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Purification, Biochemical Characterization and Applications of *Pleurotus ostreatus* ARC280 Laccase

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Authors' contributions

This work was carried out in collaboration between all authors. All authors contributed, read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To purify, characterize, and apply the laccase produced by submerged fermentation using an edible mushroom *Pleurotus ostreatus* ARC280.
Study Design: Laccase purification and characterization were designed using the most recent approaches and statistical studies of triplicate results values.
Place and Duration of Study: Department of Microbial Chemistry, Genetic Engineering and Biotechnology Division, National Research Centre, Dokki, Cairo, Egypt, between May 2011 and January 2013.
Methodology: *P. ostreatus* ARC280 laccase was purified using ammonium sulfate precipitation (40-80%), followed by gel filtration using Sephadex G100 column chromatography. The resulted pure laccase was analyzed on SDS-PAGE (12%). Laccase activity parameters such as temperature, pH, stability, metal ions and kinetic constants were studied. Laccase was applied to reduce four tumor cell lines growth and as antibacterial and antifungal agent.
Results: *P. ostreatus* ARC280 laccase was purified using ammonium sulphate followed by Sephadex G-100 chromatographic column by about 148 purification fold with Mr of 85kDa. Optimum *P. ostreatus* ARC280 purified laccase activity was recorded at 50ºC and at pH 6.0, 3.0, 4.5 for Syringaldazine (SGZ), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) and 2, 6-dimethoxyphenol (DMP) as substrates, respectively. The purified enzyme

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was more stable in alkaline pH range and retained about 37.42, 73.51, 85.65, 87.7, 88.49, 93.65, 92.86 and 100.0 % of the initial activity after 5hrs of incubation at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, respectively. Hg^{2+} caused complete inhibition at all tested concentrations; however Mn^{2+} (2.5x10-3M) caused laccase activation by about 190 and 330% after 1 and 24 hrs, respectively. K_m and V_{max} were calculated and found to be 0.074, 2.857 and 0.476 µM and 1.563, 2.500 and 2.632 µmol min-1 for SGZ, DMP and ABTS, respectively. The purified enzyme has the ability to reduce four tested cell lines growth in vitro with percentage reduction of 16.8, 23.4, 15.2 and 23.4% for HePG2, HCT116, A549 and MCF7, respectively. On the other hand, the enzyme was found to have antibacterial and antifungal activities against *Escherichia coli and Candida albicans respectively.*
Conclusion: This enzyme seems to be a prospective enzyme for further biotechnological exploitation such as anticancer and antimicrobial activity applications.

Keywords: Applications; characterization; laccase; pleurotus ostreatus; purification.

1. INTRODUCTION

Laccases (E.C. 1.10.3.2) are N-glycosylated multi copper oxidases belonging to the group of the blue copper proteins [1,2]. They catalyze the oxidation of various substrates with concomitant reduction of molecular oxygen to water [3,4]. Laccase activity has been found in plants, insects, bacteria and fungi [4]. Different groups of fungi have been reported as ligninolytic enzymes producers [5]. However, most laccases are found and studied in lignin-degrading basidiomycetes [6,7]. Among the basidiomycetes, white-rot fungi have received great attention due to their powerful production of laccase, Mn-peroxidase and lignin-peroxidase [8,9].

Laccases are multinuclear enzymes [10] and the active site of laccase contains four copper atoms which are distributed in three sites, referred to as T_1, T_2 and T_3 [11]. These copper atoms differ from each other in their paramagnetic resonance signals [12]. The laccase enzyme accepts electrons from substrates and converts them to free radicals. After receiving four electrons, the enzyme donates them to molecular oxygen to form two water molecules [13].

Recently, extensive attention has been focused on the application of laccases in different sectors [14]. The applications of laccases have arisen due to its broad substrate range, and the conversion of substrates to unstable free radicals that may undergo further non-enzymatic reactions [15]. Laccases are found to be used in dyes decolorization, bioremediation and biodegradation, paper and pulp industry, food processing industry, deodorants, toothpastes, mouthwashes and detergents [16,17]. Applications of laccases in the synthesis of complex medicinal compounds as well as heteromolecular dimers of antibiotics via phenolic oxidation [18], phenolic oxidative coupling [19] and oxidation coupled with nuclear amination were reported [20]. Laccases are also involved in enzyme-catalyzed production of anticancer drugs [21]. Antibody or antigen-conjugate laccase can be used as a marker enzyme for immunochemical assays [22]. The present study was aimed to purify and characterize a laccase from *P. ostreatus* ARC280 liquid cultures and to assess its possible antitumoral and antimicrobial activity.

2. MATERIALS AND METHODS

2.1 Microorganisms

P. ostreatus ARC280 was obtained from Agriculture Research Center (ARC), Egypt. A Gram positive bacterial strain *Bacillus mycoides,* a Gram negative bacterial strain *Escherichia coli,* and a non-filamentous fungus *Candida albicans* were selected to test the antimicrobial activity of laccase.

2.2 Media

P. ostreatus ARC280 was maintained and periodically sub-cultured on potato-dextrose agar (PDA). The liquid medium described by Tlecuitl-Beristain et al. [23] was used for laccase production with using soluble starch instead of glucose as a carbon source. The pH value was adjusted to 5.0 before autoclaving, after that the medium was inoculated with *P. ostreatus* ARC280 and incubated statically at 28ºC for 26 days, after that the filtrated medium was used as a source of enzyme for purification. *B. mycoides, E. coli* and *C. albicans* were cultivated and kept on slants of modified nutrient agar medium (g/l): peptone, 3.0; yeast extract, 1.5; meat extract, 1.5; glucose, 0.5; NaCl, 0.25 and agar, 20.0. The pH value was adjusted to 7.0 before autoclaving.

2.3 Chemicals

Laccase substrates were supplied by Sigma-Aldrich; USA: SGZ, Aldrich W404901; ABTS, Sigma A1888 and DMP, Sigma D135550. All chemicals used in this study were of analytical grade.

2.4 Laccase Assay

The enzyme activity was assayed by monitoring the rate of SGZ oxidation spectrophotometrically at 525 nm [24]. The reaction mixture (2.0 ml) contained SGZ, 0.1 µmole; citrate-phosphate buffer (pH 6.0), 90 µmoles and appropriate volume of diluted enzyme. Activity unit was defined as the change in the absorbance of 0.001/sec [7,25]. In the experiments of optimum pH and substrate specificity, the activity was determined using: 1 mM SGZ [4,24], 5mM ABTS [2, 26] and 5mM DMP [27,28]. The enzyme activity was calculated according to the method described by Annuar *et al*. [29] as follows:

$$\text{Laccase activity (Units/L)} = \frac{\Delta Abs}{\Delta t\ \varepsilon\ l} \times \frac{\text{Total assay volume}}{\text{Enzyme sample volume}}$$

Where Δt is the time of incubation (min), ΔAbs is the change in absorbance, ε is the extinction coefficient of substrates (ε_{525} = 65,000 for SGZ; ε_{436} = 36,000 for ABTS and ε_{469} = 49,600 for DMP) in units of M^{-1} cm^{-1}), and l is the cuvette diameter (1cm). Enzyme activity (Unit) was defined as the amount of enzyme that oxidized 1µmol of substrate/min. The oxidation rates were determined at room temperature (25ºC±2) in triplicate. The protein in the crude enzyme preparations was determined by the modified procedure of Lowry et al. [30]. Determination of protein in the pure enzyme preparations was made according to Layne [31] using the following equation:

$$\text{Protein (mg/ml)} = 1.55 \times A_{280} - 0.76 \times A_{260}$$

All the data was statistically evaluated according to the method described by Kenney and Keeping [32], where the means and standard deviations (Mean ± S.D.) were calculated for each experiment.

2.5 Purification of *P. ostreatus* ARC280 Laccase

Ammonium sulphate (40-80%) was used to precipitate the laccase protein produced by *P. ostreatus* ARC280 after 26 days of incubation. The precipitate was separated from the filtrate by centrifugation at 12000 rpm for 15 min in a pre-cooled rotor (HERMLE Z 323 K cooling centrifuge) at 0°C. The precipitated protein was dissolved in citrate-phosphate buffer (0.05M, pH 6.0),dialyzed and then applied to Sephadex G-100 column chromatography (3.0×26 cm) which had been equilibrated with 0.05 M citrate-phosphate buffer (pH 6.0). Five milliliters fractions were collected at a flow rate of 0.5ml/min.

2.6 Electrophoretic Analysis (SDS-PAGE)

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the development of the purification process and to determine the apparent molecular mass of the laccase enzyme. SDS-PAGE was performed with 12% polyacrylamide according to Laemmli [33]. Proteins were visualized by staining for 3 hours with Coomassie Brilliant Blue-R250. Gel was then destained with a mixture of acetic acid and ethanol (40%: 10%). Diluted protein samples were concentrated by the use of a lyophilizer (Snijders Scientific b.v., L45FM – RB, Tilburg - Holland). Low molecular weight markers; phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa) were used.

2.7 Effect of Temperature and Thermal Stability

The dependence of the activity of the purified enzyme on temperature had been assessed at different incubation temperatures (10, 20, 30, 40, 50, 60, 70 and 80ºC) at pH 6.0. For thermal stability behavior, similar aliquots of the purified enzyme were first incubated separately at different temperatures (30, 40, 50, 60, 70 and 80ºC) for different incubation periods. The residual enzyme activity was then determined by incubating each preheated enzyme aliquot with the remaining components of the standard reaction mixture, containing 0.1µmole of SGZ as the substrate.

