27.2-fold decreased with three-fold total laccase activity loss while approximately nine-fold specific activity increased (Table 2). The peak from Superdex 75 gel filtration chromatography is shown in Figure 2B. This step removes contaminating protein from the laccase and makes the total activity risen up to 60.1-fold with a yield of 3.61% and a specific activity of 617.1 U/mg. The final specific activity obtained here is fairly high compared to that reported in some other fungal laccases, e.g. 32.9 U/mg for *Pycnoporus sanguineus* (SCC 108) (Litthauer, 2007), and 50.5 U/mg for *Daedalea quercina* (Baldrian, 2004).

Purity checking by native-and reducing SDS-PAGE and native zymograms

The laccase from each step of enrichment was analyzed for purity and protein pattern by native-PAGE, with protein and enzyme activity staining (Figure 3A). Whilst the post-DEAE-cellulose laccase fraction still showed multiple components, the enriched laccase fraction (post-Superdex-75 laccase fraction) showed a single protein band on native-PAGE, suggesting a high degree of purity, with only a enzyme band seen when using ABTS as the substrate, and at the same position (R_f), supporting that the enriched laccase fraction was a pure or near pure enzyme.

For the prepared laccase, a single band equivalent to the molecular weight of 75.2 kDa was observed from SDS-PAGE electrogram while the laccase activity staining result revealed a single laccase activity zone corresponding to the band position found in the SDS-PAGE electrogram (Figure 3B). The molecular mass of this laccase is consistent to those reported in other fungal laccases, which are between 60 and 80 kDa (Mayer and Staples, 2002; Thurston, 1994).

Laccase characterization

Effect of temperature on laccase activity and thermostability

The effect of temperature on the activity of the enriched laccase is shown in Figure 3A. The optimum temperature of the purified enzyme was observed at 40°C, which is similar to that reported for the laccase from *Lentinula edodes* Lcc1 (Nagai et al., 2002), although the laccase was almost equally active over a wide range of temperatures from 0 to 40°C and declined thereafter slightly at 50°C and significantly above 60°C to being completely inactivated ay 80°C. This is in contrast to that

