Comparative study on crude and partially purified laccase from *Polyporus durus* ATCC 26726 in the decolorization of textile dyes and wastewater treatment

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Background and objective
In textile processing, 50% of the dye is released as effluent which is considered as hazardous contaminant that threats environment and human life. The use of laccase enzyme for dye decolorization is cost effective and environmentally friendly. So, the main objective of the present study is the production of microbial laccase with high yield using low-cost substrates. The study also involved comparing and evaluating the dye decolorization efficiency of both crude and partially purified laccase enzymes.
Materials and methods
Six fungal strains were tested for laccase productivity. Some agro-industrial wastes were evaluated for laccase production by the most potent fungus. The produced laccase was partially purified by acetone precipitation. Both the crude and the partially purified laccase were evaluated for decolorization of some textile dyes and two synthetic wastewater solutions.
Results and conclusion
The fungus *Polyporus durus* ATCC 26726 exhibited the highest laccase productivity among all the tested fungi. Maximal enzyme productivity (2297 U/ml) was reached after 7 days by submerged fermentation using a medium containing 60 g/l wheat bran with the addition of 1 mmol/l copper on the fifth day of fermentation. The produced laccase was partially purified by 40% acetone concentration with an 8.1-fold purification factor. Both the crude and the partially purified laccase exhibited high efficiency to decolorize reactive blue 19, acid blue 225, and reactive violet 5 dyes. The crude enzyme exhibited 100 and 96.4% decolorization activities against the synthetic wastewater solutions A and B, respectively. However, the decolorization activity of the partially purified enzyme was 100% against both solutions A and B. Hence laccase enzyme from *P. durus* ATCC 26726 could be used effectively for the removal of textile dye pollutants in both its crude and partially purified form without the need to redox mediators.

Keywords:
agro-industrial wastes, dye decolorization, laccase, *Polyporus durus* ATCC 26726, wastewater treatment

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Introduction

Laccase (EC 1.10.3.2, *p*-benzenediol : oxygen oxidoreductase) catalyzes the oxidation of aromatic compounds (particularly phenol) with the reduction of oxygen to water [1]. Although laccase enzyme is present in many sources including insects, plants, and bacteria, the most important sources are fungi [2]. White rot fungi laccase is required to degrade the lignocellulosic biomass components into low molecular weight compounds that can be easily assimilated for fungi nutrition. Laccase production by these fungi depends on the cultivation conditions by either solid-state fermentation or submerged fermentation [3]. Fungal laccase is utilized in several biotechnological applications due to its higher redox potential compared with laccase from other sources [4]. The use of agro-industrial wastes containing laccase inducers is an efficient strategy to reduce laccase production costs [5].

In textile processing, 50% of the dye is released as effluent [6]. Dyes are considered as hazardous contaminants, which threat environment and human life [7–10]. Various reports have mentioned the toxic effects of those dyes and their metals, which can cause cancers and allergies besides growth inhibition of bacteria, protozoa, algae, and plants [11]. So,

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elimination of color from textile wastewater became a very important target to textile manufacturers.

Most of the dyes are very stable against natural biodegradable metabolisms due to their high biological and chemical oxygen demand, pH, presence of metal and resistance to light, temperature, and microbial attacks [12]. Among the various techniques used for the removal of dyes such as using natural and industrial waste materials to adsorb pollutants, chemical and photochemical methods, the biological treatments are the most common techniques for the elimination of hazardous dyes [13]. Biological treatments for the decolorization of dyes are cost effective and environmentally friendly. These involve the direct use of microorganisms or the use of enzymes produced by the organisms [14–16]. Utilization of laccase enzyme for removing hazardous dyes from the industrial effluents has many advantages including low cost, more effective, and environmentally safe. So, the goal of this study is to produce a high yield of fungal laccase from low-cost substrates. The study also involved comparing and evaluating the dye decolorization efficiency of both crude and partially purified laccase enzymes.