2.8 Effect of pH Value and pH Stability

The effect of pH on the activity of the purified laccase of *P. ostreatus* ARC280 was examined using different pH values of 0.1M citrate-phosphate buffer (pH 2.6 – 7.0) with SGZ, DMP or ABTS individually as a substrate. In connection to pH stability, aliquots of the pure laccase were incubated with different buffer systems (0.1M) at different pH values ranging from pH 3.0 to 10.0 (citrate phosphate buffer (3.0–7.0); phosphate buffer (8.0) and bicarbonate buffer (9.0–10.0) for different incubation periods up to 28 h. Identical aliquots of enzyme were removed at different time intervals and assayed for residual laccase activity using SGZ as a substrate at pH 6.0.

2.9 Effect of EDTA and Different Metal salts

EDTA and different metal salts of various cations (Na^+, K^+, Mn^{2+}, Hg^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}) were added separately at a final concentrations of 2.5×10^{-3}, 5×10^{-3}, 1×10^{-2} and 5×10^{-2}M to each reaction mixture containing purified laccase. The purified enzyme was incubated with EDTA or metal salts and buffer at room temperature for different time intervals, and then SGZ was added as substrate to each reaction mixture and assayed for laccase activity using the standard assay method.

2.10 Substrate Specificity and Kinetic Constants

The activity of *P. ostreatus* ARC280 purified laccase was examined towards three different substrates (SGZ, DMP and ABTS) by incubating the purified enzyme with each individual substrate at concentrations of 1mM SGZ [24] at pH 6.0, 5mM DMP [28] at pH 4.5 and 5mM ABTS [26] at pH 3.0. The enzyme activity was assayed by the method described by Annuar et al. [29]. Kinetic constants such as K_m and V_{max} were determined using various concentrations of different substrates (SGZ, DMP and ABTS). The V_{max} values were calculated from the dependence of the activity on the concentration of the substrate and the K_m values were calculated from Lineweaver-Burk plot.

2.11 Cytotoxic Effect of Laccase on Human Tumor Cell Lines

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan [34]. Cells were suspended in RPMI 1640 medium for HePG2 (Human hepatocellular carcinoma cell line), HCT116 (Colon cell line) and MCF7 (Human caucasian breast adenocarcinoma); and DMEM for A549 (Lung carcinoma cell line). The media were supplemented with 1% antibiotic-antimycotic mixture (10,000Units/ml potassium penicillin, 10,000µg/ml streptomycin sulfate and 25µg/ml amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37ºC under 5% CO_2. Cells were batch-cultured for 10 days, then seeded at a concentration of 1×10^3 cells/well in a fresh growth medium in microtiter plastic plates at 37ºC for 24h under 5% CO_2 [35]. Media was aspirated and the cells were incubated either alone (negative control) or with different concentrations of sample. After 48h of incubation, medium was aspirated, 40 µl MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37ºC under 5% CO_2. Two hundred µl of 10% sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37ºC, to stop the reaction and dissolving the formed crystals. A positive control (Adrinamycin (Doxorubicin)) [Mw= 579.99] which composed of 100µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions [34]. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350) at 595 nm and a reference wavelength of 620nm. The percentage of change in viability was calculated according to the formula:

((Reading of extract / Reading of negative control) -1) × 100

2.12 Antimicrobial Activities

B. mycoides, E. coli, and *C. albicans* were used as model microorganisms. Cultures were prepared by inoculation of nutrient agar medium with 100µl of re-suspended overnight culture at 37ºC (1×10^7 CFU/100µl). The nutrient agar medium was poured in Petri dishes,

solidified, then wells in solidified agar plates were made and equal units of the tested laccase preparations (1.5 Units/ml) were inserted in each well, then plates were incubated at 37ºC for 7h [36].

3. RESULTS AND DISCUSSION

3.1 Purification of *P. ostreatus* ARC280 Laccase

P. ostreatus ARC280 crude laccase with 464.67U/ml, as we described in our previous work [2]**,** underwent fractional precipitation with ammonium sulphate within 40-80% saturation with a purification fold of 6.34 and a recovery of 80.63% (Table 1). The laccase obtained from ammonium sulphate precipitation was applied to Sephadex G-100 chromatographic column. Laccase from *P. ostreatus* ARC280 was successfully well purified using Sephadex G-100 column (Fig. 1). Optimum purification fold (147.74) was obtained with fraction F_{13} with a recovery percentage of 11.82% (Table 1).

Table 1. Purification of *P. ostreatus* ARC280 laccase

Fraction	Total protein (mg)	Laccase activity			
		Total units	Specific activity (Units/mg protein)	Recovery (%)	Purification fold
Crude	137.50	24250.00	176.36	100.00	1.00
$(NH_4)_2SO_4$ (40-80%)	17.50	19553.33	1117.33	80.63	6.34
Sephadex G-100 column					
F_{12}	0.17	2906.57	17097.47	11.99	96.95
F_{13}	0.11	2866.14	26055.82	11.82	147.74
F_{14}	0.11	2476.64	22514.19	10.21	127.66

In this connection, Patel et al. [37] purified the laccase produced from *P. ostreatus* HP-1 with DEAE-sepharose column to obtain13.13-fold purification with 77.63% yield of laccase enzyme. Palmieri et al. [38] achieved 85-fold purification from *P. ostreatus* POXA1 laccase isoenzyme with a final yield of 23%, and a lower yield for POXA2 isoenzyme. Murugesan et al. [39] purified laccase from *P. sajor-caju* using ammonium sulfate (70% w/v), DEAE-cellulose, and Sephadex G-100 column chromatography with an overall yield of 53% and a 10.3 purification fold. Adamafio et al. [40] purified laccase from *P. ostreatus* strain EM-1 using ammonium sulphate precipitation and gel filtration using Sephadex G-75 with a purification fold of 12.7 and a recovery yield of 21%.

3.2 SDS-PAGE Analysis

SDS-PAGE analysis indicated that the protein isolated from *P. ostreatus* ARC280 is a monomer in solution with a molecular mass of 85kDa (data not shown) which is similar to other fungal laccases. The molecular masses of *P. ostreatus* strain V-184 laccases LCC3 and LCC4 were around 80 and 82kDa, respectively [41]. In this connection, many investigators [42,1,43] reported the purification of laccase from *Scytalidium thermophilum, Paraconiothyrium variabile* and *Trichoderma harzianum* with molecular masses of 82, 84 and 79kDa, respectively. In contrast, Asgher et al. [44] and Xu et al. [45] purified laccases from *Trametes versicolor* and *Lentinus tigrinus* with relative molecular masses of 63 and 59 kDa, respectively. In this connection, *P. ostreatus* strain V-184 laccases LCC1 and LCC2 have molecular masses of about 60 and 65 kDa, respectively, [41]. The purified laccase from

P. ostreatus HP-1 and *P. ostreatus* D1 were shown to be with relative molecular masses of 68kDa and 64kDa, respectively [37,46].

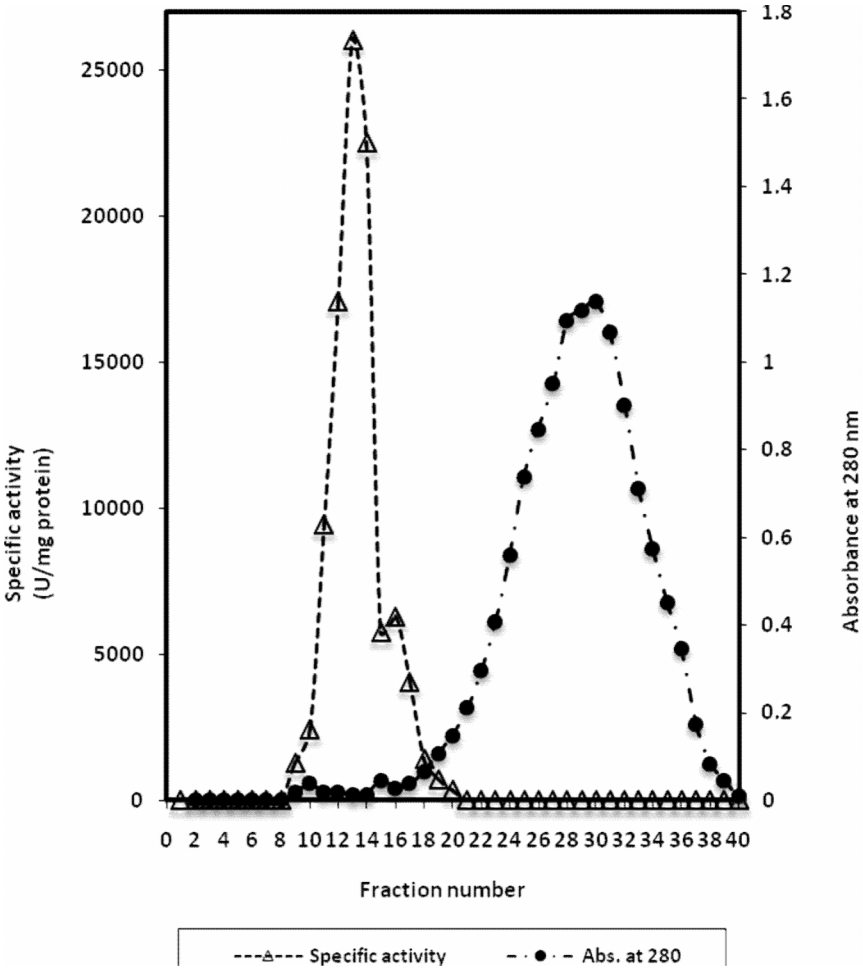


Fig. 1. Gel filtration of ammonium sulphate partially purified laccase fraction (40-80%) of *P. ostreatus* ARC280 using G-100 Sephadex column