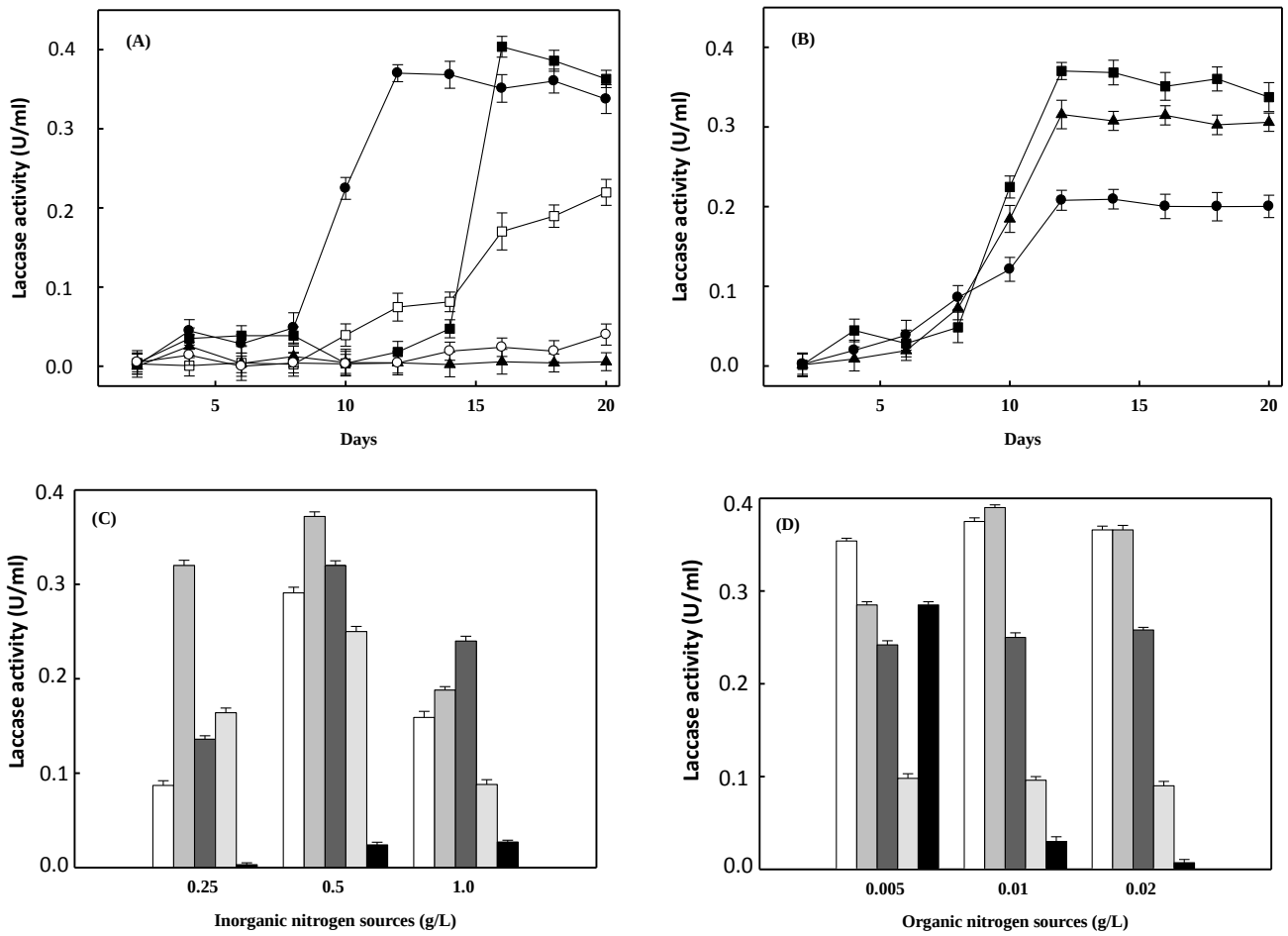

Figure 1. Laccase production by white rot fungal isolate WR77 grown in LBM with (A) the indicated 1% (w/v) rice chaff (●), bagasse (○), rice bran (■), rice straw (□) and sawdust (▲) as the carbon source and. (B) with 0.5% (●), 1.0% (■) and 2.0% (▲) (w/v) rice chaff as the carbon source. (C) Effects of various inorganic nitrogen sources and concentrations on laccase production by WR77 when grown in LBM with 1% (w/v) rice chaff and sodium nitrate (white), di-ammonium tartrate (grey), ammonium chloride (dark grey), ammonium hydrogen phosphate (light grey) or ammonium persulphate (black). (B) Effects of various organic nitrogen sources and concentration on laccase production by WR77 when grown in LBM medium with 1% (w/v) rice chaff, 0.5 g/L di-ammonium tartrate and: yeast extract (white), peptone (grey), corn steep liquor (dark grey), soybean (light grey) or urea (black). The data are shown as the mean ± 1 SD and are derived from three replicate experiments. Means with a different lowercase letter above them are significantly different ($p<0.05$; Duncan's multiple means test).

Table 2. Details of the enrichment of the laccase from white rot fungus isolate WR77.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	133.20	1,368.00	10.27	100.00	1.00
80% $(NH_4)_2SO_4$ cut	5.44	364.32	66.97	26.68	6.52
Q-sepharose	0.20	121.28	606.20	8.86	59.02
Superdex-75	0.08	49.37	617.13	3.61	60.10

the optimum temperature of most fungal laccases is usually between 50 and 70°C (Baldrian, 2006). However, a few enzymes with an optimal activity below 50°C have been described before, such as the laccase from *G. lucidum* at 25°C (Ko et al., 2001).

The thermal stability of the prepared laccase from WR77 was determined by allowing the enzyme-substrate reactions took place in different temperatures with incubation time fixing. The result is shown in Figure 4B. The enzyme was extremely stable at 40°C with no