Materials and methods

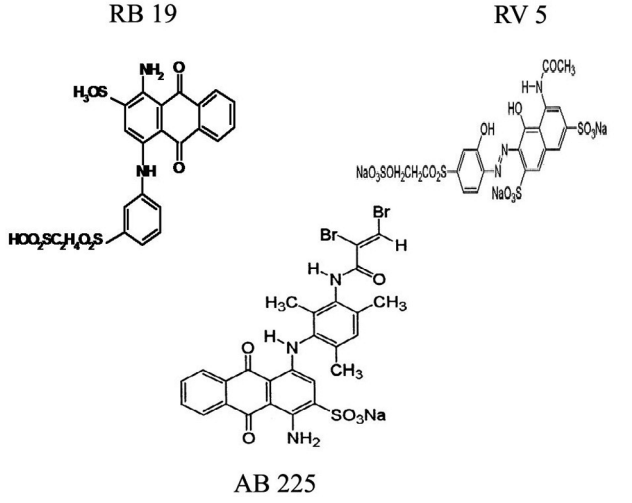
Microorganisms and media

The white rot fungi *Pleurotus ostreatus* (NRRL 3501), *Agaricus subrufescens* (DSM 23611), and *Polyporus durus* (ATCC 26726) were obtained from the Microbiological Resources Center (Faculty of Agriculture, Ain Shams University). *Penicillium pinophilum* (NRRL 1142) and *Penicillium melini* (NRRL 848) were obtained from the Northern Regional Research Laboratory (NRRL), Peoria, Illinois 61604, USA. *Aspergillus niger* was obtained from the Center of Cultures of the National Research Center, Dokki, Cairo, Egypt. The white rot fungi were regenerated on yeast malt agar medium at 30°C for 5 days, and then kept at 4°C. Other fungi were regenerated on potato dextrose agar medium at 30°C for 5 days and kept at 4°C. The basal medium used for laccase production was as follows (g/l): waste material, 60; yeast extract, 3.0; $MgSO_4.7H_2O$, 2.0; KH_2PO_4, 3.0, and glucose, 2.0.

Dyes

The dyes used in this study were Remazol Brilliant blue R (reactive blue 19 or RB 19), Sunzol brilliant violet 5 R (reactive violet 5 or RV 5), and Dystar Supralan blue 2 R (acid blue 225 or AB 225) (Scheme 1). Dyes were purchased from Sigma Chemical Co., USA.

Scheme 1



Chemical structure of dyes.

Solid substrates used in enzyme production media

The waste materials including orange peels, mandarin peels, and banana skin were washed sliced, dried at 70°C, and ground and then used in the media for laccase production. Wheat bran and sawdust were used as they were.

Laccase assay

Qualitative assay

The white rot fungi were screened for laccase production by growing them on yeast malt agar plates containing 0.02% guaiacol. The other fungi were grown on potato dextrose agar plates containing 0.02% guaiacol. The plates were then incubated in dark at 30°C for 7 days. The production of intense brown color under and around the fungal colony was considered as a positive result for guaiacol oxidation [17].

Quantitative assay

Laccase activity was based on the oxidation of the substrate 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) [18]. The reaction mixture contained 600 µl sodium acetate buffer (0.1 M, pH 4.0), 300 µl ABTS (5 mmol/l), 300 µl culture filtrate, and 1400 µl distilled water. The mixture was incubated for 2 min at 30°C. The color was read spectrophotometrically at 420 nm. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmol of ABTS per minute (ε_{420}=36,000 mol/cm).

Effect of some cultural conditions on laccase production

Solid-state fermentation

One slant was scratched with 10 ml distilled water and 2 ml of cell suspension was added to 250 ml Erlenmeyer flask containing 3 g solid substrate (waste material) and 15 ml of sterile production

media. The flasks were incubated at 35°C on a rotary shaker at 150 rpm for 7 days. After that, 25 ml sterile distilled water was added to each flask and left for 1 h on a rotary shaker at 150 rpm. Then the cells were centrifuged at 3000 rpm for 15 min and the supernatant was collected and assayed for laccase activity.

Submerged fermentation

One slant was scratched with 10 ml distilled water and 2 ml of cell suspension was added to 250 ml Erlenmeyer flask containing 3 g solid substrate (waste material) and 50 ml of sterile production media. The flasks were incubated at 35°C on a rotary shaker at 150 rpm for 7 days. After that, the cells were centrifuged at 3000 rpm for 15 min and the supernatant was collected and assayed for laccase activity.

Wheat bran concentration effect

The effect of wheat bran concentration on the production of laccase from *P. durus* was investigated. The submerged fermentation process was conducted for 7 days at 150 rpm using different concentrations of wheat bran in the medium (20–80 g/l).

Fermentation time effect

The effect of fermentation time on laccase productivity from *P. durus* was studied. The submerged fermentation process was conducted at different fermentation times (4, 7, 10, 14 days).

Effect of addition of $CuSO_4$

To investigate the effect of $CuSO_4$ on laccase production, 1 and 2 mmol/l $CuSO_4$ concentrations were added to the fermentation medium at the first and the fifth fermentation day.

Partial purification of laccase

The crude culture filtrate of laccase enzyme was precipitated with acetone at different concentrations (20, 40, 60, and 80%). The fractions were collected and assayed for laccase activity. The most active fraction was used during the study for dye decolorization.

Optimization of parameters for enhanced-reactive blue 19 decolorization

In this part, RB 19 (100 mg dissolved in 1 l of distilled water) was used to evaluate the role of laccase enzyme in its both forms (crude and partially purified forms) in dye decolorization. To improve the decolorization efficiency of crude and partially purified laccase with RB 19 dye, the effects of enzyme concentration, temperature, incubation time, and initial dye concentration were investigated. All experiments were performed in triplicate and the decolorization activity was expressed in terms of percentage as follows:

$$\text{Decolorization activity}(\%) = \left[\frac{(\text{initial dye absorbance} - \text{final dye absorbance})}{\text{initial dye absorbance}}\right] \times 100,$$

where, the initial absorbance at the maximum wavelength for RB 19 dye was 0.028.