3.3 Effect of Temperature on Laccase Activity and Stability

Tinoco et al. [47], stated that the temperature profile for all *P. ostreatus* laccases showed maximal activity between 30 and 40ºC, and the activity was drastically reduced when the reaction temperature was raised to 60ºC. In the present study, *P. ostreatus* ARC280 purified laccase displayed a maximum activity at 50ºC (Fig. 2), which is similar to the optimum temperature determined by Forootanfar et al. [1] for laccase from *Paraconiothyrium variabile, P. ostreatus* HP-1, *P. ostreatus* strain EM-1 and *P. ostreatus* [37,40,48]. On the other hand, optimum activity of the purified laccase from *Hericium coralloides* and *Trametes versicolor* IBL-04 could be detected at 40ºC [44,49], whereas the optimum activity for laccase from *Trametes hirsuta* Bm-2 is ranged from 40 to 60°C [50] and the laccase from *P. ostreatus* HP-1 was active in the temperature range of 40–70ºC [37]. In other approaches, maximal

laccase activity was observed at temperatures of 60 and 80ºC for laccase from the mushroom *Lentinus tigrinus* [45] and LacIII isozyme from the white rot fungus *Trametes* sp. HS-03 [51], respectively. Palmieri et al. [38] found that the *P. ostreatus* laccase isoenzyme POXA1 showed maximal activity in the range 45–65ºC, whereas POXC showed maximal activity in a narrower range (50 – 60ºC) and POXA2, at a lower temperature (25–35ºC).

Depending on the source of the enzyme, thermal stability varied at different temperatures. In general, typical fungal laccases exhibited a half-life of 1 h at 70ºC and below 10 min at 80ºC [43]. The present studies revealed that the *P. ostreatus* ARC280 purified laccase could sustain heating up to 30 without apparent loss of activity for 120 min, and the residual activity was regularly decreased as a function of both time of exposure and temperature. Incubating the enzyme at 40, 50 and 60ºC for 120min resulted in a loss of about 4, 20 and 40% of its activity, respectively (Fig. 3). These results indicated that the enzyme under investigation is more stable than *P. ostreatus* strain EM-1 [40] and *Paraconiothyrium variabile* [1] laccases as they retained only 22.6 and 50% of their initial activity after 60 min of incubation at 50ºC respectively, and it resembles laccase isolated from *Streptomyces cyaneus* which is reported to have retained more than 75% of its activity after incubation for 120min at 50ºC [52]. Luna et al. [53], stated that the laccase from *P. ostreatus* showed greater stability at 39ºC and pH 6, with value of residual activity of 27.5% after 15 hours of incubation. Laccase from *P. ostreatus* HP-1 was found stable for 10min at 50ºC and considerable loss of activity was observed after 12 min. In the initial 15 min of incubation, the half of the laccase activity was found to be lost at 60°C, whereas rapid inactivation of the enzyme was observed at 70ºC [37].

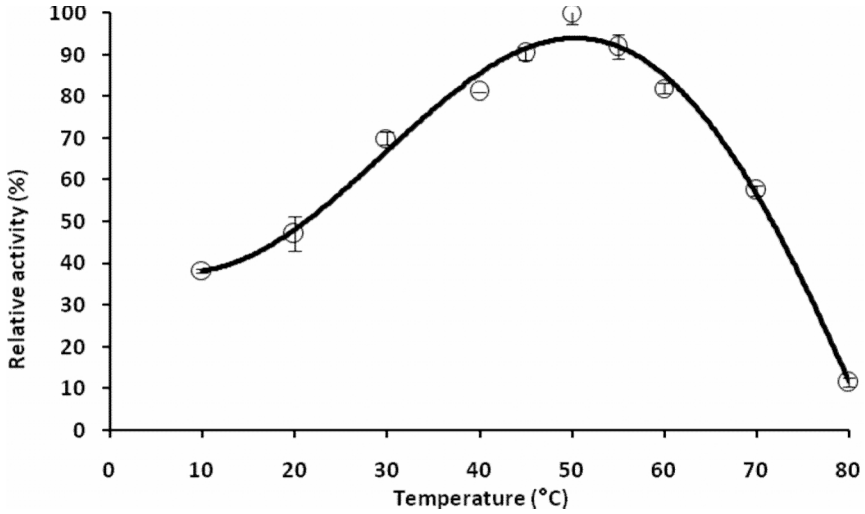


Fig. 2. Effect of reaction temperature on the activity of *P. ostreatus* ARC280 purified laccase

Reaction mixture contained: SGZ, 0.1 µmole; purified enzyme, 1.0µg protein; citrate-phosphate buffer pH 6.0, 90 µmoles; temperature, as indicated; total volume, 2ml. Values are means of three replications ± standard deviation

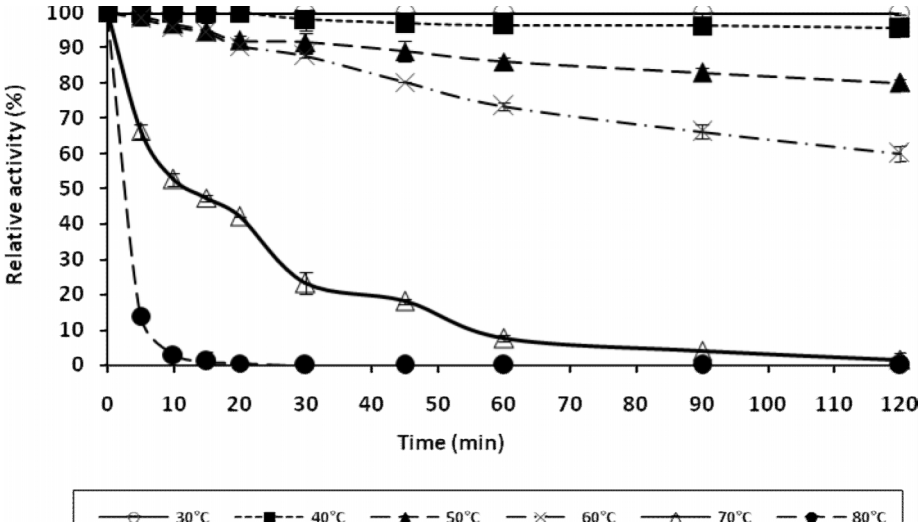


Fig. 3. Thermal stability behavior of the *P. ostreatus* ARC280 purified laccase

Reaction mixture contained: SGZ, 0.1 µmole; purified enzyme, 3.0 µg protein; citrate-phosphate buffer pH 6.0, 90 µmoles; time of exposure of the enzyme at different degrees of temperature, as indicated; total volume, 2ml. Values are means of three replications ± standard deviation

3.4 Effect of pH Value on Laccase Activity and Stability

The pH optima for laccases can vary depending on the substrate used and on its redox potential [54]. Fungal laccases are usually stable at acidic pH, although pH stability varies considerably depending on the source of the enzyme [55]. In the present study, the effect of pH on the activity of the purified laccase of *P. ostreatus* ARC280 was examined using SGZ, DMP or ABTS as substrates. The obtained results showed that, the optimum activity was recorded at pH 6.0, 4.5 and 3.0 for SGZ, DMP and ABTS, respectively (Fig. 4). In agreement with these results, Schückel et al. [56] reported that, the highest laccase activity for the oxidation of ABTS, SGZ, guaiacol and DMP was found at pH 3.0, 6.0, 5.5 and 4.0, respectively. The pH profile of *P. ostreatus* HP-1 laccase showed maximum activity at pH 4.5 when ABTS was used as a substrate [37]. The results on pH optima are consistent with previous reports that suggest that this value depends mainly on the substrates used. The optimal pH range for fungal laccases activities on substrates like DMP, guaiacol and syringaldazine, were found to be between 4.0 and 7.0 [55,57]. Patrick et al. [58] stated that, the pH profile is the result of two opposing effects: The first effect is due to the redox potential difference between a reducing substrate (phenolic compound) and the Type 1 copper center of laccase, where the electron transfer rate is favored for phenolic substrates at a high pH. The second effect is generated by the binding of a hydroxide anion to the type 2/Type 3 copper centers of laccase, which inhibits the binding of O_2, the terminal electron acceptor, and therefore inhibits the activity at a higher pH because of the increased amount of OH^- ions [59].

