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ORIGINAL ARTICLE

Optimization of physicochemical parameters and characterization laccase enzyme produced by a novel strain of *Fomes fomentarius* through solid-state fermentation

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Abstract

This present study presents that high yield of laccase (260 ± 1.8 U/mL) were obtained by indigenous fungal strain of *Fomes fomentarius* produced after 10th day of incubation at 35 °C using corn stover as a substrate. Furthermore, laccase was precipitated by 50% saturation with ammonium sulfate having 1.9 purification-fold after dialysis. Purification factor increased up to 2.2-fold (1.65 U/mg specific activity) with Sephadex G-100 gel filtration chromatography. The molecular weight of laccase was 44 kDa when estimated with sodium dodecyl sulfate polyacrylamide gel electrophoresis. Laccase maximum activity (230 ± 3 U/mL) was obtained at pH 7 and increased at 35–40 °C up to (258 ± 2.8 U/mL). The kinetic studies of laccase showed low Km (1 mM) with high Vmax values (60 mM/min). Purified laccase at a concentration of 0.25–1.00 mL/mL of lignin led to 80–90% lignin degradation. Laccase produced from *Fomes fomentarius* shown good decolorization properties (82%). Thus, *Fomes fomentarius* laccases can potentially be utilized in food, textile, pulp, and paper industries and also used in waste decontamination.

Keywords *Fomes fomentarius* · Laccase · Purification · Characterization · Corn stover

1 Introduction

The efficient enzymatic hydrolysis of cellulose, lignin, and hemicellulose is chiefly accredited to a group of fungi called white rotting fungi (WRF). Cellulases, xylanases, and pectinases produced from fungi are mainly induced by cellulose, pectin, and xylose. Lignin is abundant and complex polymer of phenylpropane linked by C–O–C and C–C bonds. These bonds are hydrolyzed by combination of three enzymes: ligninase peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase [1–3]. Laccase (benzenediol: oxygenoxidoreductase) oxidizes different compounds such as methoxy phenols, alkenes, aryl amines, aminophenols, lignin, amino phenols, and polyphen by reducing oxygen into water [4]. This property of laccase has increased their importance in different fields of biotechnology and industrial applications like textile, paper, pulp, and food industries. In this esteem, pulp bleaching and delignification are the utmost significant ones. Recently, they are attracting more consideration for emerging as biosensors for detection and removal of contaminated pollutants, biofuel cells designing, and operational as a medical diagnostic equipment. Besides, these laccases are moreover utilized in soil bioremediation from pesticides along with its types bactericides, fungicides, acaricides, insecticides, herbicides, rodenticides, and particular explosives [5].

Laccase is a tetrameric or dimeric diverse glycoprotein with different structures [6] from different species such as *Cerrena unicolor*, *Lenzites betulina*, *Schizophyllum*

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commune, *Trametes versicolor*, *Flammulina velutipes*, and *Pleurotus ostreatus* [7–9]. Laccase belongs to the multi-copper oxidase family containing four Cu + /2 + centers per monomer distributed in three redox sites. Laccases have the advantage of not requiring H_2O_2 for substrate oxidation like peroxidases and they have a broader substrate spectrum [10]. Although different phenols, including syringaldazine, 2,4,6 tris dimethylaminomethyl phenol, and guaiacol, are typical substrates of laccases, laccases can also oxidize electron donor substrates such as ABTS [5] and this ability of laccase was exploited in the current study. Recently, lignin and cellulose degrading enzyme production has been increased due to utilization of agricultural wastes such as corn cob, bamboos, tree leaves, cottonseed hull, and coffee shells [8].

