The Protein Journal (2024) 43:115–128
https://doi.org/10.1007/s10930-023-10169-7

Biochemical Characterization of Laccase from *Spirulina CPCC-695* and Their Role in Estrone Degradation

Neha Sami[1] · Bushra Afzal[1] · Durdana Yasin[1] · Tasneem Fatma[1]

Accepted: 13 November 2023 / Published online: 21 December 2023

Abstract
The addition of exogenous endocrine disrupting compounds (EDCs) like estrone, in the food chain through the aquatic system, disrupts steroid biosynthesis and metabolism by altering either the genomic or non-genomic pathway that eventually results in various diseases. Thus, bioremediation of these compounds is urgently required to prevent their addition and persistence in the environment. Enzymatic degradation has proven to be a knight in shining armour as it is safe and generates no toxic products. The multicopper oxidases (E.C. 1.10.3.2 benzenediol: oxygen oxidoreductase), laccase with the potential to degrade both phenolic and non-phenolic substrates has recently gained attention. In this study, the laccase was purified, characterized, and used to study estrone degradation. The culture filtrate (crude laccase) was concentrated and precipitated using cold-acetone and dialyzed against tris buffer (50 mM) giving a four-fold partially purified form, with 45.56% yield and 204.14 U/mg as specific activity and a single peak at 250–300 nm. The partially purified laccase was approximately 80 kDa as estimated by SDS-PAGE preferred ABTS as substrate. Both crude and partially purified laccase showed maximum activity at pH 3.0, 40 °C, and 4 mM ABTS. Kinetic constants (Km, Vmax) of crude and partially purified laccase were found to be 0.83 mM; 494.31 mM/min, and 0.58 mM; 480.54 mM/min respectively. Iron sulphate and sodium azide inhibited laccase maximally. Crude and partially purified laccase degradation efficiency was 87.55 and 91.35% respectively. *Spirulina* CPCC-695 laccase with efficient estrone degradation ability renders them promising candidates for EDCs bioremediation.

Keywords Laccase · Oxidoreductases · Crude · Estrone · Endocrine disrupting compounds · *Spirulina* CPCC-695

1 Introduction

Among all the natural and synthetic endocrine disrupting compounds (EDCs) that persist in the environment and affect the endocrine system of living organisms, steroid hormones like estrone (E1) is known to exhibit higher-estrogenicity. It is one of the chemically stable natural fat-soluble estrogens endogenously present in both men and women, however, significantly higher levels are found in females of reproductive age and play an important role in the oestrous cycle. Furthermore, its excessive usage as a drug to overcome fertility issues in humans and cattle, along with its use for increasing milk and meat yield in cattle and poultry has worsened the situation. A large amount of estrone gets excreted through faeces and urine (in free form or as inactive glucuronides and sulfate conjugates) that are flushed down from home toilets, municipal, pharmaceutical industry, and hospitals effluents through which it enters wastewater treatment plants (WWTPs) and then released into the aquatic environment [1]. Since their complete removal is still a question thus they have been detected in surface waters, ground waters, and even in drinking waters, thus categorised as an emerging pollutant [2]. Consequently, the entry of exogenous hormones in the form of EDCs in the food chain, interferes with the steroid biosynthesis and metabolism of the organism, either as inhibitors of relevant enzymes or at the level of their expression. Effective and appropriate techniques for removing estrone in wastewater treatment processes continue to be a challenge of high environmental and public health importance [3].