Effect of enzyme concentration

A measure of 1 ml of crude laccase with two different concentrations (1×, 2×) and 1 ml of partially purified enzyme with concentrations 1× and 2× each was added separately to 1 ml of the dyestuff solution at pH 4.0. The mixtures were left for 60 min at 60°C. The decrease in dye absorbance for the filtrate was measured at 580 nm using a double-beam spectrophotometer Thermo Electron Corporation Unican 300 (England).

Effect of temperature

In this part, a series of experiments were conducted for decolorization of RB 19 at different temperatures (30, 40, 50, 60, and 70°C) for 60 min at pH 4.0.

Effect of reaction time

The effect of crude and partially purified laccase on RB 19 decolorization was investigated at optimum reaction conditions at different time intervals (30, 60, 90, and 120 min).

Effect of dye concentration

In this experiment, the maximum decolorization activity of laccase enzyme on different concentrations of RB 19 (100–1000 mg/l) was investigated at optimum reaction conditions.

Effect of laccase enzyme on some other dyes

In this part, the ability of both crude and partially purified laccases for the decolorization of other dyes (RV 5 and AB 225) was investigated at optimum conditions.

Enzymatic decolorization of synthetic wastewater

The decolorization activity of both the crude and the partially purified laccase on synthetic wastewater A (100 mg/l RB 19, 100 mg/l AB 225, 90 g/l sodium sulfate, and 20 g/l sodium carbonate) and B (100 mg/l

RB 19, 100 mg/l RV 5, 90 g/l sodium sulfate, and 20 g/l sodium carbonate) was investigated at optimum conditions [19].

Results and discussion

Qualitative assay for some fungal strains for laccase production

By using the agar plate assay, positive results for guaiacol oxidation appeared with *P. ostreatus* and *P. durus* only (Table 1 and Fig. 1) since an intense brown color was appeared under and around the fungal colonies, which indicated the presence of laccase enzyme in these fungi. As reported by Pointing [20], the qualitative assays are powerful tools used in screening fungi for lignocellulose degrading enzyme production.

Effect of some cultural conditions on laccase production

Solid-state fermentation on some waste materials

The laccase positive fungi *P. ostreatus* and *P. durus* were used for laccase production by solid-state fermentation for 7 days using some waste materials such as sawdust, wheat bran, orange peels, mandarin peels, banana skin, and rice straw. As shown in Fig. 2, the maximal laccase productivity (1940 U/gds) was obtained from *P. durus* using wheat bran as a substrate. Lower enzyme yield was obtained from *P. durus* when rice straw and banana skin were used (142 and 117 U/gds, respectively). However, no laccase productivity was observed with sawdust, orange peels, and mandarin peels. On the other hand, maximum laccase production by *P. ostreatus* was also observed with wheat bran (1300 U/gds) and the other waste materials showed no laccase productivity. From these results, the fungus *P. durus* will be used for laccase production during this work.

Solid-state fermentation

Submerged fermentation for laccase production using some waste materials

In this part, the previously used waste materials will be tested in laccase productivity from *P. durus* by using the submerged fermentation technique. The data in Fig. 3 illustrated that wheat bran gave the highest laccase productivity (1806 U/ml) in submerged fermentation. However, a very low enzyme productivity was obtained

Table 1 Qualitative assay for some fungal strains for laccase production

Fungi	Plate test (guaiacol oxidation)
Penicillium pinophilum (NRRL 1142)	Negative
Penicillium melini (NRRL 848)	Negative
Aspergillus niger	Negative
Pleurotus ostreatus (NRRL 3501)	Positive
Polyporus durus (ATCC 26726)	Positive
Agaricus subrufescens (DSM 23611)	Negative

Figure 1

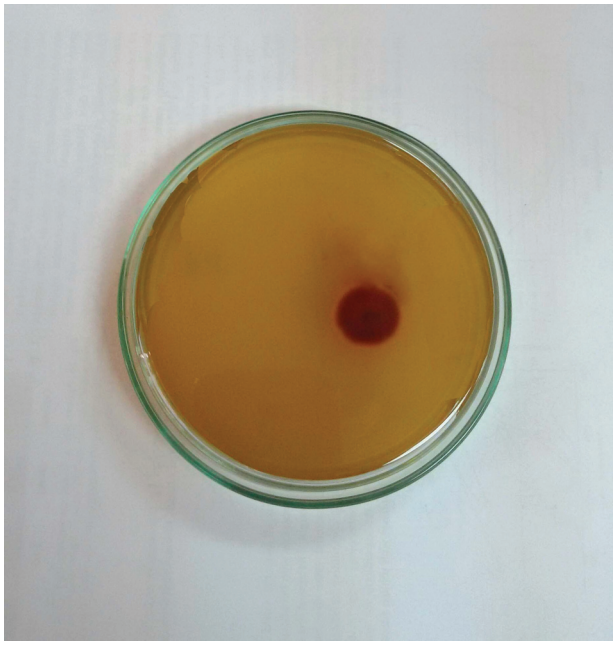
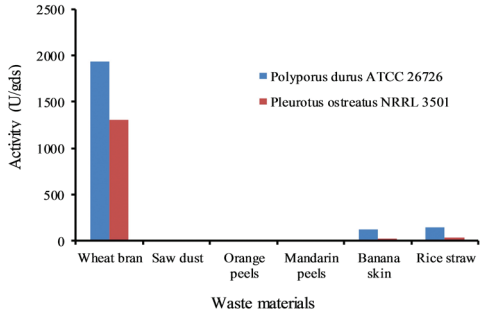