Developing countries like Pakistan have a major problem of disposing of these wastes and causing environmental pollution. Recently, manganese-dependent peroxidases and polyphenol oxidases have been utilized in bioremediation of industrial wastes, de-lignification, pollutant oxidation, development of biosensors, lignin hydrolysis for bioethanol production, bio-finishing, biofuels, bio-finishing, bio-bleaching, beverage processing, and detergent manufacturing [5, 6]. Solid state fermentation (SSF) process is the most appropriate technique for enzyme production in comparison to submerged fermentation because SSF provides the natural environment for fungal growth [11], because solid-state fermentation (SSF) is economically viable and practically acceptable technology because of the small size of the fermenter, reduced stirring, lower cost of sterilization, and reduced downstream processing [12].

Pakistan produces a huge amount of lignocellulosic wastes like corn cobs and corn stover. SSF using corn stover is an attractive option for enzyme production from fungal cultivation. Industrial importance of laccase makes it possible to design a study for its production, purification, and utilization in industry. *Fomes fomentarius* which secretes laccase in high titers under optimum physical and nutritional conditions was selected for this study.

2 Materials and methods

2.1 Collection of *F. fomentarius* strain and agro-industrial waste

A fine culture of indigenous white rot fungal strain *F. fomentarius* was available in the Department of Biochemistry and Molecular Biology, University of Gujrat, Pakistan, and was utilized in this study. The corn stover was obtained from local fields in Gujrat, Pakistan, and washed, dried in oven at 60 °C, grinded into 0.1 mm mesh size, and finally kept in polythene bags [12]. ABTS was imported from Sigma (Sigma-Aldrich, Germany) and other materials of analytical laboratory scale were utilized in this research.

2.2 Spore suspension development for *F. fomentarius*

For the development of spore suspension of fungus (10^7–10^8 spores/mL), *F. fomentarius* was cultured for 5 days at 30 ± 1 °C in conical flasks (250 mL) in a medium of basal salt solution [13]. Main ingredients of this basal media in 1L were $(NH_4)_2SO_4$ 10 g, KH_2PO_4 4 g, $MgSO_4$ 0.5 g, and $CaCl_2$ 0.5 g. After the preparation of basal media, it was autoclaved and then inoculated with fungal spores in laminar flow hood.

2.3 Laccase production in pre-optimized SSF process

Triplicate flasks contained 10 (g) corn stover and 50% moistened with modified Krik's salt nutrient medium followed by autoclaved, then added 5 mL of newly prepared *F. fomentarius* spores and incubated at 30 °C for 10 days in a static incubator (MIR-254, Sanyo, Japan).

2.4 Laccase extraction

The production of laccase through *F. fomentarius* was done in 250-mL conical flasks using corn stover waste under pre-optimized conditions of growth. These flasks containing spore culture were placed at 30 ± 1 °C in static incubator for 10 days. Extracellular laccase was obtained according to modified method by [9] using 100 mL of D_2O in solid medium (5 days) and set the flask in an incubator shaker at 120 rpm for 30 min. Extracted enzymes were purified using filter paper and then filtrate were centrifuged at 3000 *g* for 10 min at 4 °C to get clear biomass cell debris-free supernatant. Supernatants were used to check laccase activity.

2.5 Laccase activity and protein contents estimation

The extract of crude enzyme was used to check the laccase activity by Han et al. [14] using the UV–Visible spectrophotometric method at 415 nm by measuring the absorbance from the oxidation rate of 1 Mm 2,2 azinobis-[3-ethyltiazoline-6-sulfonate] ABTS in a 50 mM sodium acetate buffer having pH 4.2.

2.6 Fractional precipitation for laccase purification

Crude laccase extract was purified up to a homogenity level by ammonium sulfate precipitation and Sephadex G-100 gel filtration chromatography. The active fractions

of laccase were subjected to $(NH_4)_2SO_4$ precipitations by placing in an ice bath followed by the continuous addition of $(NH_4)_2SO_4$ crystals to obtain 30% capacity and placed overnight at 4 °C. The fraction saturated with 30% ammonium sulfate was stirred at 5000 *g* at 4 °C for 30 min. Finally, to attain 50% ammonium sulfate saturation, more $(NH_4)_2SO_4$ crystals were supplemented in the collected supernatant and kept at 4 °C for 6 (h) followed by centrifugation as described above. The 50 mM sodium malonic acid buffer (2.05 g/500 mL malonic acid and 0.915 g/500 mL sodium malonate, pH 4.5 and pKa 5.05) was used to dissolve the pellets and same buffers were used after sealing well in a dialysis bag (12,000 Da) to remove extra salt and the dialysate was finally freeze dried.