Enzymatic degradation can prove to be a boon as they are cost-efficient and don't produce toxic end-products. Laccase (p-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) are the blue multicopper oxidases that play an important role in the degradation of phenolic and non-phenolic substrates with

✉ Tasneem Fatma
fatma_cbl@yahoo.com

[1] Cyanobacterial Biotechnology Laboratory, Department of Biosciences, Jamia Millia Islamia, New Delhi, India

or without mediators [4]. It is basically a glycoprotein that exists in various forms, mostly the monomeric form, but some also occur in multimeric, heterodimeric, and homodimeric forms and possess a molecular mass between 50 and 140 kDa, that solely depends on the organism type [5]. Laccase consists of three copper centers (Type I, II, and III) consisting of four copper ions and two active sites. Type I copper center (T1) is located in a surface exposed cleft that forms the first active site and oxidises phenols, and aliphatic or aromatic amines [6]. It provides the intense blue colour of protein, shows absorption maxima at 605 nm, and a slight hyperfine coupling in the electron paramagnetic resonance (EPR) spectrum. Type II copper center (T2) is colourless, and shows no sharp band in absorbance spectroscopy, as it only features nitrogen and oxygen ligands and gives a normal EPR spectrum. Type III copper centre (T3) is binuclear, i.e., has a pair of the copper atom, and gives a weak absorbance of around 330 nm, but usually lacks EPR spectrum due to the anti-ferromagnetic coupling between the two metals [7]. T2 and T3 collectively form the tri-nuclear cluster (TNC) of laccases (the second active site) situated in the protein core that receives electrons from T1. When catalysing the oxidation of a wide range of xenobiotics, electrons transfer from the compounds to the T1, then from the T1 site to the TNC, and eventually to a dioxygen molecule bound between the two T3 copper ions that concurrently reduce molecular oxygen to water [8] (Fig. 1). Thus, based on colour and the presence or absence of absorbance peak at 600 nm, laccases have been classified as atypical and typical laccases. The former are either colourless or pale yellow, lacking the absorbance peak, and the latter exhibit blue colour and the typical absorbance peak [9]. As compared to other enzymatic systems, laccase require no additional/expensive co-substrate or co-factor. Due to their strong oxidative power and formation of water as the final product, laccases are regarded as versatile eco-friendly enzymes with promising EDCs degradation ability.

Laccase has been reported in plants, fungi, and some bacteria where they perform diverse physiological functions, lignin degradation, pigmentation, pathogenesis, melanin production and spore coat resistance [10–12]. Fungi are the most efficient laccase producers and has been commercially exploited [4]. Recently, laccase activity has also been reported from cyanobacteria like *Phormidium valderianum, Oscillatoris boryana* [13], and *Spirulina* CPCC-695 [14]. Among all these cyanobacterial species, *Spirulina* possesses the highest possible protein content (60–70% dry weight) and is rich in other value-added compounds, such as PUFAs and pigments. Further, they can grow at high pH values, under mixotrophic and heterotrophic conditions, and can be harvested using easy and cheaper methods. Since they can be used as bio-adsorbent for heavy metals without generating any toxic end products [15]. Thus, in our previous studies, laccase has been purified from *Arthrospira maxima* [16] and *Spirulina platensis* CFTRI [17, 18] that has been used for bioremediation of anthraquinonic dyes like Reactive Blue and Remazol Brilliant Blue R.

Except for our preliminary findings, there is no report on role of cyanobacterial laccases in estrone degradation. In our previous studies, it has been reported that *Spirulina* CPCC-695