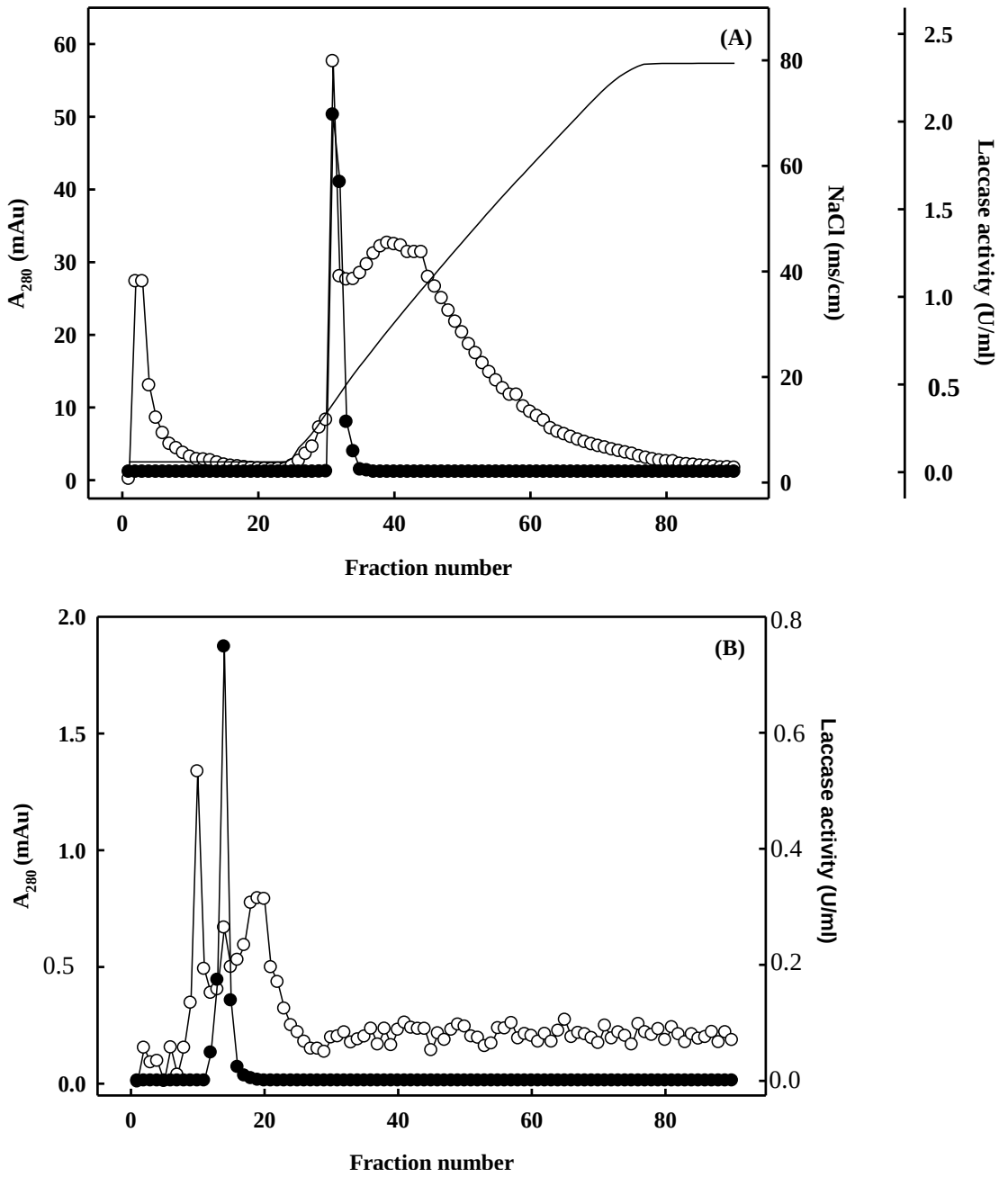

Figure 2. (A) Q-sepharose anion exchange column chromatography enrichment of the fractionWR77 extracellular laccase from the 80% saturation $(NH_4)_2SO_4$ cut fraction, showing the absorbance at 280 nm (○), laccase activity (●) and NaCl (–). (B) Superdex 75 gel filtration chromatography enrichment of the WR77 extracellular laccase from the post-Q-sepharose fraction showing the absorbance at 280 nm (○) and laccase activity (●). Profiles shown are representative of 3 different enrichments.

detectable loss of activity after 120 min. However, at 50°C, the enzyme lost activity from 30 min onwards, whilst at 60 and 70°C, the enzyme activity rapidly declined in 120 and 45 min respectively. This thermal stability is in line with other fungal laccases that have been reported, such as *Marasmius quercophilus* strain 17 (Farnet et al., 2000) and *Trametes* sp. (Smirnov et al., 2001).

Effect of pH on laccase activity and stability

From Figure 5, the reaction rate was slightly higher at pH 4 which might be considered as optimum, but the rate