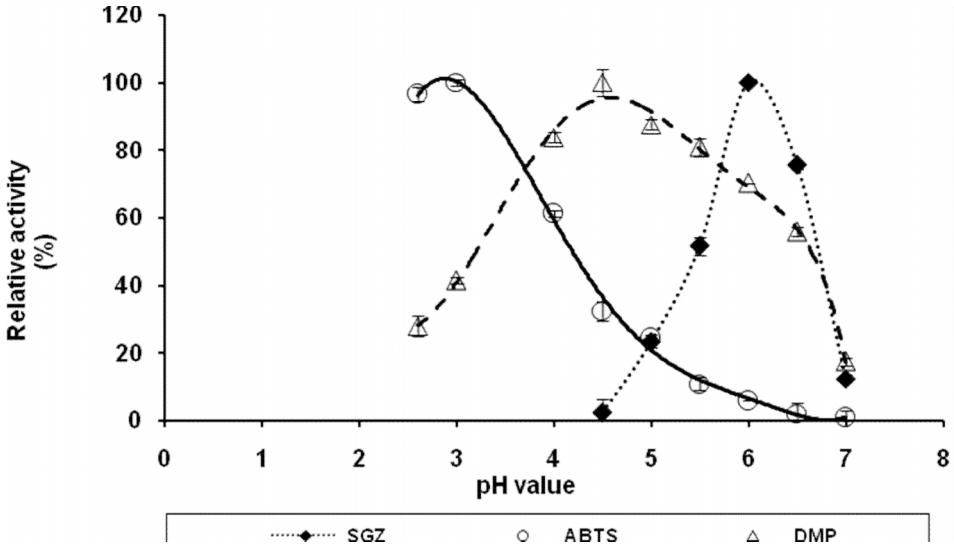


Fig. 4. Effect of pH value on the activity of the *P. ostreatus* ARC280 purified laccase

Reaction mixture contained: substrate (SGZ, 0.1 µmole; DMP, 0.5 µmole or ABTS, 0.5 µmole); purified enzyme, 1.0 µg protein; citrate-phosphate buffer pH (as indicated), 90 µmoles; total volume, 2ml. Values are means of three replications ± standard deviation

The results obtained, indicated that the purified laccase from *P. ostreatus* ARC280 is stable at different pH values and more stable at alkaline range and it retains about 37.42, 73.51, 85.65, 87.70, 88.49, 93.65, 92.86 and 100.00 % of the initial activity after 5 h of incubation at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, on the other hand, the enzyme retains about 11.97, 37.84, 55.74, 65.87, 70.63, 76.19, 88.98 and 82.54 % after incubation for 28h at the same pH values, respectively (Fig. 5). In contrast, Guo et al. [51] found that, LacI, LacII, and LacIII isozymes from the white rot fungus *Trametes sp.* HS-03 had considerable stability behavior at pHs of 2.5 to 4.5, 3.5 to 5.5 and 2.0 to 4.5, respectively, after incubation for 4 h, and at pH values higher than 7.0, all the laccase isozymes lost their activity very rapidly. Luna et al. [53], stated that the laccase from *P. ostreatus* showed greater stability at pH 7, with value of residual activity of 42.1% after 15 hours of incubation.

3.5 Effect of the Nature of Buffering System

Concerning the nature of the buffer systems, the results obtained indicated that the optimum laccase activity of *P. ostreatus* ARC280 was recorded with citrate-phosphate buffer system (406.67U/ml), followed by citrate buffer (331.67U/ml), succinate buffer (325.00U/ml) and maleate buffer (260.00U/ml). The lowest laccase activity could be detected with phthalate-NaOH buffering system (228.33U/ml).

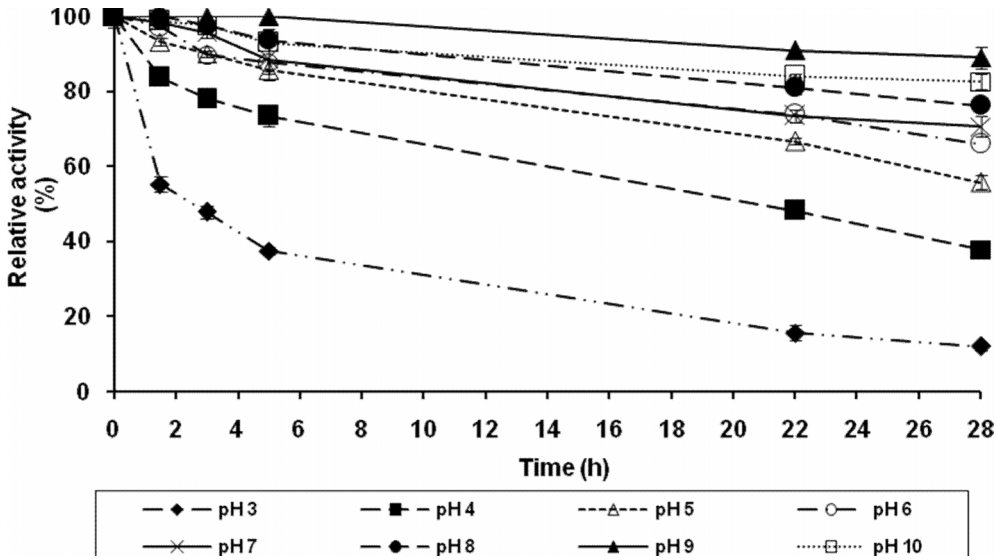


Fig. 5. pH stability of the *P. ostreatus* ARC280 purified laccase

Reaction mixture contained: SGZ, 0.1 µmole; purified enzyme, 1.0µg protein; buffers (citrate phosphate buffer (pH 3.0 - 7.0), phosphate buffer (pH 8.0) and bi-carbonate buffer (pH 9.0 - 10.0)), 90 µmoles; incubation time of the enzyme in the absence of substrate, as indicated; total volume, 2 ml. Values are means of three replications ± standard deviation

3.6 Effect of EDTA and Different Metal Salts

Chelating agent EDTA inhibited the enzyme only at higher concentrations which was in accordance with the earlier studies by other researchers [37]. Results obtained in this study indicated that the addition of EDTA at concentrations of 2.5×10^{-3}, 5×10^{-3}, 1×10^{-2} and 5×10^{-2}M caused slight inhibition to laccase activity according to the strength of EDTA concentrations used to give residual activities of 100.00, 98.42, 94.21 and 92.63%, respectively after 1h of incubation (Fig. 6a) and 99.32, 93.62, 86.32 and 81.05%, respectively after 24h (Fig. 6b). In agreement with our results, Zapata-Castillo et al. [50] found that the activity of *Trametes hirsuta* Bm-2 laccase is partially resistant to EDTA (10 mM). On the other hand, Asgher et al. [44] reported that EDTA inhibited the purified laccase enzyme from *Trametes versicolor* IBL-04. Pozdnyakova et al. [46], stated that EDTA, a weak inhibitor of fungal laccases, reduced the activity of the laccase from *P. ostreatus* D1 enzyme by 60% at a concentration of 100mM. Few exceptions such as laccases of *Marasmius quercoplhilus* [60] and *Phellinus ribis* [61] have been described, which are only inhibited by high concentrations of EDTA.

The obtained results indicated that, the addition of $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$ at a concentration of 5×10^{-2} M caused complete inhibition of laccase activity, while as $HgCl_2$ caused complete inhibition at all tested concentrations. On the other hand, when $MnCl_2.4H_2O$ was added at concentrations of 5×10^{-3} and 2.5×10^{-3}M caused laccase activation by about 50 and 190 % increase, respectively (Fig. 6a). The prolonged exposure (24h) of the purified enzyme to metal salts indicated that laccase is more stable at the lowest metal ions concentration (2.5×10^{-3} M). It gives 330% activation when incubated with Mn^{+2} at the previously mentioned concentration (Fig.6b).

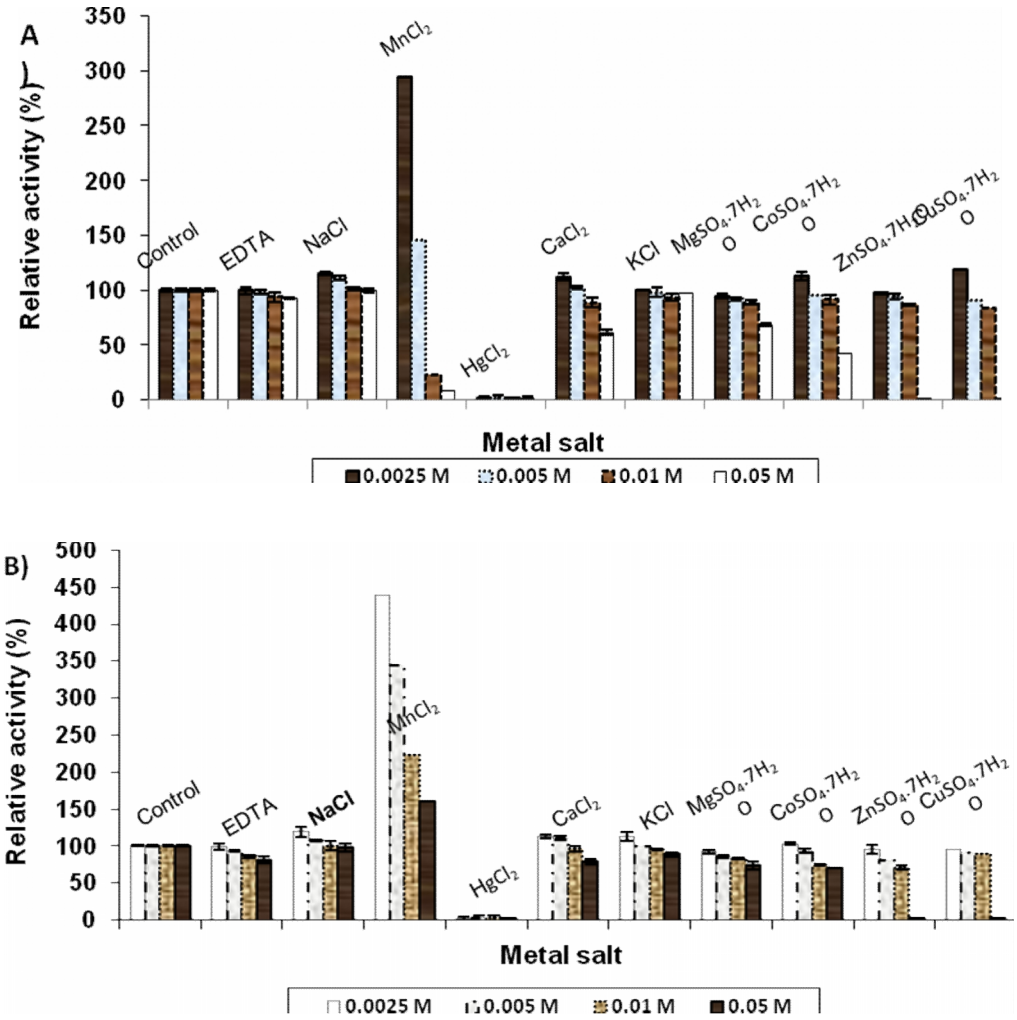


Fig. 6. Effect of EDTA and different metal salts on the *P. ostreatus* ARC280 purified laccase. A) After 1 h and B) after 24 h of incubation