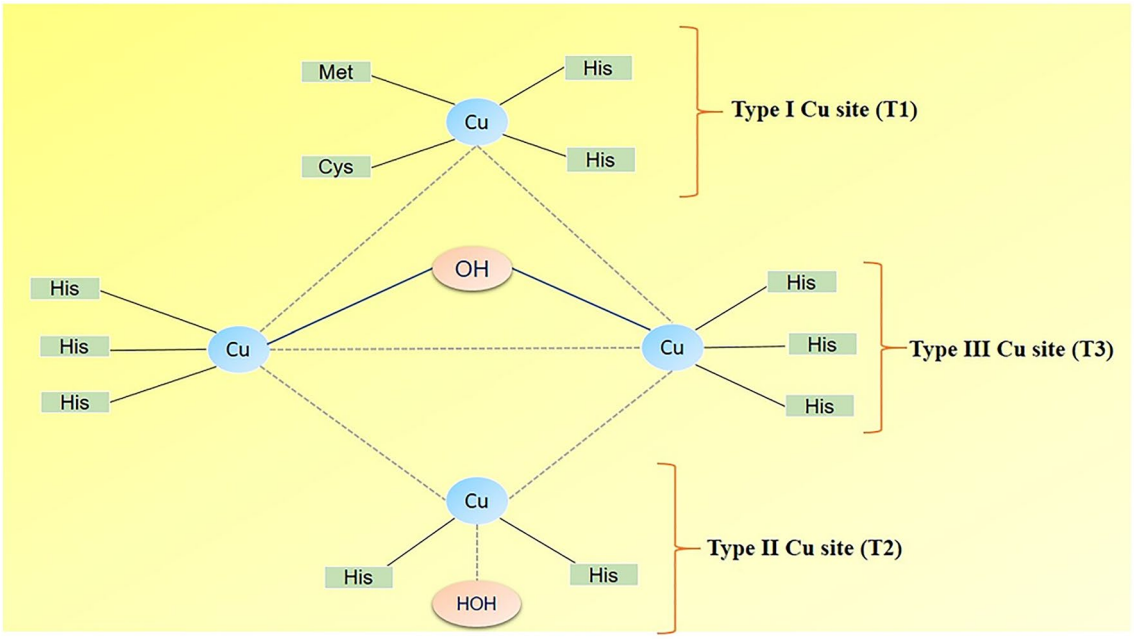

Fig. 1 Structure of copper reaction center of laccase enzyme

exhibited maximum laccase activity compared to other cyanobacterial species and also showed appreciable estrone degradation ability [14, 19]. Thus, in the present study the crude and partially purified laccase enzyme was isolated from *Spirulina* CPCC-695 and their estrone degradation ability was studied. It involved the isolation, purification, and characterization of laccase (crude and partially purified) from *Spirulina* CPCC-695 and the determination of their estrone degradation potential.

2 Materials and Methods

2.1 Chemicals and Cultivation of Cyanobacteria

All the chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA). The freshwater cyanobacterium *Spirulina* CPCC-695 was procured from the University of Madras and grown in BG-11 positive medium. The cultures were maintained at 27 ± 2 °C under a 12:12 light:dark photoperiod supplied by cool white fluorescent tubes at 25 µmol photons min^{-1} light intensity [20].

2.2 Laccase Production, Isolation and Purification

For laccase production, cyanobacterial cultures were induced with 100 µM guaiacol and harvested after four days of growth. Laccase was isolated by centrifuging the spent medium at 6000 rpm for 15 min and then filtering the supernatant so obtained through Whatman No.1 filter paper which was further used as the crude enzyme.

For purification, the crude culture filtrate was concentrated using Amicon® Ultra-15 Centrifugal Filter Unit (Millipore; Billerica/MA, USA) and reduced to 10 times its present volume. This was followed by cold-acetone precipitation in which 4 times chilled acetone was added and then stored at − 20 °C for 1 h. The mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. After centrifugation, the supernatant was discarded and the residual pellet was air-dried to remove any acetone residues. The pellet was then dissolved in a minimal amount of 50 mM tris buffer (pH 8.0) and extensively dialyzed against the same buffer using the Sigma-Aldrich dialysis tubing cellulose membrane of pore size 10 kDa at 4 °C for 24 h (St. Louis, Missouri, USA). The fraction obtained after dialysis was the partially purified form of the protein. The total protein concentration (mg/ml) was estimated using bovine serum albumin as a standard [21]. Total activity, specific activity, purification fold and yield of the protein after each purification steps were calculated according to the given formulae:

$$TotalActivity(U) = Enzyme\ activity \times Total\ volume$$

$$Specificactivityv(U/mg) = \frac{Enzyme\ activity}{Protein\ concentration}$$

$$PurificationFold = \frac{Specific\ activity\ of\ each\ step}{Specific\ activity\ of\ the\ first\ step}$$

$$Yield(\%) = \frac{Total\ activity\ of\ each fraction}{Total\ activity\ of\ crude\ extract} \times 100\%$$

UV-absorbance spectrum of crude and partially purified laccase was analysed by scanning from 200 to 700 nm at room temperature on a Motras Scientific Spectrophotometer (India).