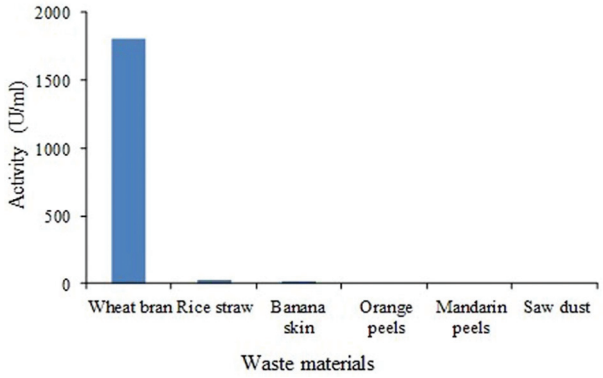
Plate assay of *Polyporus durus* (ATCC 26726).

Figure 2



Laccase production by the positive laccase fungi after 7 days.

Figure 3



Survey on some waste materials for laccase production by *Polyporus durus* (ATCC 26726) by submerged fermentation after 7 days.

with rice straw and banana skin (24 and 15 U/ml, respectively). No laccase productivity was observed with sawdust, orange peels, and mandarin peels in submerged fermentation. Since wheat bran gave the highest laccase yield in both solid-state and submerged fermentation, it will be used throughout this study for laccase production by the submerged fermentation technique because it gave a laccase productivity higher than that of solid-state fermentation. The physical integrity of wheat bran serves as a supporting material and it provides the fungus with an environment similar to its natural habitat, so it enhances fungus growth. Wheat bran also is an abundant source for hydroxycinnamic acids, particularly ferulic, and *p*-coumaric acids which stimulate laccase production [21,22]. Many authors also reported a high laccase yield with wheat bran [3,23–27].

Effect of wheat bran concentration on laccase production by submerged fermentation

The highest laccase production yield (1806 U/ml) was reached at a wheat bran concentration of 60 g/l. At higher concentrations, the media is thicker which might limit the cell growth and enzyme productivity. For this reason, this wheat bran concentration will be favorable for laccase production (Fig. 4). Some authors used higher concentrations of wheat bran for laccase production (100 g/l) [23,25] and other authors used lower wheat bran concentrations (20 g/l [27] and 40 g/l [3], and 50 g/l [26]).

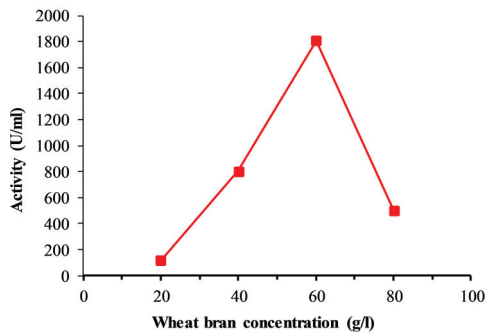
Effect of fermentation time on laccase production by submerged fermentation

When the submerged fermentation was carried out at different times (4, 7, 10, 14 days), it was observed that the maximal laccase production was reached after 7 days of fermentation (Fig. 5). After this period, the enzyme productivity was lowered. This might be due to the cells' digestion by autolysis caused by the proteolytic enzymes in the cells [24] and also due to nutrient depletion in the medium. This high laccase yield in a short period would be advantageous in industrial applications over other fungal laccases which require longer production periods [27]. Many other researchers reached the maximum level of laccase after a longer fermentation period (14 and 20 days) [24,28].

Effect of addition of $CuSO_4$

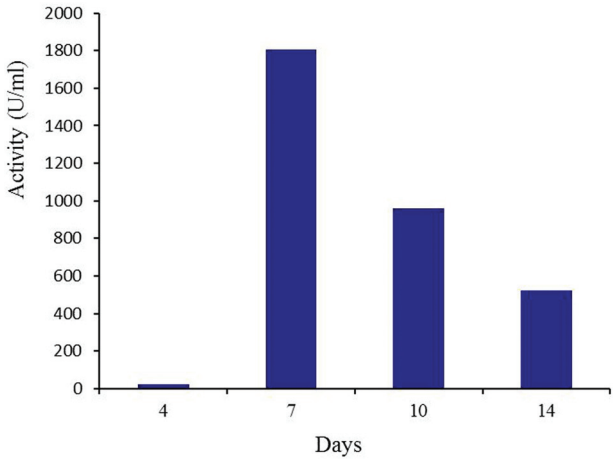
The enzyme activity was increased from 1806 to 2297 Ul/ml when 1 mmol/l $CuSO_4$ was added to the fermentation medium on the fifth day of fermentation. However, the addition of $CuSO_4$ on the first fermentation day led to the inhibition of enzyme productivity (Fig. 6). This might be due to