Reaction mixture contained: SGZ, 0.1 µmole; purified enzyme, 1.0µg protein; EDTA or metal salt, (as indicated) and citrate phosphate buffer pH 6.0, 90 µmoles; total volume, 2ml. Values are means of three replications ± standard deviation

The activation effect caused by copper ions on laccase activity was mentioned with several reports on the metal ion requirements of laccases [40,62]. The positive response to copper is because laccases contain at least two types of copper centres; the first is a mononuclear centre that serves as the site for substrate oxidation and the second is a trinuclear centre where the reduction of oxygen to water occurs [63]. Despite this fact, not all laccases are stimulated by copper ions. For instance, laccase from *P. ostreatus* strain 10969 was reported to be inhibited by copper ions [64] in agreement with the present results. The observed ability of manganese ions to activate *P. ostreatus* ARC280 laccase is in general agreement with the findings on laccases from other sources [40,62].

According to Lundell and Hatakka [65], manganese ions participate either as reducing or oxidizing agents in laccase-catalyzed reactions. In the present investigation, Mn^{2+} caused an increase in the enzyme activity. The potent effect of different Mn^{+2} concentrations range (1.0×10^{-5}M - 5.0×10^{-3}M) were tested. Laccase activation was increased by increasing Mn^{2+} concentration up to 1.0×10^{-3}M which gave about 240 and 445% activity increase after incubation for 1 and 24h, respectively (Fig. 7). Laccase activity then decreased by increasing Mn^{2+} concentration above this value. In agreement with current results, Adamafio et al. [40] reported the stimulatory effect of manganese on *P. ostreatus* strain EM-1 laccase contrasts sharply with the reported inhibition of *Daedalea quercina* laccase by Mn^{2+} [66].

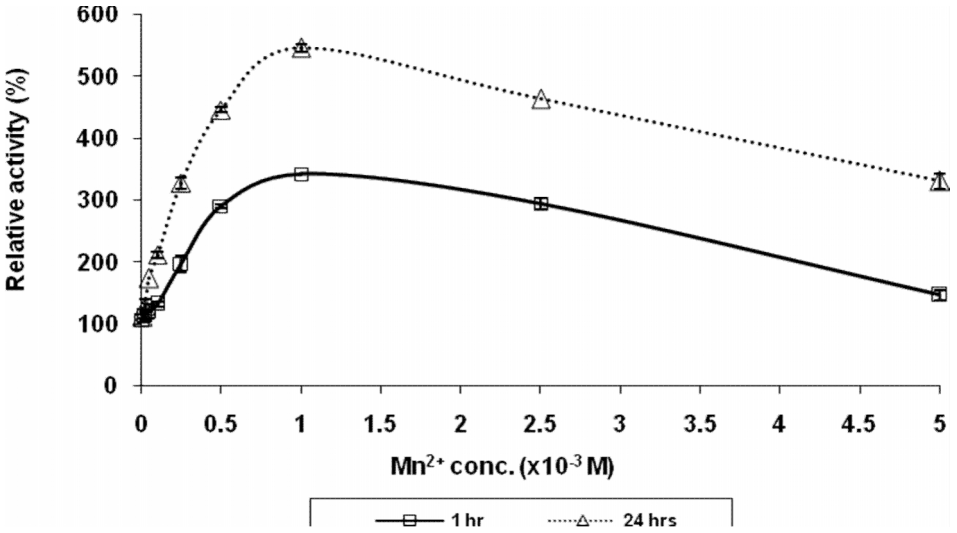


Fig. 7. Effect of different concentrations of Mn^{2+} on the *P. ostreatus* ARC280 purified laccase

Reaction mixture contained: SGZ, 0.1 µmole; purified enzyme, 1.0 µg protein; Mn^{2+}, (as indicated) and citrate phosphate buffer pH 6.0, 90 µmoles; total volume, 2ml. Values are means of three replications ± standard deviation

3.7 Substrate Specificity and Kinetic Constants

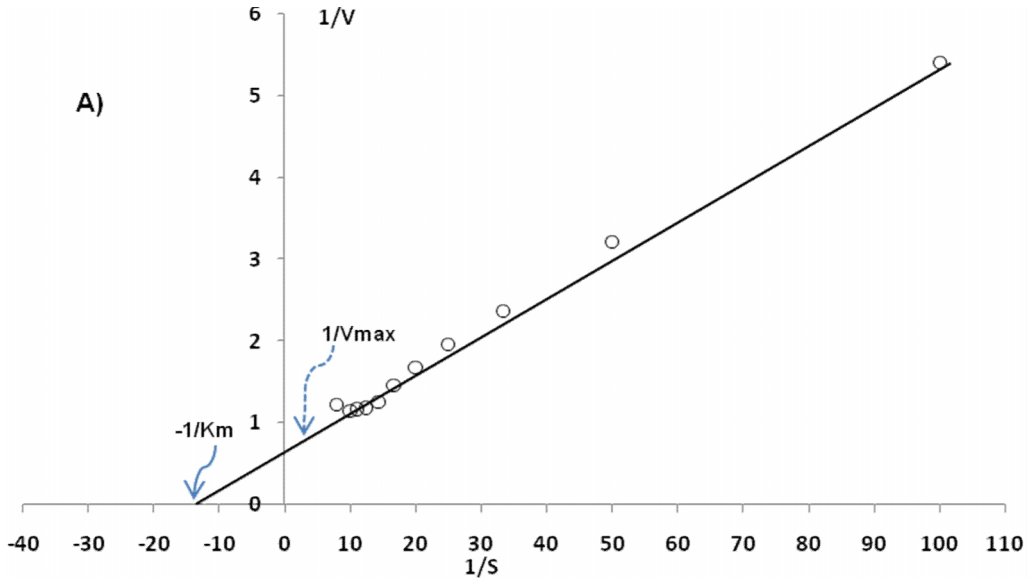
The reaction rate and the substrate affinity greatly varied depending on the nature of substrate. There is a difference in terms of reactivity of the enzyme towards different compounds. Tinoco et al. [47] stated that ABTS and SGZ were shown to be good substrates for laccases from all the *P. ostreatus* strains, and the apparent affinity constants (K_m) also showed significant differences between the different strains, from 8 to 80mmol l^{-1} for ABTS, from 12 to 52µmol l^{-1} for SGZ and from 0.46 to 6.61mmol l^{-1} for guaiacol. In this connection, Patel et al. [37] found that the K_m values for *P. ostreatus* HP-1 laccase with ABTS and DMP were 46.51 and 400mM, respectively. The laccase activity against SGZ was much higher than that against ABTS and DMP, respectively. Results also revealed that SGZ was the best substrate for the purified laccase of *P. ostreatus* ARC280 (Table 2).

Table 2. Substrate specificity and kinetic constants for the oxidation of various substrates by the *P. ostreatus* ARC280 purified laccase

Substrate	Optimal pH	Extinction coefficient (M^{-1} cm^{-1})	Relative activity (%)	K_m (µM)	V_{max} (µmol min^{-1})	K_m/ V_{max}
SGZ	6.0	65000	100.00	0.074	1.563	0.047
DMP	4.5	49600	8.05	2.857	2.500	1.143
ABTS	3.0	36000	50.57	0.476	2.632	0.181

Reaction mixture (2ml) contained: substrate, 0.1 µmole SGZ, 0.5 µmole DMP or 0.5 µmole ABTS; purified enzyme, 1.0 µg protein and citrate phosphate buffer pH (as indicated), 90 µmoles

K_m and V_{max} were determined using various concentrations of different substrates (SGZ, DMP and ABTS) with the purified *P. ostreatus* ARC280 laccase (Figs. 8a, b and c). K_m and V_{max} were calculated and found to be 0.074, 2.857 and 0.476µM and 1.563, 2.500 and 2.632 µmol min^{-1} for SGZ, DMP and ABTS, respectively (Table 2). In agreement with our results, Stoilova et al. [67] found that laccase from *Trametes versicolor* has K_m value for SGZ substrate lower than the value of ABTS substrate and stated that, the K_m index for SGZ substrate is lower than the value of the index established for ABTS substrate, pointing out the greater affinity of the investigated laccase to SGZ. In contrast, Baldrian [55] stated that in general, laccases are known to possess very wide range substrate affinities; however the most of the laccases characterized show greater affinity for ABTS. In this connection, Asgher et al. [44] reported that on using ABTS as a substrate, the enzyme showed V_{max} of 780 Units/ml with a corresponding K_m value of 73µM. Zapata-Castillo et al. [50] found that the values of apparent K_m are 68 and 164µM and V_{max} values are 14 and 4.65 Units/ml for ABTS and DMP, respectively for *Trametes hirsuta* Bm-2 laccase.