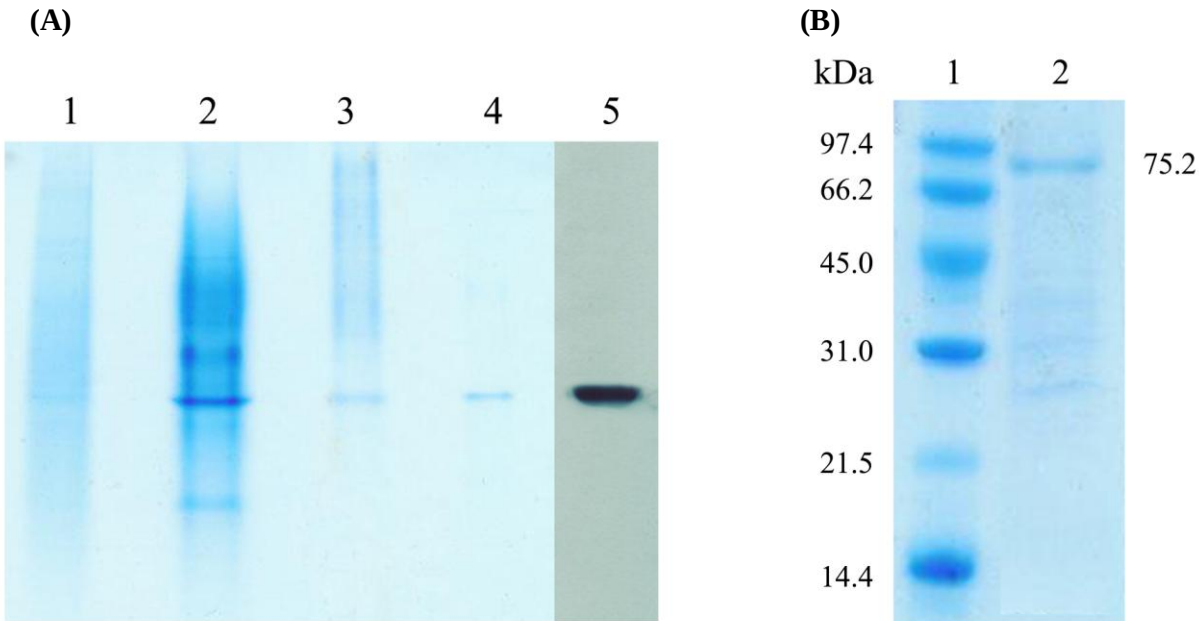

Figure 3. (A) Coomassie blue stained native-PAGE analysis of the WR77 laccase fractions from each step of the enrichment and stained for protein by coomassie blue (Lanes 1-4) or for laccase enzyme activity (Lane 5). Lane 1, crude enzyme (20 μg of protein); Lane 2, ammonium sulfate cut fraction (20 μg of protein); Lane 3, post-Q-sepharose laccase fraction (10 μg of protein); Lanes 4 & 5, post-Superdex-75 laccase fraction (10 μg of protein). (B) The SDS-PAGE analysis of the prepared laccase: Lane 1, Low molecular weight protein markers and Lane 2, prepared laccase (10 μg of protein). Gels shown in (A) and (B) are representative of 3 separate enrichments.

was still high over pH 4-11 range. Decreasing of the activity (pH 3 and 2) was occurred in the same buffer as the high laccase activity seen (pH 4), thus it was unlikely to be buffer effect but true pH effect. The same argument could be hold for the activity declination at pH 12. Similar broad optimal pH has been reported for laccases from several fungi, such as *Lentinula edodes* Lcc1 (Nagai et al., 2002). Fungal laccases typically exhibit optimal pH in the acidic range, whereas the optimal pH for ABTS oxidation are generally lower than 4.0 (Baldrian, 2004). From the result, the laccase activity had stayed high at 4.0-11.0 pH range. The broad pH stability of WR77 could be a very useful characteristic for various industrial applications.

Effect of metals and reagents

The effects of various ions on the laccase activity are shown in Table 3. The purified enzyme was strongly inhibited by Hg^{2+} and Fe^{2+} in a dose-dependent manner reaching as high as 97-98% inhibition at 5.0 mM in both cases. The enzyme was also moderately inhibited by 10 mM Zn^{2+} (77.8% of control activity), whereas EDTA showed no significant inhibitory effect. Cu^{2+} ions at 10 mM showed a weak stimulation of the laccase activity (9.7%). Baldrian and Gabriel (2002) reported the highly negative effect of Hg^{2+} at more than 0.1 mM on the stability of a purified laccase from the white-rot fungus *Pleurotus ostreatus*, but the activity increase in the presence of 0.05-50.0 mM Cu^{+2}. Baldrian (2004) found that laccase from the white-rot fungus *Daedalea quercina* was inhibited by 1 mM Mn (9% of control activity) and 10 mM Hg^{2+} (7% of control activity), whereas 1 mM EDTA exhibited only a slight inhibitory effect (90% of control activity). The addition of 10 mM Cu^{2+} increased the enzyme activity by 17%. In addition, Nagai et al. (2002) reported that a purified laccase from *Lentinula edodes* was inhibited by 55% in the presence of 1 mM Hg^{2+}, and it was activated by 40% in the presence of 10 mM Cu^{2+}. Couto et al. (2005) claimed that the effect of metal ions on laccase activity depended on laccase sources. Metal ions can also influence other factors that affect biodegradation, thus the activation or inhibition of proteolytic enzymes by metals can also change the turnover rate of extracellular enzymes (Palmieri et al., 2001).