2.3 Laccase Activity

Laccase (EC 1.10.3.2) activity was measured according to the protocol of Bourbonnais et al. [22] based on the oxidation of the substrate 2,2′-azino–bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS). ABTS oxidation rate was determined by taking absorbance at 420 nm. The reaction mixture contained 800 µl sodium citrate buffer (0.1 M, pH 4.0 at 27 °C), 100 µl ABTS (2 mM), 100 µl culture filtrate. The mixture was then incubated for 10 min at room temperature and the absorbance was measured at 420 nm. One unit of activity was defined as activity of an enzyme that catalyses the conversion of one mole of ABTS per minute.

$$Laccase\ Activity(U/L) = \frac{A_{420} \times Total\ volume \times Incubation\ time}{Sample\ volume \times (3.6 \times 10^4)}$$

2.4 Physicochemical Characterization of the Crude and Partially Purified Laccase

2.4.1 Molecular Weight Determination and Zymography Analysis

The crude and partially purified laccase enzyme were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [23]. The molecular mass of purified laccase was determined using medium range (14.3–97.4 kDa) molecular weight markers (GeNei™, Genei Laboratories Private Limited, Bangalore, India). For detection of laccase activity, zymography technique under non-denaturing and non-reducing conditions with (SDS-PAGE) was used [23]. The samples were loaded into the wells along with native dye (without sodium dodecyl sulfate and β-mercaptoethanol) which were then electrophoresed at 50 V through the stacking gel and at 100 V through the resolving gel using Power Supply-EPS 1001 (Amersham Pharmacia Biotech, UK). The gel was then placed in ABTS solution (2 mM) prepared in citrate buffer (0.1 M, pH 4.0). An appearance of a green–blue band at room temperature will indicate the presence of laccase enzyme [24].

2.4.2 Effect of Temperature and pH

The optimum temperature for the crude and partially purified laccase enzyme was determined using ABTS as substrate in citrate buffer (pH 4.0). The effect of temperature (20–80 °C) on their activity were determined by incubating the reaction mixture at these temperatures for 10 min. To determine the pH optima of the enzymes, ABTS solution (2 mM) was prepared in Britton-Robinson buffer having pH in the range 2.0–11.0 and the reaction mixture was incubated at optimum temperature for 10 min. Buffers with respective pH were used as control.

For determination of thermal stability, enzyme samples (both crude and partially purified) were incubated for 1 h at different temperatures (30–80 °C) at optimum pH. Samples were taken at the interval of 15, 30, 45 and 60 min, and relative enzyme activity was determined. Similarly, to determine the pH stability, the enzymes were incubated in 0.1 M Britton-Robinson buffer (pH 2.0–7.0) for 1 h and relative activity was calculated.

2.4.3 Effect of Substrate Concentration and Kinetics

ABTS (2–20 mM) was used as the substrate to examine its effect on the activity of the crude and the partially purified enzyme. Control was prepared for each concentration by using the same substrate concentration but adding dead enzyme.

The kinetic parameters were determined for crude and partially purified laccase at the optimal temperature and pH by measuring the initial reaction velocity at 0–6 mM ABTS. Michaelis–Menten constant (Km) and maximum reaction velocity (Vmax) were calculated from Lineweaver–Burk plot [25], which were further used to calculate the catalytic constant or turnover number (Kcat) and specificity constant (Kcat/Km). The accuracy of the obtained values was confirmed by comparing them to the values obtained through Michaelis–Menten curve fit using GraphPad Prism 8.0.1 software.