2.7 Gel filtration chromatography

Sephadex G-100 column (2 × 25) was used to obtain active fraction of laccase to enhance purification factor [15]. The 600 µL/run of sample in elution buffer of pH 4.5 with flow rate of 0.31 mL/min was used and around twenty active fractions were obtained and checked for laccase activity.

2.8 Molecular mass determination of laccase

The molecular weight determination of laccase was carried out by running on both native and SDS-PAGE. SDS-PAGE utilized 10% of polyacrylamide to run laccase using the method described by [16], using pre-stained broad range (21–116 kDa) low molecular mass protein markers. After completing the electrophoresis, PAGE was stained with Coomassie Brilliant Blue.

2.9 Purified laccase characterization

Active purified enzyme fraction was assessed for characterization of their pH, temperature, substrate concentration, and activators/inhibitors to check the stability and nature of laccase.

2.10 Characterization of parameters

2.10.1 pH

Change in pH for laccase activity was assessed at 25 °C using various buffers of 50 mM (sodium tartrate buffer of pH 2–3, malonate and succinate buffer of pH 4–5, citrate and sodium phosphate buffer of pH 6–7, sodium phosphate and potassium carbonate buffer of pH 8–9) of varying pH that were used in the study [17]. The corresponding laccase stability towards pH was evaluated by incubating purified laccase for 2 h at different pH before adding it into the assay mixture.

2.10.2 Temperature

Purified laccase thermal stability was checked by changing temperature from 25 to 70 °C with an interval of 5 °C, using ABTS as substrate. Laccase was incubated at different temperatures without substrate before carrying out laccase assay. For stability studies, purified laccase was placed at different temperature before normal activity assay [18].

2.10.3 Determination of kinetic constants Vmax and Km

Enzyme kinetic parameters like maximum velocity (Vmax) and Michaelis–Menten constant (Km) were determined through plotting reciprocal graph substrate concentration versus velocity of reaction after obtaining activity value at 420 nm in triplicate conical flasks by investigating the effect of different concentrations of ABTS as assay substrate in 1–5 mM concentration range.

2.10.4 Activators/inhibitors

The effects of different concentrations (1–5 mM) of metal ions (Cu^{2+}) on purified laccase were assessed through pre-incubating the laccase at 45 °C in the presence of 100 µL of 1 mM activators/inhibitors for 10 min. Purified laccase without any metal ion was used as control, and after incubation, enzyme activity was assessed.

2.11 Lignin determination

Lignin is determined by Klason lignin method [19] in which 0.15 g of stored residue was taken in the conical flask, then add 1.5 mL 72% H_2SO_4 and heated it at 30 °C. Add 42 mL deionized water after 1 h, and autoclaved at 121 °C for 1 h. Cool them, filter, and save both filtrate and residue. Dry the residue in oven and weigh this residue and subtract this residue weight from taken sample that determined the insoluble lignin and checked the filtrate absorbance at 205 nm by a spectrophotometer, and determine the soluble form of lignin by the formula given below

$$A_{SL=}U_{ABS} \times V_{FILTRATE} \times DILUTION/\xi \times Oven\ Dry\ Weight\ (ODW)$$

Table 1 Purification summary for laccase produced by *F. fomentarius*

Sr. No	Purification steps	Total volume (mL)	Total enzyme activity (IU)	Specific activity (U/mg)	Purification (fold)
1	Crude Laccase	100	41400	0.75	1
2	$(NH_4)_2SO_4$ (50% saturation)	15	9230	1.15	1.5
3	Dialysis	10	5250	1.45	1.9
4	Sephadex-G100 gel filtration	7	1792	1.65	2.2

%Lignin ext free = %Acid Insoluble Lignin + %Acid Soluble Lignin

2.12 Decolorization with laccase

Decolorization potential of laccase was checked by the method of Afreen et al. [18]. Two dye solutions Brilliant blue R and Remazol were incubated at 30 °C with pH of 6.0. Samples were checked after every 24 h and absorbance was taken at 495 nm.