Determination of kinetic parameters

The prepared laccase from WR77 showed different kinetic behaviors depending on the substrate concentrations. From the Lineweaver-Burk plot (Figure 6), the K_m and V_{max} values were of 447.93 μM and 104.17 mmol/min/mg protein, respectively. The K_m value of this

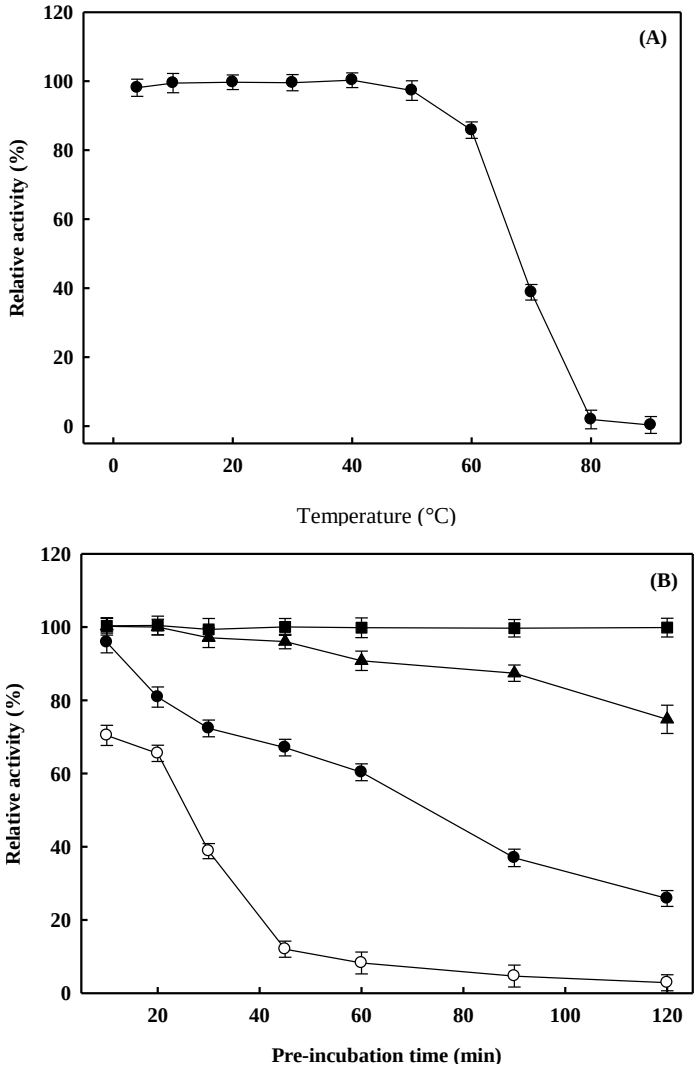

Figure 4. The (A) optimal temperature and (B) thermostability of the prepared laccase from WR77 as determined in 100 mM sodium acetate buffer pH 5.0: at (■) 40°C, (▲) 50°C, (●) 60°C and (○) 70°C. The data are shown as the mean ± 1 SD and are derived from three replicate experiments. Means with a different lowercase letter above them are significantly different ($p<0.05$; Duncan's multiple means test).

WR77 laccase is relatively low compared to the reported values of 38, 108, 130 and 207 μM from *Daedalea quercina* (Baldrian, 2004), *Lentinula edodes* Lcc1 (Nagai et al., 2002), *Pycnoporous sanguineus* (SCC 108) (Litthauer et al., 2007) and *Phellinus ribis* (Min et al., 2001), respectively. Therefore, the laccase from WR77 possesses low K_m and thus high substrate affinity it is.