Figure 4



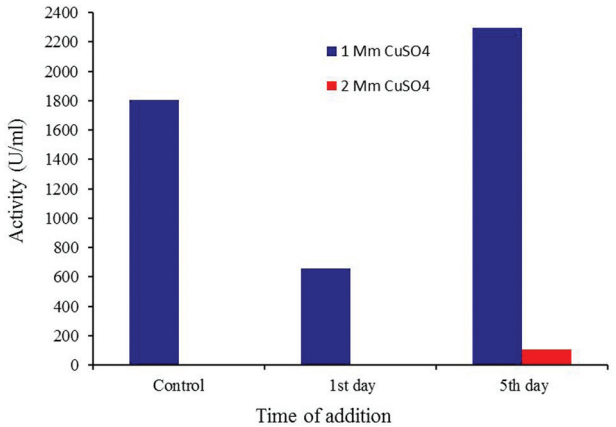
Effect of wheat bran concentration on laccase production by *Polyporus durus* (ATCC 26726) by submerged fermentation after 7 days.

Figure 5



Effect of fermentation time on laccase production from *Polyporus durus* (ATCC 26726) by submerged fermentation.

Figure 6



Effect of $CuSO_4$ in media on laccase production by *Polyporus durus* (ATCC 26726) after 7 days of fermentation.

the suppression of cell growth as previously reported by other authors [24]. On the other hand, addition of 2 mmol/l of $CuSO_4$ on the fifth fermentation day resulted in a considerable decrease in enzyme activity (108 U/ml) compared with the control (1806 U/ml). Other investigators also observed maximum laccase activity with 1 Mm concentration of $CuSO_4$ and reported that both growth and enzyme productivity were suppressed by using higher concentrations of $CuSO_4$ [24,29]. As laccase is a multicopper oxidase, the presence of copper in the medium might allow the synthesis of the enzyme. However, high concentrations of copper were extremely toxic to microbial cells [23,30,31]. The effect of copper on laccase production was previously studied in other white rot fungi (*Trametes versicolor* and *P. ostreatus*) by several authors [23,32].

Partial purification of laccase

Partial purification of laccase by acetone yielded four enzyme fractions (data not shown). The most active fraction was obtained at 40% acetone concentration with an 8.1-fold purification compared with the crude culture filtrate and a specific activity of 6966 U/mg protein. This fraction will be used in comparison with the crude enzyme in the decolorization of some dyes.

Decolorization of reactive blue 19 by crude and partially purified laccase

Synthetic dyes are widely used in textile industries, but they cause serious environmental problems due to their carcinogenic effect [33]. In this part, the effect of the crude and partially purified laccase on synthetic dyes decolorization will be studied. The dye RB 19 had been widely used as a model compound in decolorization studies [34]. Palmieri *et al.* [35] reported that RB 19 decolorization by extracellular laccase was dependent on the reaction media conditions such as temperature, pH, and enzyme concentration.

Effect of enzyme concentration

As indicated in Fig. 7, the lower concentration of the crude enzyme (917 U/mg protein) gave higher decolorization activity (64%) than the high enzyme concentration (21% decolorization). This result is similar to that of Şaşmaz *et al.* [36] who also observed the maximum decolorization activity of Rem Blue RR and Dylon Navy 17 (64.8 and 75.4%, respectively) at low concentrations of crude laccase. On the contrary, the partially purified laccase exhibited the higher decolorization activity (59%) at the higher enzyme concentration. Lower decolorization activity at higher concentrations in case of the crude enzyme might be attributed to the crowding around the enzyme by other proteins in the crude culture filtrate which might delay the decolorization process. However, in the case of the partially purified enzyme increasing the enzyme concentration increased the decolorization efficiency as the proteins are very little and more specific compared with the crude enzyme.