2.13 Statistical analysis

All the experiments were performed in triplicates. Standard error and means were calculated for each treatment and used to draw the figures where needed.

3 Results and discussion

3.1 Production of laccase

A higher yield laccase was extracted from indigenous white rotting fungal strain of *F. fomentarius* through solid-state bioprocessing of corn stover under pre-optimized fermentation growth conditions. Maximum laccase activity (260 ± 1.8 U/mL) was obtained when corn stover was inoculated with 5 mL of freshly prepared inoculum of *F. fomentarius* at 35–40 °C. The amount and nature of microelements/nutrients in fermentation growth substrate had a strong effect on extracellular ligninolytic enzyme production. *F. fomentarius* produced laccase activity (260 ± 1.8 U/mL) in 10 days as compared to other reported WRF strains producing significantly lower laccase activities after 10 and 17 days, respectively [20, 21]. It was reported [22] that maximum laccase activity in the crude extract of *Aspergillus* sp. HB-RZ4 was obtained after 9 days of incubation.

3.2 Laccase purification

A purification summary of laccase produced by *F. fomentarius* is shown in Table 1. In this study, three different stages were opted to purify the enzyme from other wastes of polysaccharides, pigments, and proteins. The crude cell-free supernatant laccase solution was analyzed through salting out with $(NH_4)_2SO_4$ fractionation with specific and unit activity of 1.15 U/mg and 9230 U/15 mL, respectively. After Sephadex G-100 gel filtration column, laccase activity maximally refined to homogeneity level with specific activity of 1.65 and purification up to 2.2-fold (Table 1). In an earlier study, [23] reported 50 to 80% ammonium sulfate saturation and chromatographic separation further responsible for the recovery of enzyme.

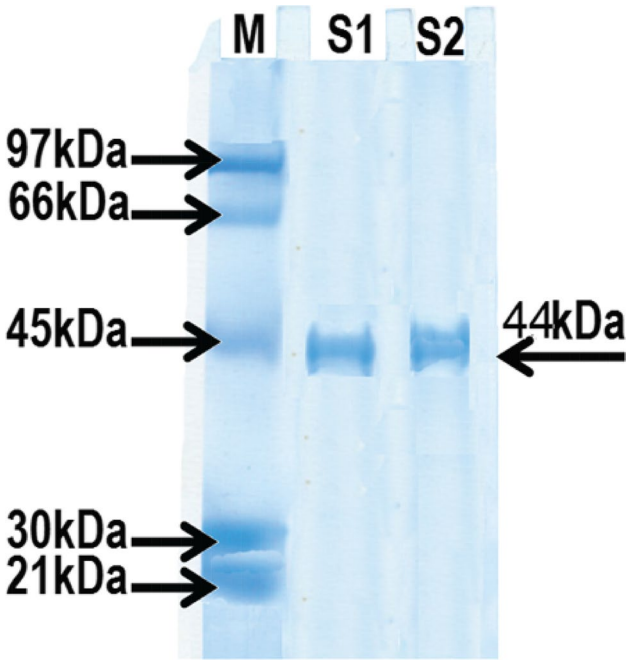

Fig.1 Molecular mass determination of purified *F. fomentarius* laccase by SDS-PAGE. [(Lane M, Molecular weights in kDa of standard marker; lane 1; lane 2, Purified laccase (44 kDa)]