Dye decolorization

The white rot fungus WR77 showed good decolorizing results to Ambifix Blue H3R (approximately 98% removal within 8 days) (Figure 7A) contrasting to the Ambifix Yellow H3R and Ambifix Red HE3B (very slowly processed, reached 24% removal at 10 days) or Ambifix Red HE3B (reached 50% at 18 days). This could be due to either enzyme inhibition (by some products generated in the decolorization process) or substrate inhibition (Johann et al., 2007). Moreover, the crude laccase (5 U/ml) exhibited good ability in decolorizing the Ambifix Blue H3R and MG (Figures 7B and 7C), attaining 65% within 15 min and 80% within 24 h, respectively. However, the Ambifix Yellow H3R and Ambifix Red HE3B were not decolorized by the crude prepared enzyme (data not shown), which is probably due to the complexity

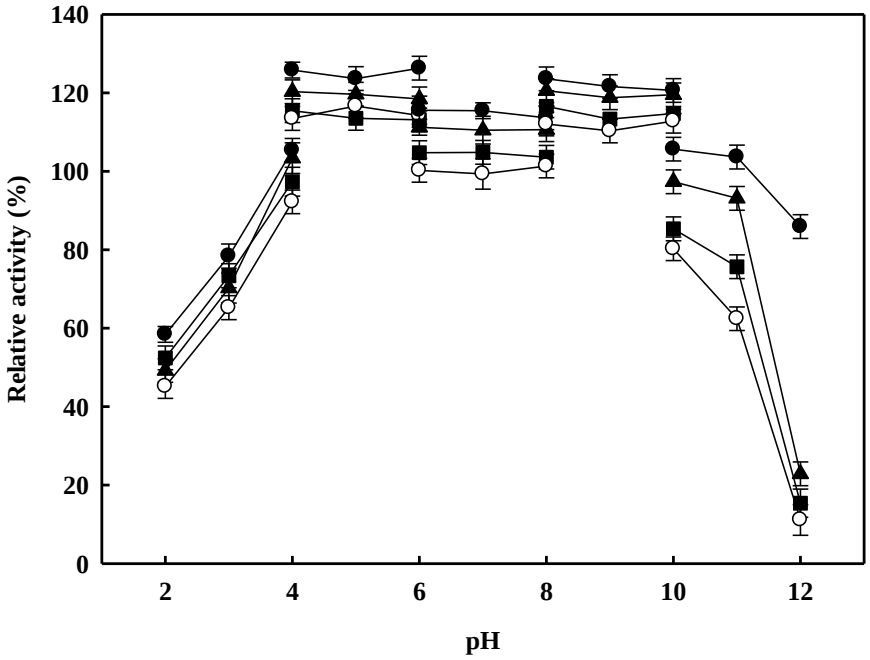

Figure 5. Effect of pH on the activity and stability of the prepared WR77 laccase, shown as the relative activity after incubation for 45 min (●), 60 min (▲), 90 min (■) and 120 min (○). The buffers used (all 20 mM) were glycine-HCl pH 2-4, sodium acetate pH 5-6, potassium phosphate pH 7-8, Tris-HCl pH 9-10 and glycine–NaOH pH 11-12. The data are shown as the mean ± 1 SD and are derived from three replicate experiments. Means with a different lowercase letter above them are significantly different ($p<0.05$; Duncan's multiple means test).

Table 3. Effect of divalent metal cations and EDTA on the activity of the prepared laccase from the white rot fungal isolates WR77.

Reagent	Relative laccase activity (%)[a]			
	0.5 mM	1 mM	5 mM	10 mM
Control[b]	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00
$CuSO_4$	98.8 ± 0.50	100.0 ± 1.04	100.4 ± 0.15	109.7 ± 0.87
$ZnSO_4$	99.2 ± 0.37	99.1 ± 0.11	92.0 ± 0.07	77.8 ± 0.22
$CaCl_2$	100.1 ± 0.32	100.4 ± 0.42	100.8 ± 0.11	100.6 ± 0.93
$MnCl_2$	100.7 ± 1.34	99.1 ± 0.53	99.7 ± 1.49	99.4 ± 0.18
$HgCl_2$	42.8 ± 0.19	21.8 ± 1.27	2.3 ± 0.02	2.6 ± 0.35
$FeCl_2$	66.4 ± 0.20	9.7 ± 1.08	2.8 ± 0.05	1.4 ± 0.03
EDTA	99.1 ± 0.56	98.3 ± 0.62	99.0 ± 1.30	98.7 ± 0.29

[a]The relative activity was determined by measuring laccase activity at 30°C in 100 mM sodium acetate buffer pH 5.0 after pre-incubation at 30 °C for 30 min with the indicated individual cations or reagents. Results are shown as the average of 3 independent assays, each performed in triplicate. [b]The activity assayed in the absence of cations or reagents was taken as 100%.

of dye structures.