2.5 Catalytic Characterization of the Crude and Partially Purified Laccase

2.5.1 Substrate Specificity

The substrate specificity of the laccase enzymes was studied for both phenolic (o-cresol, p-cresol, phenol, syringaldazine and guaiacol) and non-phenolic substrates (ABTS), to check whether the purified laccase is capable of degrading both types of pollutants or not. Substrates were added to the reaction mixture at a concentration of 4 mM and relative enzyme activity was calculated.

2.5.2 Effect of Metal Ions

To determine the effect of different metal ions on enzymes activity, they were preincubated for 15 min with 4 mM of each of these metal ions including $MgSO_4$, $MnSO_4$, $FeSO_4$, KCl, NaCl, $CaCl_2$ and $BaCl_2$. Then, substrate (4 mM ABTS) was added and enzyme activity was assayed. The control was not exposed to metal ions.

2.5.3 Effect of Inhibitors

The effect of inhibitors like ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), sodium azide, dithiothreitol (DTT), cysteine and urea on laccase activity was studied at a concentration of 4 mM. Control was prepared having no inhibitor.

2.6 Estrone Degradation Ability of Laccase Enzymes

The degradation ability of laccase was checked according to the method of Sei et al. [26] with some modifications. Reaction mixture (3 ml) was prepared in a 10 ml test tube containing 20 ppm estrone, 100 mM citrate buffer (pH 3.0), 200 U/l enzyme. Reaction was performed at 30 °C for 24 h. Amount of estrone was quantified every 6 h. Absorbance was taken at 266 nm. Degradation efficiency was calculated according to the given relation:

$$Degradation\ efficiency(\%) = [(A_c - A_t) \div A_c] \times 100$$

where, A_c is the initial concentration of estrone and A_t is the concentration of estrone at time t.

Amount of estrone was calculated using the regression equation. The standard curve of estrone was plotted by recording absorbance at 266 nm against acetone as blank. The regression equation so obtained was used to calculate the amount of estrone. The calibration curve was described by the equation given below with regression co-efficient (r^2) being 0.9894.

$$Y = 0.02791 \times X + 0.1238$$

where, Y is absorbance at 266 nm and X is the amount of estrone in ppm.

2.7 Statistical Analysis

Experiments were conducted in triplicate, and the mean values and standard deviations (SD) were calculated from the different replicates ($n = 3$). Statistical analysis was performed by using the Graph Pad Software 8.1, San Diego, California,

USA. Before carrying out the statistical tests the normality of the data obtained was checked using the Shapiro–Wilk *W* test, at α level of 0.05 (Null hypothesis is that the data are normally distributed, which is rejected if *p*-value < 0.05 and is not rejected if *p*-value > 0.05). All the data were normally distributed and gave a straight line in Q-Q Normal plot. Further, to confirm the validity of the variability of results, data were subjected to unpaired t-test analysis (in case of means of two groups) and one way ANOVA (in case of means of more than two groups). $p < 0.05$ was considered as significant.

3 Results

3.1 Purification of Laccase Enzyme

The filtered spent medium of four-day-old *Spirulina* CPCC-695 culture grown in BG-11 medium was used as the extracellular crude laccase enzyme. During purification, acetone precipitation of 10 times concentrated spent medium (crude enzyme) followed by dialysis resulted in a colourless protein having four-fold purity with a yield of 45.56% and a specific activity of 204.14 U/mg (Table 1).

The absorbance pattern obtained at successive steps of purification exhibited presence of laccase with peaks in the range of 250–300 nm, however no peaks were observed at 600 nm. The partially purified extracellular laccase exhibited a very broad absorbance spectrum with a shoulder peak around 330 nm. Also, the absorbance decreased from crude to partially purified enzyme due to decrease in the protein concentration (Fig. 2).