3.3 SDS-PAGE

Characterization of laccase was done with 10% SDS PAGE and laccase was found to be a 44 kDa protein when compared with standard molecular weight markers (Fermentas Ladder SME#21–116 kDa) as shown in Fig. 1. One band over the SDS-PAGE indicated that laccase was a single unit enzyme with molecular

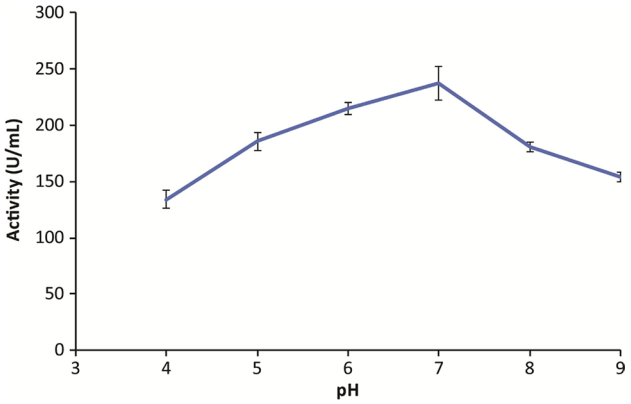

Fig. 2 Effect of different pH on the activity of laccase produced by *F. fomentarius*

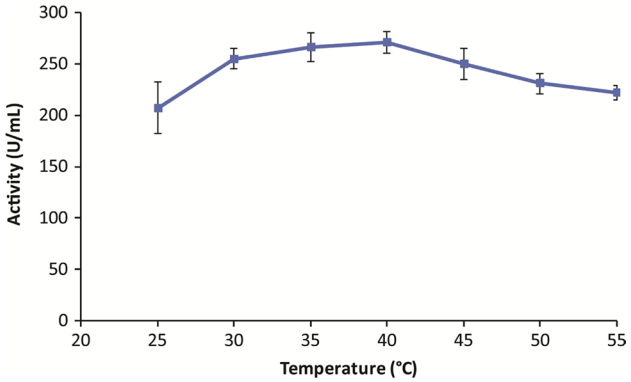

Fig. 3 Effect of different temperatures on the activity of laccase produced by *F. fomentarius*

weight ranges from 40 to 100 kDa when extracted from bacterial and fungi strains [24]. Our results showed that similarity with molecular mass of laccases from *Ganoderma lucidum*-CDBT1 (43 kDa) [25], *Bacillus subtilis* WPI (55 kDa) [26], *Spirulina platensis* CFTRI (66 kDa) [27], *Tricholoma giganteum* AGHP (66 kDa) [28] have a molecular mass very close to that of *F. fomentarius*.

3.4 Characterization

3.4.1 pH

The influence of pH on enzyme activity was checked from pH range of 3–9 and optimum activity of laccase was obtained at pH 7 (230 ± 3 U/mL) for ABTS oxidation produced by *F. fomentarius*. Laccase denatured with further rise in pH as shown in (Fig. 2) laccase which was produced through *Mauginiella* sp. at acidic pH 2.4 was stable and stayed stable at this pH (90%) after 24 h of incubation [29]. Our results showed compatibility with *Bacillus aquimaris* AKRCO2-produced crude laccase extract, and revealed its optimum activity at pH of 7 [30], and more refine comparative results under various ranges of pH were also observed [31, 32].

3.4.2 Temperature

Thermal stability of laccase was observed following the oxidation of 1 mM concentration of ABTS. Purified laccase revealed its maximum activity at 40 °C (258 ± 2.8 U/mL) shown in Fig. 3 which was higher as compared to previously reported results of pH. White rotting fungi produced laccase and revealed laccase activity at various optimum temperature ranges from 40 to 65 °C [33].