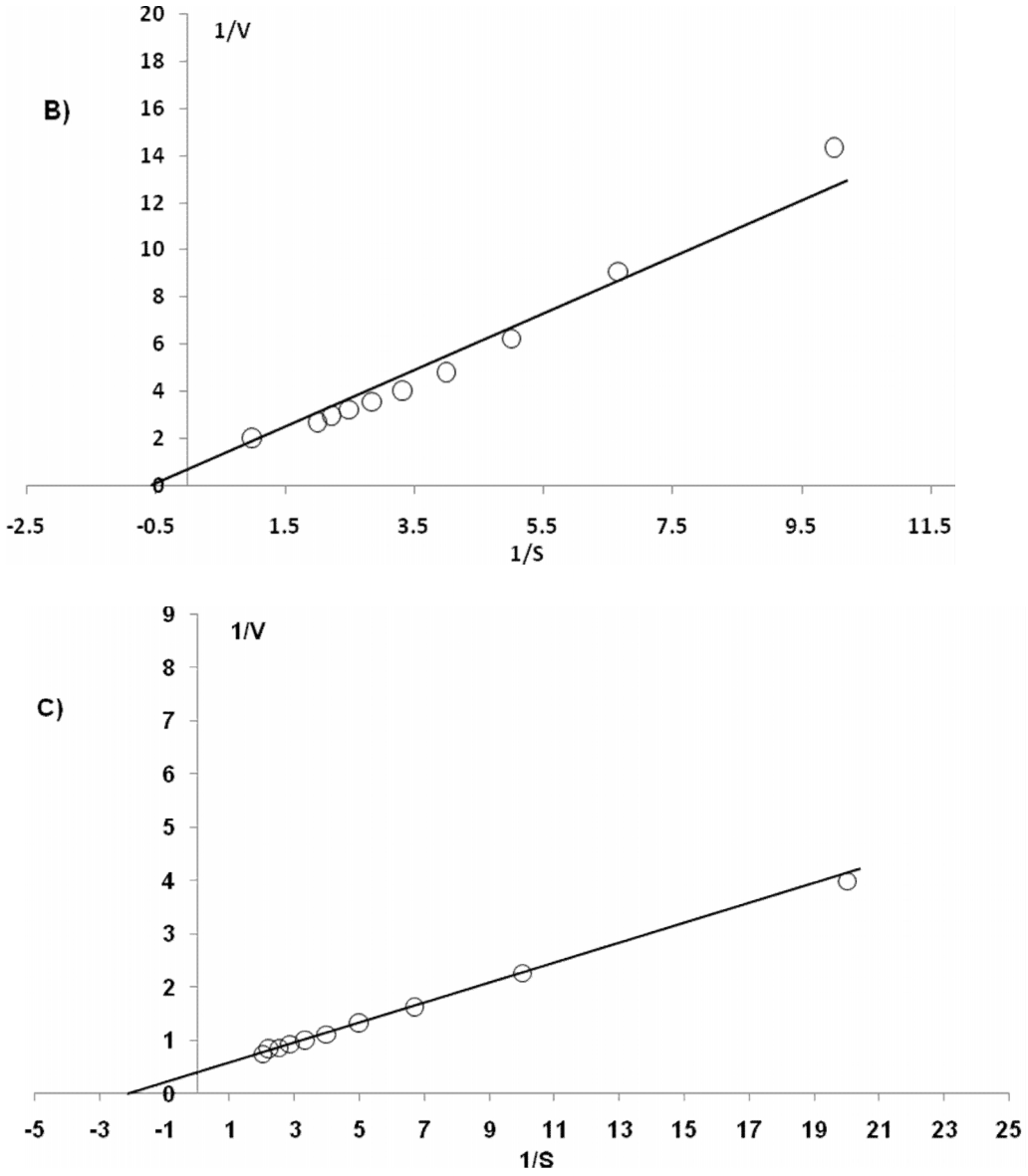


Fig. 8. Lineweaver–Burk plot of the reciprocal of initial velocities and different laccase substrates concentrations

A) SGZ: Syringaldazine (4-hydroxy-3,5- dimethoxy-benzaldehyde azine), B) DMP: 2, 6-dimethoxyphenol and C) ABTS: 2, 2´-azino-bis-3-ethylbenzthia-zoline-6-sulfonic acid

3.8 Anti-Proliferative (Anticancer) Activity

A few of laccases were reported to manifest some bioactivities such as anti-proliferative activity toward tumor cells [68]. Potential successes in cancer treatment using bioactive metabolites isolated from medicinal mushrooms have shown as biological immunotherapeutic agents stimulating the immune system against cancer cells. These

bioactive metabolites also act as an effective source of anticancer agents, capable of interfering with cellular signal transduction pathways linked to cancer development and progression [69].

In the present study, the in-vitro bioassay cytotoxic effect of *P. ostreatus* ARC280 laccase crude enzyme on the growth of four human tumor cell lines namely HePG2, HCT116, A549 and MCF7 revealed that, the crude laccase enzyme extract of *P. ostreatus* ARC280 at a concentration of 100 µg/ml, has anti proliferative activity against HePG2 and MCF7 cell lines growth in-vitro with percentage reduction of 33.4 and 45.9%, respectively. In contrast, the *P. ostreatus* ARC280 crude laccase enzyme extract have no anti proliferative activity against HCT116 and A549 cell lines growth in-vitro (Table 3).

Table 3. In-vitro bioassay of *P. ostreatus* ARC280 laccase on human tumor cell lines

Sample	Anticancer activity (%) at conc. of 100µg/ml			
	HePG2[a]	HCT116[b]	A549[c]	MCF7[d]
Crude laccase	33.4	0	0	45.9
Pure laccase	16.8	23.4	15.2	23.4
Negative control	0	0	0	0

[a] *Human hepatocellular carcinoma cell line*
[b] *Colon cell line*
[c] *Lung carcinoma cell line*
[d] *Human caucasian breast adenocarcinoma*

On the other hand, the treatment of the four human tumor cell lines previously mentioned with *P. ostreatus* ARC280 purified laccase (100µg/ml) resulted in the reduction of different tested cell lines growth in-vitro with a percentage reduction of 16.8, 23.4, 15.2 and 23.4 % for HePG2, HCT116, A549 and MCF7, respectively (Table 3). The highest antitumor activity was recorded with the *P. ostreatus* ARC280 crude laccase enzyme extract toward MCF7 cell line growth in-vitro (45.9%) when compared with the negative control. From the obtained results it could be concluded that, *P. ostreatus* ARC280 laccase can be used for developing the therapy of different types of tumors. In this connection, Hu et al. [70] reported that the purified laccase from an edible mushroom, *Agrocybe cylindracea* has highly potent antiproliferative activity against hepatoma HepG2 cells and breast cancer MCF-7 cells, where at the concentrations of 1.25, 2.5, 5.0, and 10µM, inhibited proliferation of HepG2 cells by 7.8, 30.2, 46.4, and 78.5%, respectively, and MCF7 cells by 7.2, 22.7, 41.3, and 70.6%, respectively.

3.9 Anti-Microbial Activity

Beyond the chemical compounds that can be used as antimicrobial agents, enzymes are also interesting as antimicrobial agents. Furthermore, because of their proteinaceous nature, enzymes are considered to be environmentally safe [71]. In this connection, Ibrahim et al. [72] stated that, the effect of enzymes as antimicrobial agents is mainly due to the electrochemical mode of action to penetrate cell wall of microorganisms, thereby causing leakage of essential metabolites and physically disrupting other key cell functions.