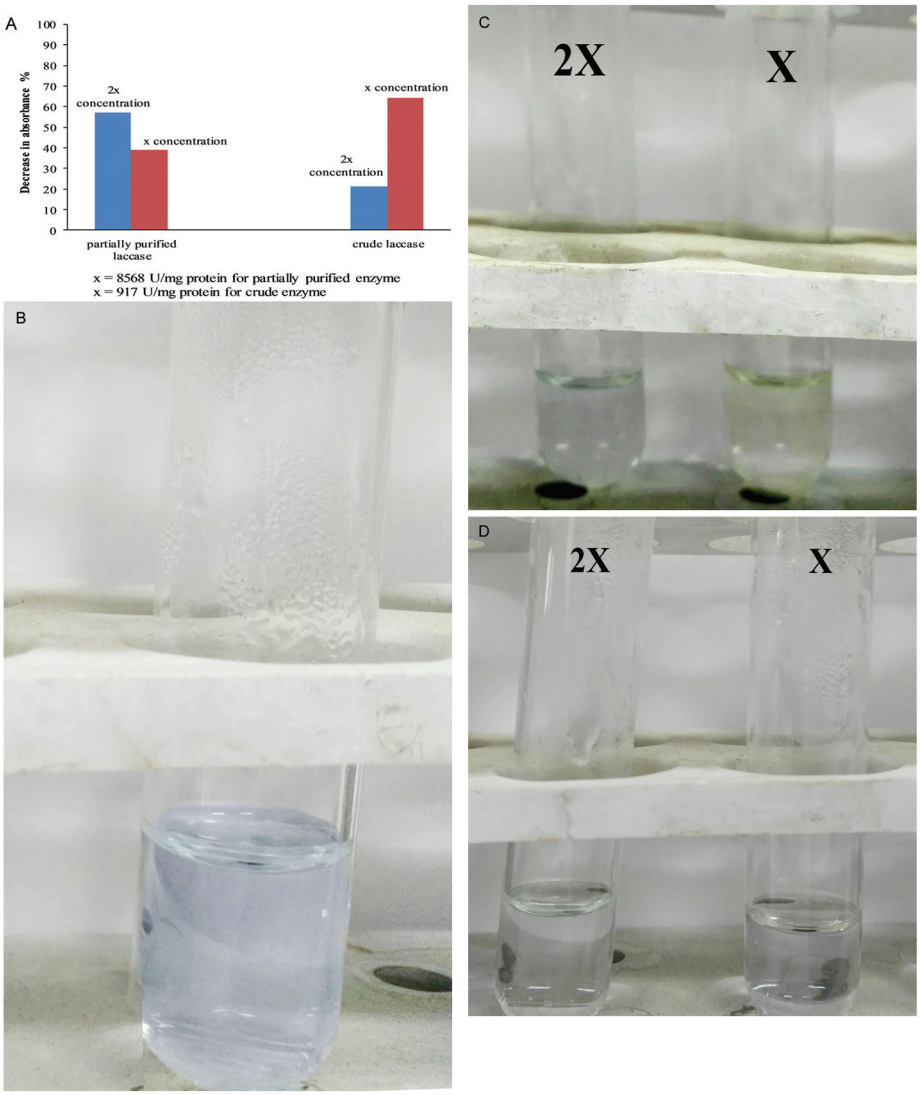
Effect of temperature

The optimum temperature can depend on the type of dye and the strain used to produce laccase enzyme [36]. The temperatures reported in the literature that are used in decolorization reactions vary from 40 to 50°C [37]. In this experiment, the effect of temperature on RB 19 dye decolorization activity of crude and partially purified laccase was investigated at pH 4.0 for 60 min. As indicated in Fig. 8, the maximum decolorization activity of crude laccase (75%) on RB 19 dye was achieved at 40°C. This optimum temperature is similar to that obtained by Kuddus *et al.* [38]. On the other hand, the partially purified enzyme exhibited 100% decolorization activity at 50°C. This result was in agreement with that reported by other researchers who reached the maximum decolorization efficiency on RB 171 and RB 5 at 50°C [19]. Other researchers investigated higher optimum temperature for the decolorization of RB 5 and RBBR by crude laccase (60°C) [39]. However, some researchers reported a lower optimum temperature of 30 and 35°C [36,37].

Effect of reaction time

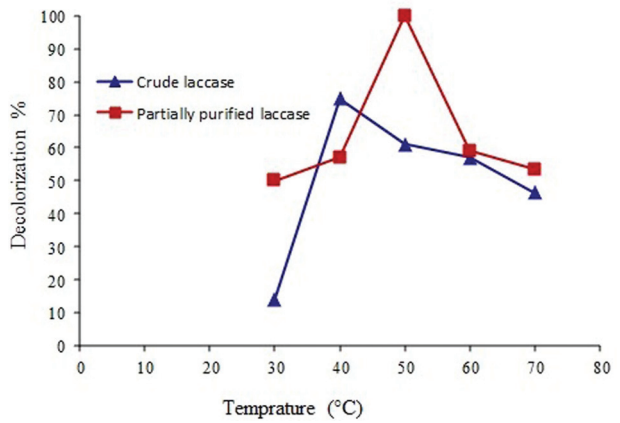
Incubation time was detected as an important factor for the decolorization process [19]. The decolorization activity of crude and partially purified laccase on RB 19 was investigated at the optimum temperature of each enzyme preparation. Figure 9 represents the effect of time on the percentage decrease in dye absorbance. For the crude enzyme, 100% decolorization activity was obtained after 90 min incubation. However, the partially purified laccase exhibited 100% decolorization activity after 30 min only. This may be due to the interruption of the laccase enzyme by other proteins in the crude enzyme solution. This decolorization time for the partially purified laccase is too much shorter than that reported by other authors for laccase from other microorganisms (36–94% decolorization of some dyes within 12–24 h by *Pseudomonas putida* laccase, 40% Congo red decolorization within 240 min by *Cotylidia pannosa* laccase, and 33.7, 57.5% RB 19 after 1 and 24 h, respectively) [27,37,38].