Thus, the white rot fungus WR77 was better in decolorizing the Ambifix Yellow H3R and Ambifix Red HE3B while the crude enzyme couldn't decolorize both of these two dyes. It is possible that this cause by biosorption effect of the fungal mycelia. Moreover, intact fungal mycelia can also possibly provide more suitable conditions than in vitro environments (e.g. presences of some cations, coenzymes, high energy substance supplementations, and even other enzymes that have ability to enhance the degrading reaction). Along these lines, Swamy and Ramsay (1999) suggested that mycelium-associated activities play an important role in the dye decolorization in *Trametes versicolor*. It has also been shown that the decolorization of an astrazone dye by isolated fungal pellets of *Funalia trogii* involves

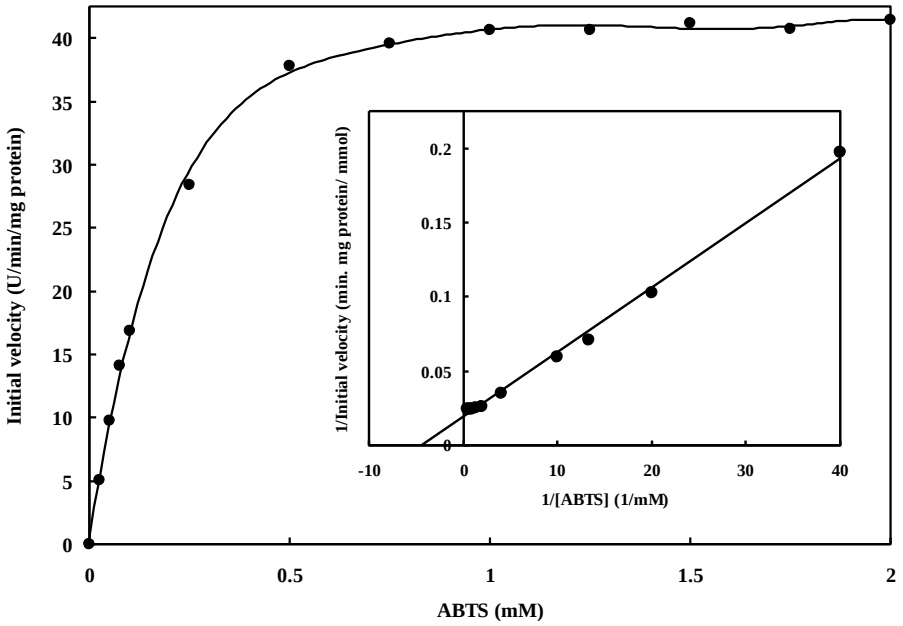

Figure 6. Lineweaver-Burk plot of the prepared WR77 laccase with ABTS as the substrate.