Table 4. Anti-microbial activity of *P. ostreatus* ARC280 laccase

Sample	Diameter of inhibition zone (mm)		
	B. mycoides	***E. coli***	***C. albicans***
Crude laccase	43	30	27
Partial purified laccase ($(NH_4)_2SO_4$ precipitation)	17	23	25
Purified laccase	33	35	37

Table 5. A comparison of purified laccase properties from *P. ostreatus* ARC280 with other reported *P. ostreatus* strains

Source	M.w. (kDa)	Temperature (°C)	Thermal stability (Residual activity)	pH stability (Residual activity)	pH optimum		K_m		References
P. ostreatus ARC280	85 kDa	50	96, 80, 60% after 120 min at 40, 50, 60°C	88.49, 93.65, 92.86 and 100.00 % after 5 h at pH 7.0, 8.0, 9.0 and 10.0	ABTS DMP SGZ	3.0 4.5 6.0	ABTS DMP SGZ	0.476 µM 2.857 µM 0.074 µM	The present study
P. ostreatus HP-1	68	50	50 % after 15 min at 60°C	ND	ABTS DMP Guaiacol O-dianisidine	4.5 3.5 5.5 3.5	ABTS DMP Guaiacol O-dianisidine	46.51 mM 400mM 100mM 23.52 mM	Patel et al. [37]
P. ostreatus D1	64	ND	ND	ND	ABTS DMP SGZ Pyrocatechol	4.0 4.0 7.0 8.0	ABTS DMP SGZ Pyrocatechol	0.11 mM 0.43 mM 0.0087 mM 3.65 mM	Pozdnyakova et al. [46]
P. ostreatus 10969	40	50	10% after 10 min at 60°C	10% after 60 min at pH 7.0	ABTS	4.0	ABTS	0.31 mmol / l	Liu et al. [74]
P. ostreatus strain EM-1	78	50	22.6 % after 60 min at 50°C	ND	DMP	5.0	ND		Adamafio et al. [40]
P. ostreatus lacc POXA1	61	45–65	$t_{1/2}$= 200 min at 60°C, pH 7.0	$t_{1/2}$= 24 h at pH 3.0	ABTS DMP SGZ	3.0 3-5 6.0	ABTS DMP SGZ	0.09 mM 2.1 mM 1.3 mM	Palmieri et al. [38, 48]
P. ostreatus lacc POXC		50 – 60	$t_{1/2}$= 30 min at 60°C, pH 7.0	$t_{1/2}$= 30 min at pH 3.0	ABTS Guaiacol DMP SGZ	3.0 6.0 3-5 6.0	ABTS Guaiacol DMP SGZ	2.8 mM 1.2 mM 2.3 mM 2.0 mM	Palmieri et al. [38, 48]
P. ostreatus lacc POXA2	67	25–35	$t_{1/2}$= 10 min at 60°C, pH 7.0	$t_{1/2}$= 2 h at pH 3.0	ABTS Guaiacol DMP SGZ	3.0 6.0 6.5 6.0	ABTS Guaiacol DMP SGZ	1.2 mM 3.1 mM 7.4 mM 1.4 mM	Palmieri et al. [38, 48]
P. ostreatus	ND	ND	27.5 % at 39°C after 15 h	42.1 % at pH 7 after 15 h	ND		ND		Luna et al. [53]
P. ostreatus strains	ND	ND	ND	ND	ND		ABTS SGZ Guaiacol	8 - 80 mmol / l 12 - 52 µmol / 1 0.46 - 6.61 mmol / l	Tinoco et al. [47]

In the present study, the results of antimicrobial activity indicated that the pure laccase produced by *P. ostreatus* ARC280 gave the highest Gram-negative antibacterial and antifungal activities against *E. coli* (35 mm) and *C. albicans* (37 mm) respectively, expressed as the width of inhibition zone. Optimum Gram positive antibacterial activity against *B. mycoides* (43 mm) was found with the crude preparation of *P. ostreatus* ARC280 laccase (Table 4) above. In agreement with the present results, Arul Diana Christie and Shanmugam [73] reported that the laccase enzyme could inhibit both Gram positive and Gram negative bacteria.

Table (5) above summarizes the properties of purified laccase from *P. ostreatus* ARC280 and other reported *P. ostreatus* strains. The table indicates the stability behavior of *P. ostreatus* ARC280 laccase under study compared with some of other reported *P. ostreatus laccases.*

4. CONCLUSION

In view of the results obtained, it can be concluded that *P. ostreatus* ARC280 laccase seems to be a prospective enzyme for further biotechnological exploitation such as anticancer and antimicrobial activity applications.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Forootanfar H, Faramarzi MA, Shahverdi AR, Yazdi MT. Purification and biochemical characterization of extracellular laccase from the ascomycete *Paraconiothyrium variabile*. Bioresour Technol. 2011;102:1808-14.
2. Hassan MM, Elshafei AM, Haroun BM, Elsayed MA, Othman AM. Biochemical characterization of an extracellular laccase from *Pleurotus ostreatus* ARC280. J Appl Sci Res. 2012;8(8):4525-36.
3. Yaropolov AI, Skorobogatko OV, Vartanov SS, Varfolomeyev SD. Laccase: Properties, catalytic mechanism and applicability. Appl Biochem Biotechnol. 1994;49:257–80.
4. Elshafei AM, Hassan MM, Haroun BM, Elsayed MA, Othman, AM. Optimization of laccase production from *Penicillium martensii* NRC345. Adv Life Sci. 2012;2(1):31-7.
5. Gianfreda L, Rao MA. Potential of extra cellular enzymes in remediation: A review. Enzyme Microb Technol. 2004;35:339-54.
6. Thurston CF. The structure and function of fungal laccases. Microbiol. 1994;140:19-26.
7. Elsayed MA, Hassan MM, Elshafei AM, Haroun BM, Othman AM. Optimization of cultural and nutritional parameters for the production of laccase by *Pleurotus ostreatus* ARC280. Br Biotechnol J. 2012;2(3):115-32.
8. Martínez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillen F, et al. Biodegradation of lignocellulosics: Microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol. 2005;8(3):195–204.
9. Halaburgi VM, Sharma S, Sinha M, Singh TP, Karegoudar TB. Purification and characterization of a thermostable laccase from the ascomycetes *Cladosporium cladosporioides* and its applications. Proc Biochem. 2011;46:1146-52.

10. Piontek K, Antorini M, Choinowski, T. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90–Å resolution containing a full complement of coppers. J Biol Chem. 2002;277:37663-9.
11. Solomon EI, Sundaram UM, Machonkin TE. Multicopper oxidases and oxygenases. Chem Rev. 1996;96:2563–605.
12. Gianfreda L, Xu F, Bollag JM. Laccases: A useful group of oxidoreductive enzymes. Bioremediat J. 1999;3:1–25.
13. Bourbonnais R, Paice MG. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. 1990;267(1):99-102.
14. Maciel MJM, Silva AC, Ribeiro HCT. Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota: A review. Electron J Biotechnol; 2010. DOI: 10.2225/vol13-issue6-fulltext-2.
15. D'acunzo F, Galli C, Masci B. Oxidation of phenols by laccase and laccase-mediator systems: Solubility and steric issues. Eur J Biochem. 2002;269:5330-5.
16. Shekher R, Sehgal S, Kamthania M, Kumar A. Laccase: Microbial Sources, Production, Purification, and Potential Biotechnological Applications: A review. Enzyme Research; 2011. DOI:10.4061/2011/217861.
17. Markussen EK, Jensen PE. Stabilisation of granules comprising active compounds. WO2006053564 A2; 2006.
18. Agematu H, Kominato K, Shibamoto N, Yoshioka T, Nishida H, Okamoto R, et al. Transformation of 7- (4-hydroxy phenyl acetamido) cephalo-sporanic acid into a new cephalosporin antibiotic, 7-[1-oxaspiro (2.5) octa-6-oxo-4,7-diene-2-carboxamido] cephalosporanic acid, by laccase. Biosci Biotechnol Biochem. 1993;57:1387–8.
19. Anyanwutaku I, Petroski R, Rosazza J. Oxidative coupling of aureolic acids and hydroquinone catalyzed by copper oxidases. Implications for the molecular mechanism of action of Aureolic acids. Bioorg Med Chem. 1994;2:543–51.
20. Mikolasch A, Matthies A, Lalk M, Schauer F. Laccase induced C-N coupling of substituted *p*-hydroquinones with *p*-aminobenzoic acid in comparison with known chemical routes. Appl Microbiol Biotechnol. 2008;80:389–97.
21. Kunamneni A, Plou FJ, Ballesteros A, Alcalde M. Laccases and their applications: A patent review. Recent Pat Biotechnol. 2008;2:10-24.
22. Dwivedi UN, Singh P, Pandey VP, Kumar A. Structure–function relationship among bacterial, fungal and plant laccases: A review. J Mol Catal B: Enzym. 2011;68:117–28.
23. Tlecuitl-Beristain S, Sánchez C, Loera O, Robson-Geoffrey D, Díaz-Godínez G. Laccases of *Pleurotus ostreatus* observed at different phases of its growth in submerged fermentation: Production of a novel laccase isoform. Mycol Res. 2008;112:1080-4.
24. Minussi RC, Miranda MA, Silva JA, Ferreira CV, Aoyama H, Marangoni S, et al. Purification, characterization and application of laccase from *Trametes versicolor* for colour and phenolic removal of olive mill wastewater in the presence of 1-hydroxybenzotriazole. Afr J Biotechnol. 2007;6(10):1248-54.
25. Arıca MY, Altıntas B, Bayramoğlu G. Immobilization of laccase onto spacer-arm attached non-porous poly (GMA/EGDMA) beads: Application for textile dye degradation. Bioresour Technol. 2009;100:665–9.
26. Uthandi S, Saad B, Humbard MA, Maupin-Furlow JA. LccA, an archaeal laccase secreted as a highly stable glycoprotein into the extracellular medium by *Haloferax volcanii.* Appl Environ Microbiol. 2010;76(3):733–43.
27. Muzariri CC, Mapingire J, Mazorodze J, Mandikutse L. Isolation and screening of microorganisms for potential application in remediation of effluent water from the pulp and paper Industry. 2nd WARFSA/WaterNet Symposium: Intergrated Water Resources Management: Theory, Practice, Cases; Cape Town; 2001.