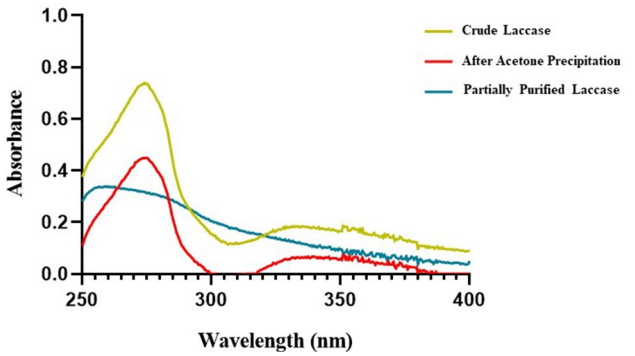

Fig. 2 Absorption spectra of crude laccase, after acetone precipitation and the partially purifiedlaccase form of *Spirulina* CPCC-695

3.2 Physicochemical Characteristics of Crude and Partially Purified Laccases

3.2.1 Molecular Weight and Activity Analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the laccases enzyme exhibited a molecular weight of approximately 80 kDa. During zymography (substrate gel electrophoresis) when the native gel was placed in ABTS solution (2 mM) in citrate buffer (0.1 M), a blue–green colour band appeared that confirmed the presence of laccase (Fig. 3a and b).

3.2.2 Optimal Temperature and pH

Laccase activity was maximum at 40 °C which then declined at higher temperatures. With the increase in temperature, the laccases activity significantly decreased ($F = 1.361$; significant at $p < 0.05$). The enzyme, however, retained almost 60% activity at 60 °C (Fig. 4a). Similarly, an increase in pH also significantly decreased the laccase activity ($F = 1.083$; significant at $p < 0.05$). The enzyme activity was much higher under acidic conditions, i.e., from pH 2.0 to pH 6.0 than in

Table 1 Summary of purification of laccases from *Spirulina* CPCC-695

Purification step	Protein concentration[a] (mg/ml)	Total Activity[b] (U)	Specific Activity[c] (U/mg)	Purification fold[d]	% Yield[e]
Crude	2.2	98.57	44.8	1	100
First Concentration	1.5	92.98	61.99	1.39	94.33
Acetone precipitation	0.75	55.23	73.64	1.64	56.05
Partially Purified	0.22	44.91	204.14	4.56	45.56