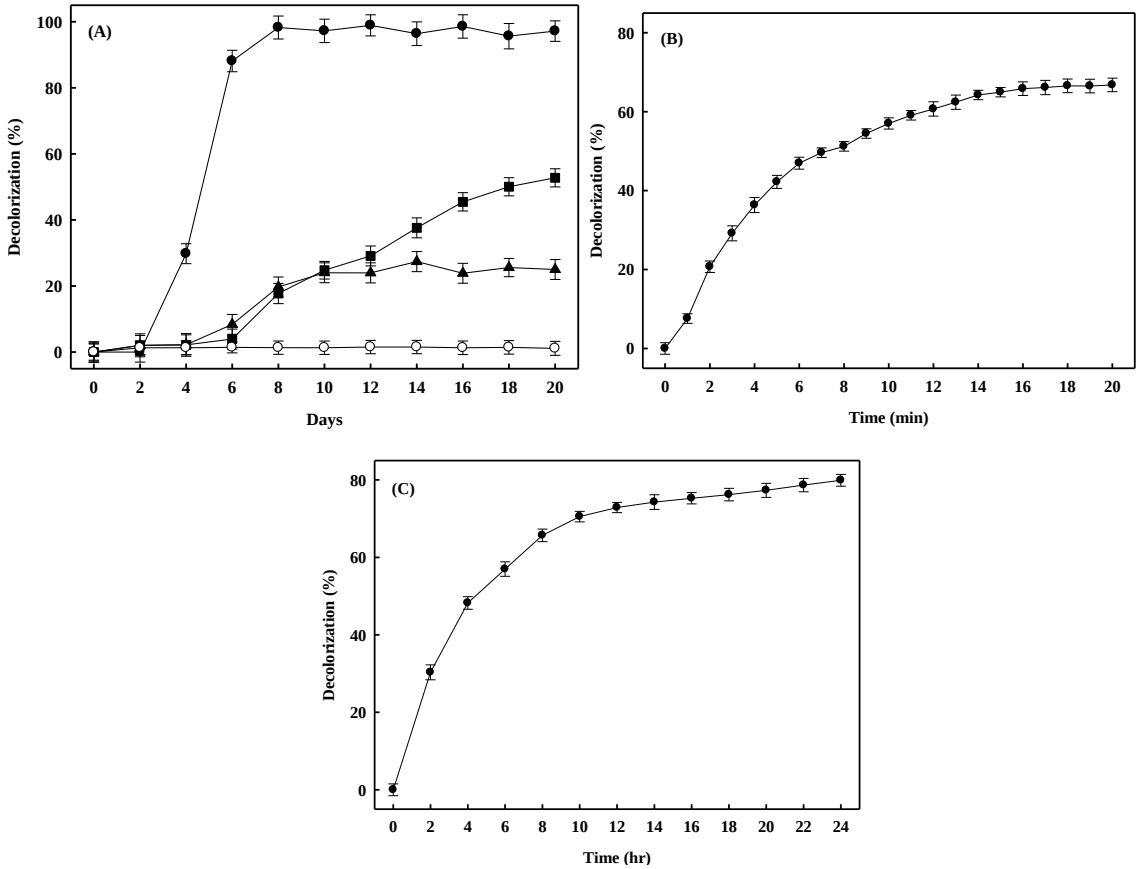

Figure 7. Dye decolorization of Ambifix Blue H3R (●), Ambifix Yellow H3R (■) and Ambifix Red HE3B (▲) and MG (○), by white rot fungal strain WR77, all from an initial concentration of 200 mg/L. Dye decolorization of (B) Ambifix Blue H3R and (C) MG by the crude WR77 laccase, both at an initial concentration of 200 mg/L. The data are shown as the mean ± 1 SD and are derived from three replicate experiments. Means with a different lowercase letter above them are significantly different ($p<0.05$; Duncan's multiple means test).

mechanisms other than biosorption by mycelia and degradation by secreted enzymes in the culture liquid (Cing and Yesilada, 2004).

Svobodova et al. (2008) demonstrated that *I. lacteus* cultivated in a liquid medium could fade out 88.5 and 98.6% of azo dyes RO16 and RBBR within 24 h, respectively, whereas, in contrast, no dye decolorization was detected by the crude culture filtrate. This indicated that the dye decolorization observed in complete fungal cultures may not occur when the secreted enzyme is used alone. However, the crude enzyme from WR77 showed good ability to decolorize the MG, but not by the fungal cultures (Figure 6A). Perhaps, most of the fungi cannot tolerate in initially high MG concentration owing to its toxicity (Cha et al., 2001). Compared with laccases from other fungi; including *Phanerochaete chrysosporium* (Chagas and Durrant, 2001), *Cunninghamella elegans* (Cha et al. 2001), and *Coriolus versicolor* (Levin et al., 2004); the MG decolorization efficiency of the crude laccase from WR77 was very high, with about 160 mg/L MG removal within 1 h at 30°C and pH 5.0. Thus, the white rot fungus WR77 showed a high laccase production ability and dye decolorization. This new discovered laccase can be a good candidate for industrial application.

Conclusions

The isolate WR77 of white rot fungi exhibits very close relation to or being a member of *Polyporous pseudobetulinus*. Among all 30 isolates, this one exhibits highest laccase production. By univariate analysis, it was found that the 1% (w/v) rice chaff, 0.5 g/L di-ammonium tartrate, and 0.01 g/L peptone are the optimal carbon, inorganic, and organic nitrogen composition for laccase production, respectively. In sequential purification with ammonium sulphate precipitation, Q-sepharose anion-exchange chromatography, and Superdex G-75 gel filtration chromatography; it was found that laccase was concentrated with the specific activity of 617.12 U/mg. The enzyme has molecular mass approximately 75.2 kDa, stable at pH 4-11 range with the optimum pH and temperature of 4 and 40°C, respectively. The enzyme possesses relatively highS substrate affinity. Its activity is strongly inhibited by Hg^{2+} and Fe^{2+} ions whereas weakly stimulated by 10 mM Cu^{2+} ions. Moreover, WR77 showed good ability in synthetic dyes (Ambifix Blue H3R, Ambifix Yellow H3R and Ambifix Red HE3B) decolorization which can reduce the dyes from 200 mg/l by 98% in 8 days, 24% in 10 days and 50% in 18 days, respectively. The crude laccase (5 U/ml) can decolorize Ambifix Blue H3R by 65% within 15 min and Malachite Green by 80% within 24 h. Thus, new laccase has been successfully identified and, from this study, it has good potential in applying to decolorize dyes in industrial waste water. Further studies are still needed for extending this enzyme to other applications such as other pollutant eradications and paper bleaching.

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