28. Saparrat MCN, Guillén F, Arambarri AM, Martínez AT, Martínez MJ. Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida.* Appl Environ Microbiol. 2002;68(4):1534–40.
29. Annuar MSM, Adnan S, Vikineswary S, Chisti Y. Kinetics and energetics of azo dye decolorization by *Pycnoporus sanguineus*. Water Air Soil Pollut. 2009;202:179–88.
30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265-75.
31. Layne E. Spectrophotometric and turbidimetric methods for measuring proteins. Meth Enzymol. 1957;3:447-55.
32. Kenney JF, Keeping ES. The standard deviation and calculation of the standard deviation. In: Mathematics of statistics, Pt.1,3rd ed. Princeton, NJ, Van Nostrand. 1962;77-80.
33. Laemmli UK. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature. 1970;227:680-5.
34. Mosmann T. Rapid colorimetric assays for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55-63.
35. Thabrew MI, Hughes RD, McFarlane IG. Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. J Pharm Pharmacol. 1997;49:1132-5.
36. Zhou J. Study and development of a 'smart' wound dressing technology which can detect and inhibit/kill the colonisation of pathogenic bacteria. Ph. D. thesis, Department of Chemistry, University of Bath, United Kingdom; 2011.
37. Patel H, Gupte S, Gahlout M, Gupte A. Purification and characterization of an extracellular laccase from solid-state culture of *Pleurotus ostreatus* HP-1. 3 Biotech. 2013;4:77–84, DOI 10.1007/s13205-013-0129-1.
38. Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G. A Novel White Laccase from *Pleurotus ostreatus*. J Biol Chem. 1997;272(50):31301–7.
39. Murugesan K, Arulmani M, Nam IH, Kim YM, Chang YS, Kalaichelvan PT. Purification and characterization of laccase produced by a white rot fungus *Pleurotus sajor-caju* under submerged culture condition and its potential in decolorization of azo dyes. Appl Microbiol Biotechnol. 2006;72:939–46.
40. Adamafio NA, Sarpong NS, Mensah CA, Obodai M. Extracellular laccase from *Pleurotus ostreatus* strain EM-1: Thermal stability and response to metal ions. Asian J Biochem. 2012;7(3):143-50.
41. Mansur M, Arias ME, Copa-Patiño JL, Flärdh M, González AE. The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities. Mycologia. 2003;95(6):1013-20.
42. Younes SB, Sayadi S. Purification and characterization of a novel trimeric and thermotolerant laccase produced from the ascomycete *Scytalidium thermophilum* strain. Comp Biochem Physiol B Biochem Mol Biol. 2011;73:35-42.
43. Sadhasivam S, Savitha S, Swaminathan K, Lin FH. Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. Process Biochem. 2008;43:736-42.
44. Asgher M, Iqbal HMN, Asad MJ. Kinetic characterization of purified laccase produced from *Trametes versicolor* IBL-04 in solid state bio-processing of corncobs. Bio Resources. 2012;7(1):1171-88.
45. Xu LJ, Wang HX, Ng TB. A laccase with HIV-1 reverse transcriptase inhibitory activity from the broth of mycelial culture of the mushroom *Lentinus tigrinus*. J Biomed Biotechnol; 2012. Article ID 536725. DOI:10.1155/2012/536725.

46. Pozdnyakova NN, Turkovskaya OV, Yudina EN, Rodakiewicz-Nowak YA. Yellow Laccase from the fungus *Pleurotus ostreatus* D1: Purification and characterization. Appl Biochem Micro. 2006;42(1):56–61.
47. Tinoco R, Pickard MA, Vazquez-Duhalt R. Kinetic differences of purified laccases from six *Pleurotus ostreatus* strains. Lett Appl Microbiol. 2001;32:331–5.
48. Palmieri G, Giardina P, Marzullo L, Desiderio B, Nitti B, Cannio R, Sania G. Stability and activity of phenoloxidase from lignolytic fungus *Pleurotus ostreatus*. Appl Microbiol Biotechnol. 1993;39:632–6.
49. Zou YJ, Wang HX, Ng TB, Huang CY, Zhang JX. Purification and characterization of a novel laccase from the edible mushroom *Hericium coralloides*. J Microbiol. 2012;50(1):72-8.
50. Zapata-Castillo P, Villalonga-Santana ML, Tamayo-Cortés J, Rivera-Muñoz G, Solís-Pereira S. Purification and characterization of laccase from *Trametes hirsuta* BM-2 and its contribution to dye and effluent decolorization. Afr J Biotechnol. 2012;11(15):3603-11.
51. Guo W, Yao Z, Zhou C, Li D, Chen H, Shao Q, et al. Purification and characterization of three laccase isozymes from the white rot fungus *Trametes* sp. HS-03. Afr J Biotechnol. 2012;11(31):7916-22.
52. Arias ME, Arenas M, Rodríguez J, Soliveri J, Ball AS, Hernández M. Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. Appl Environ Microbiol. 2003;69(4):1953–8.
53. Luna L, Meneses M, Mendoza G, Montalvo C, Loera O. Effect and enzyme activity of Pleurotus ostreatus on cell wall of barley straw. Livestock Research for Rural Development. 2013; 25, Article 223. Retrieved June 8, 2014, from http://www.lrrd.org/lrrd25/12/luna25223.htm.
54. Soden DM, O'Callaghan J, Dobson AD. Molecular cloning of a laccase isozyme gene in the heterologous *Pichia pastoris* host. Microbiology. 2001;148:4003-14.
55. Baldrian P. Fungal laccases-occurrence and properties. FEMS Microbiol Rev. 2006;30(2):215-42.
56. Schückel J, Matura A, Van Pée KH. One-copper laccase-related enzyme from *Marasmius sp*.: Purification, characterization and bleaching of textile dyes. Enzym Microb Tech. 2011;48(3):278-84.
57. Wang HX, Ng TB. Purification of a laccase from fruiting bodies of the mushroom *Pleurotus eryngii*. J Appl Microbiol Biotechnol. 2006;69(5):521-5.
58. Patrick F, Mtui G, Mshandete AM, Johansson G, Kivaisi A. Purification and characterization of a laccase from the basidiomycete *Funalia trogii* (Berk.) isolated in Tanzania. Afr J Biochem Res. 2009;3(5):250-8.
59. Munoz C, Guillen F, Martinez AT, Martinez MJ. Laccase isoenzymes of *Pleurotus eryngii*: Characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. Appl Environ Microbiol. 1997;63:2166–74.
60. Farnet AM, Criquet S, Gil G, Ferre E. Purification of a new isoform of laccase from *Marasmius quercophilus* strain isolated from cork oak litter (*Quercus suber* L.). Micologia. 2002;94:735-40.
61. Min KL, Kim YH, Kim YW, Jung HS, Hah YC. Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. Arch Biochem Biophys. 2001;392(2):279–86.
62. Stajić M, Persky L, Hadar Y, Friesem D, Duletic-lausevic S, Wasser SP, et al. Effect of copper and manganese ions on activities of laccase and peroxidases in three *Pleurotus* species grown on agricultural wastes. Appl Biochem Biotechnol. 2006;168:87-96.

63. Desai SS, Nityanand C. Microbial laccases and their applications: A review. Asian J Biotechnol. 2011;3:98-124.
64. Liu L, Lin Z, Zheng T, Lin L, Zheng C, Lin Z, et al. Fermentation optimization and characterization of the laccase from *Pleurotus ostreatus* strain 10969. Enzyme Microb Technol. 2009;44:426-33.
65. Lundell T, Hatakka A. Participation of Mn(II) in the catalysis of laccase, manganese peroxidase and lignin peroxidase from *Phlebia radiate*. FEBS Letts. 1994;348:291-6.
66. Baldrian P. Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. Appl Microbiol Biotechnol. 2004;63(5):560-3.
67. Stoilova I, Krastanov A, Stanchev V. Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. Adv Biosci Biotechnol. 2010;1:208-15.
68. Zhang GQ, Tian T, Liu YP, Wang HX, Chen QJ. A laccase with anti-proliferative activity against tumor cells from a white rot fungus *Abortiporus biennis*. Process Biochem. 2011;46:2336–40.
69. De Silva DD, Rapior S, Fons F, Bahkali AH, Hyde KD. Medicinal mushrooms in supportive cancer therapies: An approach to anti-cancer effects and putative mechanisms of action. Fungal Divers. 2012;55:1-35.
70. Hu DD, Zhang RY, Zhang GQ, Wang HX, Ng TB. A laccase with antiproliferative activity against tumor cells from an edible mushroom, white common *Agrocybe cylindracea*. Phytomedicine. 2011;18:374–9.
71. Aldred N, Phang I, Conlan Y, Clare SL, Vancso AS. The effects of a serine protease, Alcalase, on the adhesives of barnacle cyprids (*Balanus amphitrite*). Biofouling. 2008;24(2):97-107.
72. Ibrahim NA, Gouda M, Elshafei AM, Abdel-Fatah OM. Antimicrobial activity of cotton fabrics containing immobilized enzymes. J Appl Polymer Sci. 2007;104:1754-61.
73. Arul Diana Christie S, Shanmugam S. Analysis of fungal cultures isolated from anamalai hills for laccase enzyme production effect on dye decolorization, antimicrobial activity. IJPAES. 2012;2(3):143-8.
74. Liu L, Lin Z, Zheng T, Lin L, Zheng C, Lin Z, Wang S, Wang Z. Fermentation optimization and characterization of the laccase from *Pleurotus ostreatus* strain 10969. Enzyme Microb. Technol. 2009;44:426-433.

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