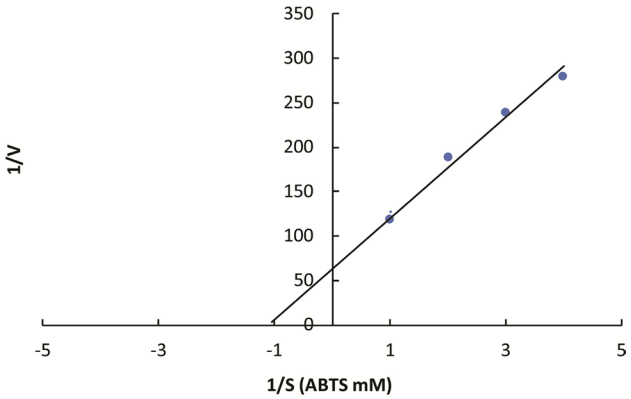

Fig. 4 Reciprocal plot of 1/[S] Vs 1/[V] for determination of K_M and V_{max} of laccase produced by *F. fomentarius*

Our results showed similarity with [34] who reported that laccase produced from *Myrothecium verrucaria* showed maximum activity at 40 °C. Laccase isoforms (LacC1 or LacC2) produced from *Cerrena unicolor* 137 displayed maximum activity at 55 or 50 °C [35] while laccase from *Panus tigrinus* revealed maximum activity at 65 °C with pH 7 [36, 37]. *Trametes trogii* produced two isoforms of laccase and revealed maximum activity at 50 °C [38].

3.4.3 Enzyme kinetics (Vmax and Km)

Maximum velocity (Vmax) and Michaelis–Menten constant (Km) values for purified laccase were analyed by different concentrations of substrate (ABTS). Enzyme activity values were taken at different substrate concentration and a reciprocal graph was drawn between V and S shown in Fig. 4. Purified laccase enzyme revealed Km value (1 mM) and velocity of reaction (60 mM/min) respectively through ABTS substrate. A low Km and high value of Vmax exposed strong affinity of substrate

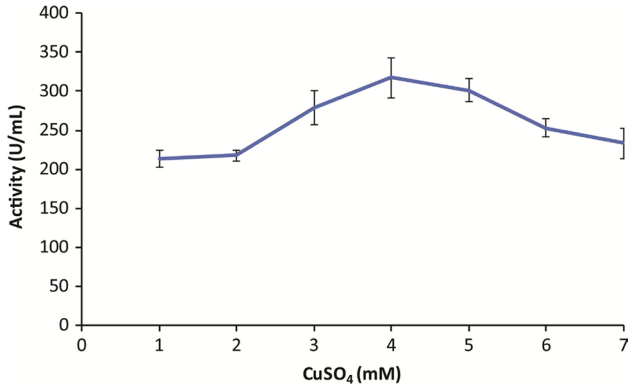

Fig. 5 Effect of activators/inhibitors on purified laccase produced by *F. fomentarius*

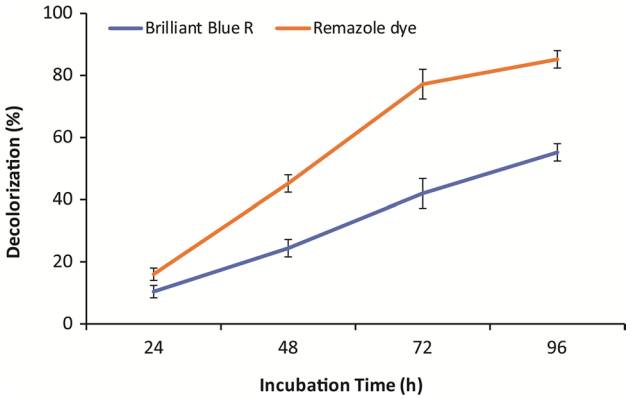

Fig. 7 Decolorization of Dyes with laccase

with enzyme which was found to be similar in case of *Schyzophyllum commune* IBL-06. Very low K_M and high V_{max} values were observed for *Schyzophyllum commune* IBL-06 with respect to *Cerrena maxima* [39] and *Pycnoporus sanguineus* [40].