Figure 7



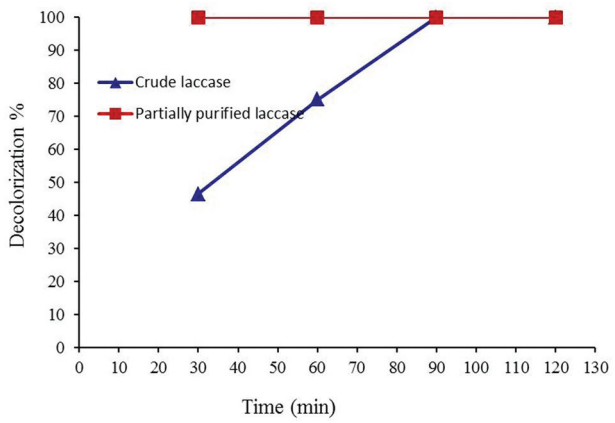
(a) Effect of enzyme concentration on percentage decrease in RB 19 dye absorbance, (b) RB 19 dye, (c) RB 19 dye decolorization by crude laccase with two different concentrations, (d) RB 19 dye decolorization by partially purified laccase with two different concentrations. RB 19, reactive blue 19. The initial absorbance of RB 19=0.028.

Figure 8



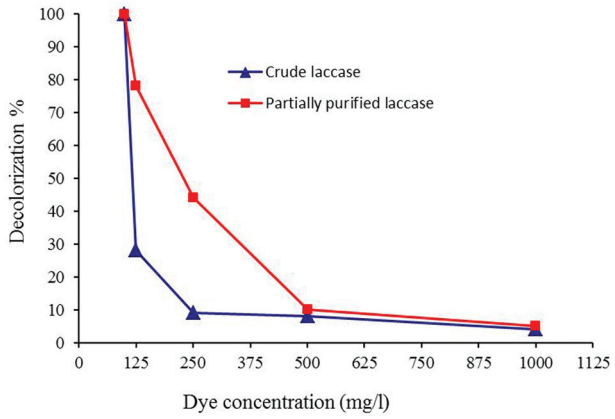
Effect of temperature on reactive blue 19 dye decolorization.

Figure 9



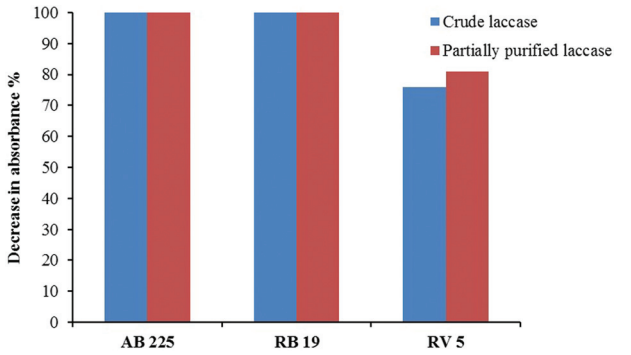
Effect of time on reactive blue 19 dye decolorization.

Figure 10



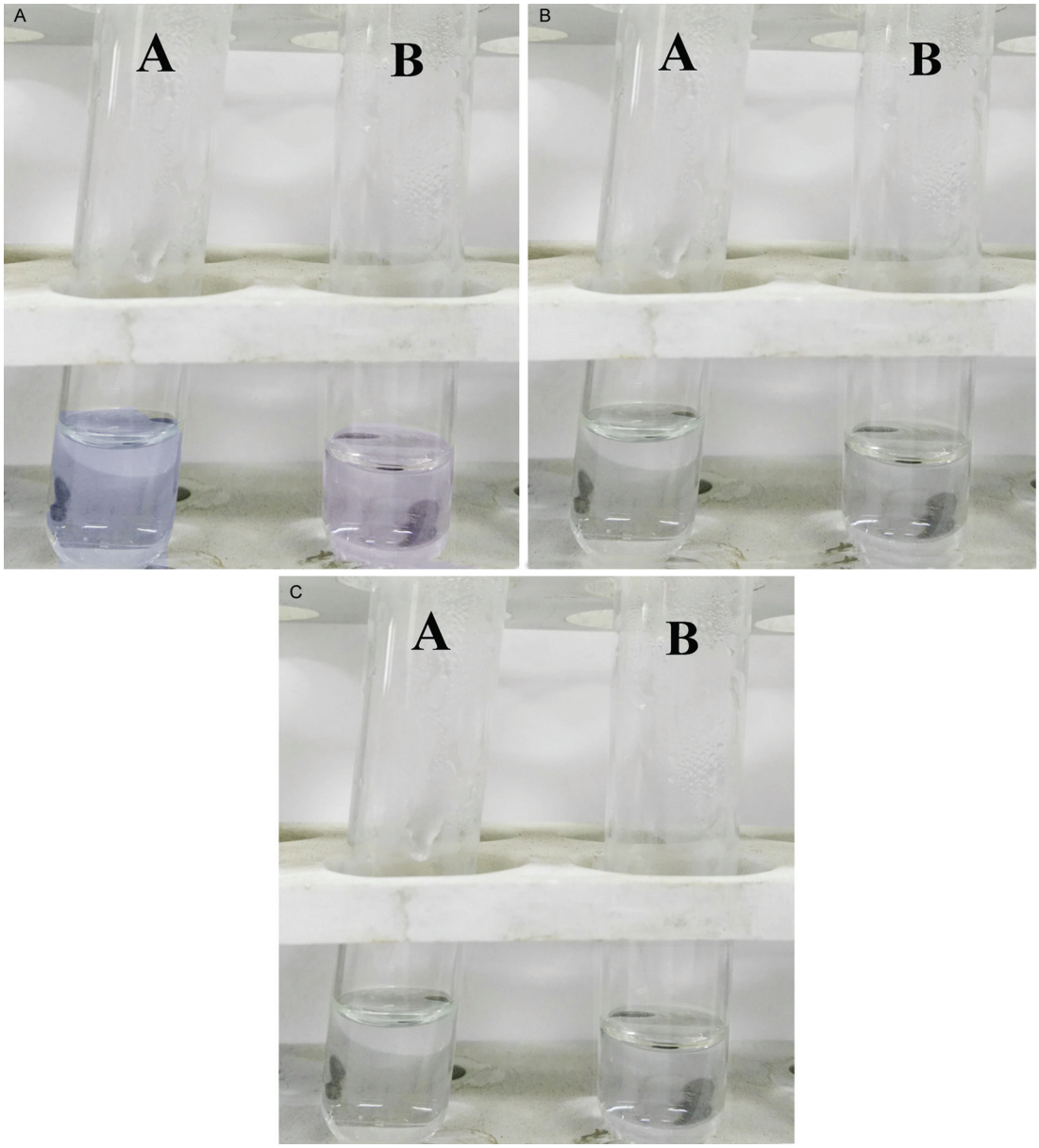
Effect of initial dye concentration on reactive blue 19 dye decolorization.