[a]Calculated through Lowry analysis

[b]Total activity = Enzyme activity x total volume

[c]Specific activity = Enzyme activity/protein concentration

[d]Purification fold = Sp. activity of each step/ Sp. activity of first step

[e]% Yield = Total activity of each fraction/Total activity of crude extract

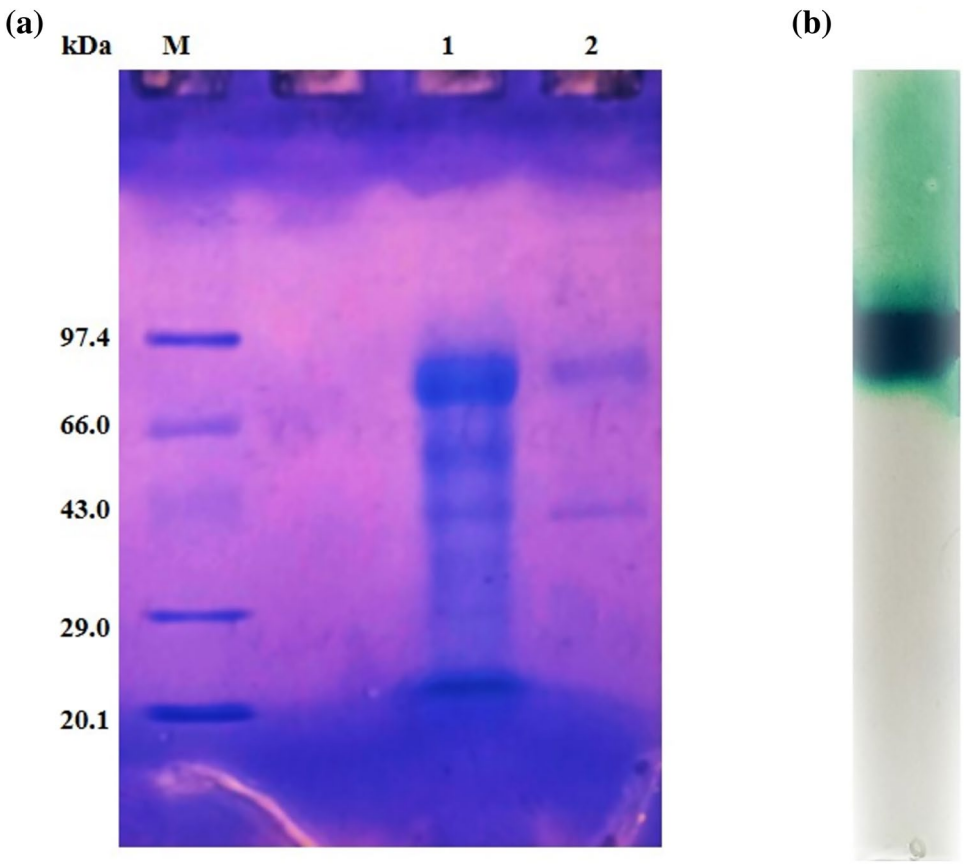

Fig. 3 **a** SDS-PAGE profile of laccases isolated from *Spirulina* CPCC-695 M: Marker, Lane 1:Crude laccase Lane 2: Partially purified laccase **b** Zymogram-analysis of partial-lypurified laccase

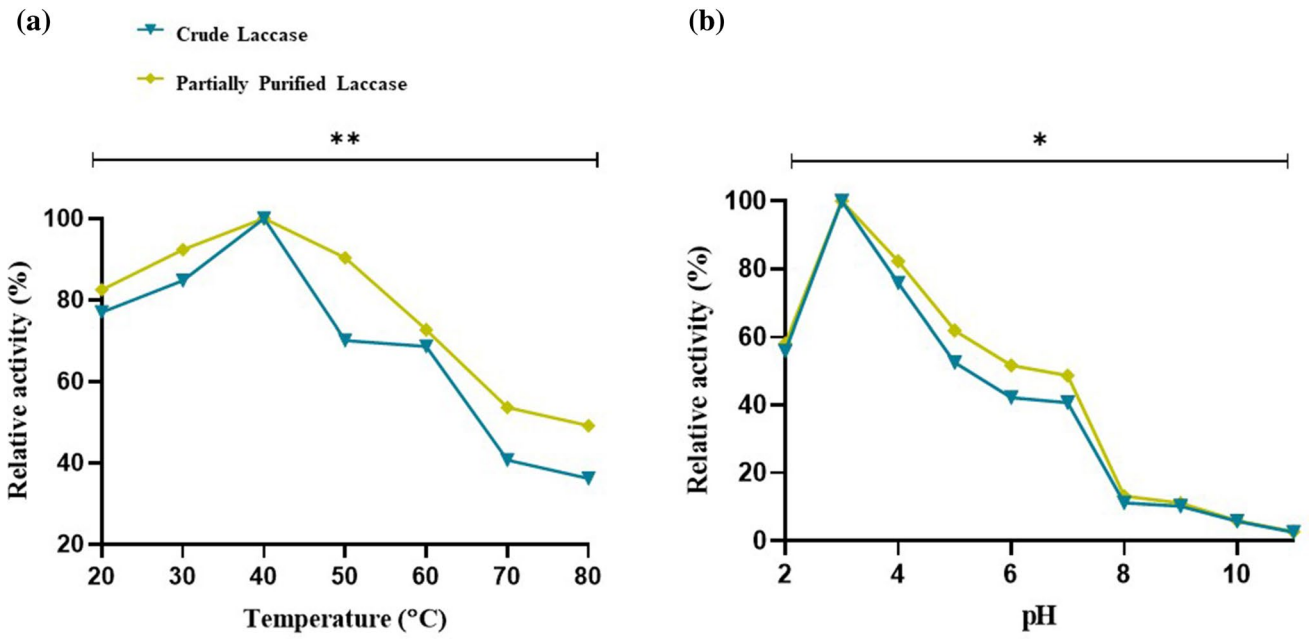

Fig. 4 **a** Effect of temperature on the relative laccase activity of *Spirulina* CPCC-695 **b** Effectof pH on laccase activity of *Spirulina* CPCC-695. Values are means of three replicates withstandard deviation and asterisks (*) indicate level of significant at $p < 0.05$