3.4.4 Activators/inhibitors

Various organic and inorganic compounds are used to check the activity of laccase and it was revealed that 4 M $CuSO_4$ showed maximum activity (Fig. 5). Increase in laccase activity using Cu^{2+} ions revealed that Cu^{2+} was a cofactor of laccase enzyme active sites. Laccase was inhibited in *Sinorhizobium meliloti* CE52G with $CuSO_4$ [41]. However, this result was not applicable to all laccase that EDTA inhibited the enzyme activity like *Sinorhizobium meliloti* CE52G and *Marasmius quercophilus* [42]. Similarly, laccase activity was inhibited with cystein (0.1 mM), EDTA (1 mM), $CuCl_2$ (10 mM), $FeCl_3$(1 mM), reduced glutathione (0.1 mM), and mecaptoethanol (0.1 mM), while laccase revealed maximum inhibition with sodium azide [43–45].

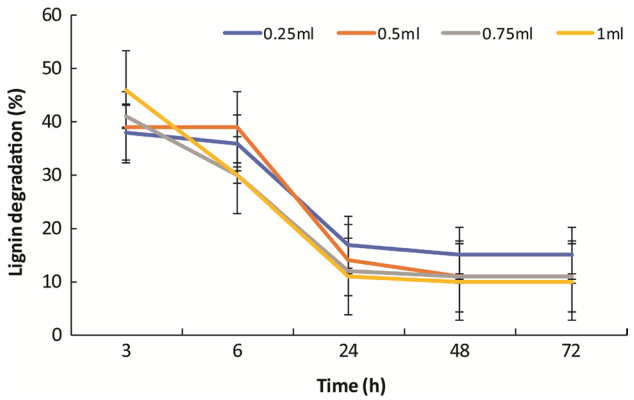

Fig. 6 Lignin degradation percentage of corn stover at various intervals of time and enzyme concentration

3.5 Enzymatic hydrolysis and lignin determination

These purified laccase were further detected for lignin degradation capability of biomass and it showed that at higher concentration of enzyme produced maximum lignin (90.40% ± 0.8) degradation after 48 h. The highest rate of lignin degradation was achieved between 24 and 48 h (90.4% ± 0.8) and above this time period showed that degradation of lignin became constant (Fig. 6).

3.6 Decolorization with laccase

Decolorization potential of laccase was higher with Remazol dye as compare to Brilliant blue R4 dye. Maximum decolorization of laccase was achieved with Remazol dye 82% and Brilliant blue R4 55% shown in Fig. 7. These results showed little similarity with [18] that laccase showed 84% decolorization potential for Reactive Blue 4 dye and 49% for Remazol Brilliant Blue R within 96 h. It was reported [46] that decolorization rate for indigo carmine is 70%, malachite green 48%, crystal violet 33%, and congo red 31% at pH 7.0.

4 Conclusion

Current study revealed that *F. fomentarius* showed maximum yield of laccase (260 ± 1.8 U/mL) using corn stover as a substrate after the 10th day of incubation. Laccase was precipitated with 50% $(NH_4)_2SO_4$ saturation followed by dialysis having purification 1.9-fold with specific activity 1.45 U/mg, then followed by Sephadex G-100 gel filtration column chromatography which increases purification-fold up to 2.2 along with specific activity 1.65 U/mg. The laccase gives maximum activity at temperature 35–40 °C and pH 7. The laccase molecular weight was 44 kDa and

revealed maximum activity for 2,2 azino bis 3 ethyl thiazoline 6 sulfate (ABTS) and oxidization of ABTS exhibited kinetic constants Km of 1 mM with Vmax values of 60 mM/mins. Laccase gives maximum activity at 4 mM Cu^{2+} ions, revealing that Cu^{2+} is a cofactor of laccase enzyme found in their active sites. Laccase is involved in degradation of lignin and decolorization of dyes which makes it a good candidate for biotechnological applications.

Declarations

Conflict of interest The authors declare no competing interests.

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