Figure 11



Effect of laccase on other dyes decolorization. The initial absorbance of AB 225=0.085; RB 19=0.028; and RV 5=0.04.

Figure 12



(a) Synthetic wastewater solutions A and B, (b) enzymatic decolorization of synthetic wastewater solutions by crude laccase, and (c) enzymatic decolorization of synthetic wastewater solutions by partially purified laccase where, the initial absorbance of RB 19=0.028.

Effect of initial dye concentration

The decolorization of dye is dependent on the initial dye concentration and the chemical structure of the dye [36]. The decolorization activity of laccase enzyme on different concentrations of RB 19 (100–1000 mg/l) was investigated at the optimum reaction conditions. The results (Fig. 10) illustrated that the maximum decolorization activity (100%) of both the crude and the partially purified laccase was achieved with a dye concentration of 100 mg/l. As the dye concentration increases, the decolorization efficiency decreases. Many researchers also reported that the percentage of decolorization decreased at higher dye concentrations [39,40]. This might be due to the complete saturation of laccase with dyes at higher concentrations.

Effect of laccase enzyme on decolorization of other dyes

Figure 11 shows the effect of both crude and partially purified laccases on RB 19 and two other dyes (RV 5 and AB 225) at optimum conditions. Both enzyme preparations exhibited 100% decolorization activity with RB 19 and AB 225. However, with RV 5 at the same reaction conditions, the decolorization activities were 76 and 81% for the crude and the partially purified laccase, respectively. The dissimilarities in dye decolorization might be due to the source of enzyme and the structural differences of dyes, especially the position of substituents on the aromatic ring which is known as steric effect, which could prevent the binding of dye molecule in the active site of laccase [36,41].

Enzymatic decolorization of synthetic wastewater

The most currently existing processes for wastewater treatment are ineffective and uneconomical. Therefore, the use of laccases seems an attractive solution due to their ability to degrade dyes of diverse chemical structures [42], including synthetic dyes currently used in the industry [43]. As shown in Fig. 12 both the crude and the partially purified laccase could effectively decolorize the two synthetic wastewater solutions (A and B). The crude enzyme exhibited decolorization activities of 100 and 96.4% against solutions A and B, respectively. On the other hand, the decolorization activity of the partially purified enzyme was 100% against the two solutions A and B. This variation in dye decomposition might be due to the structural differences of dyes in the mixture. This high decolorization efficiency of laccase enzyme in the absence of redox mediators might be due to that some fungi could produce low molecular weight compounds that can act as redox mediators [44]. The decolorization effect of laccase against synthetic wastewaters was previously investigated by other researchers [19,45].

Conclusion

The fungus *P. durus* proved to be a strong producer for laccase enzyme. The use of wheat bran as an agro-industrial waste reduced the production cost which is an important factor in industrial applications. Both the crude and the partially purified laccase from *P. durus* could be used effectively for decolorizing textile dye effluents and wastewater in a short time without the need for redox mediators. However, the partially purified laccase had better decolorization properties than the crude one. Hence, the partially purified laccase from *P. durus* is highly recommended to be used in the environmental biotechnology field for textile pollutants and wastewater treatments.

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Conflicts of interest

There are no conflicts of interest.

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