alkaline conditions, i.e., from pH 8.0 to pH 11.0. Maximum activity was observed at pH 3.0 (Fig. 4b).

Incubation of enzyme for 60 min at 30, 40, 50, 60, 70 and 80 °C at optimal pH (3.0) to check its thermal stability showed that laccase was highly stable at 40 °C (relative activity of 100%) even after 60 min exposure (crude: $F = 6.131$, partially purified: $F = 6.345$; significant at $p < 0.05$) (Fig. 5a and b). Thermal stability of partially purified laccase at 50 °C after 60 min was higher, i.e., 73.46% than the crude laccase (70.68%) that decreased to 58.31 and 56.92% respectively at 60 °C.

Similarly, incubation of enzyme at pH 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 at optimal temperature (40 °C) showed constant highest activity at the optimum pH 3.0, i.e., the enzyme possessed highest stability even after 60 min exposure. Alkaline condition exhibited adverse effects on the enzyme stability (crude: $F = 4.502$, partially purified: $F = 5.022$; significant at $p < 0.05$) (Fig. 6a and b).

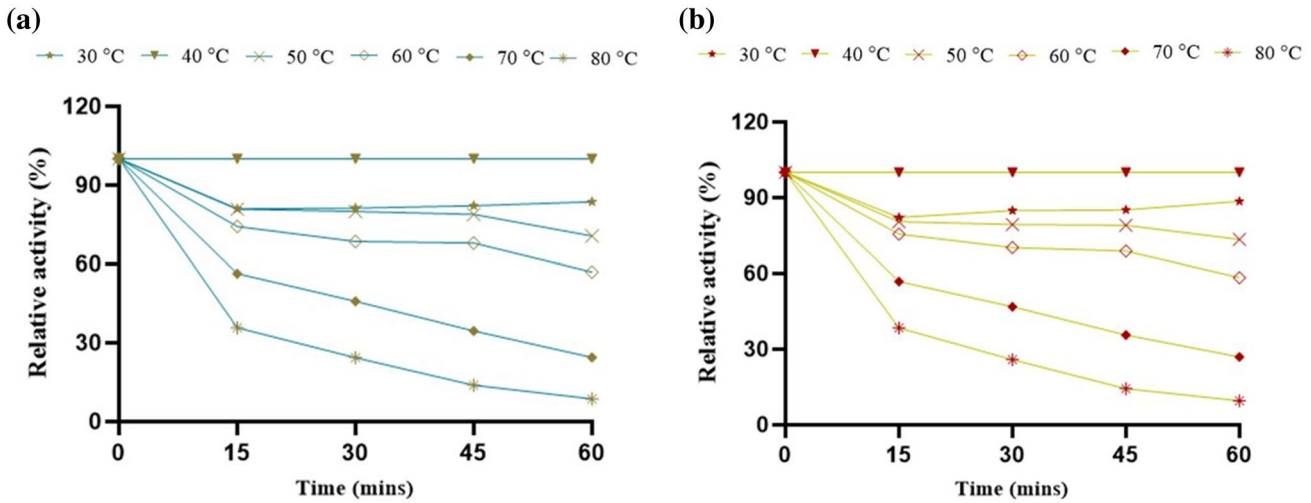

Fig. 5 Thermal stability of **a** crude laccase **b** partially purified laccase of *Spirulina* CPCC-695. Values are means of three replicates with standard deviation and asterisks (*) indicate level of significant at $p < 0.05$

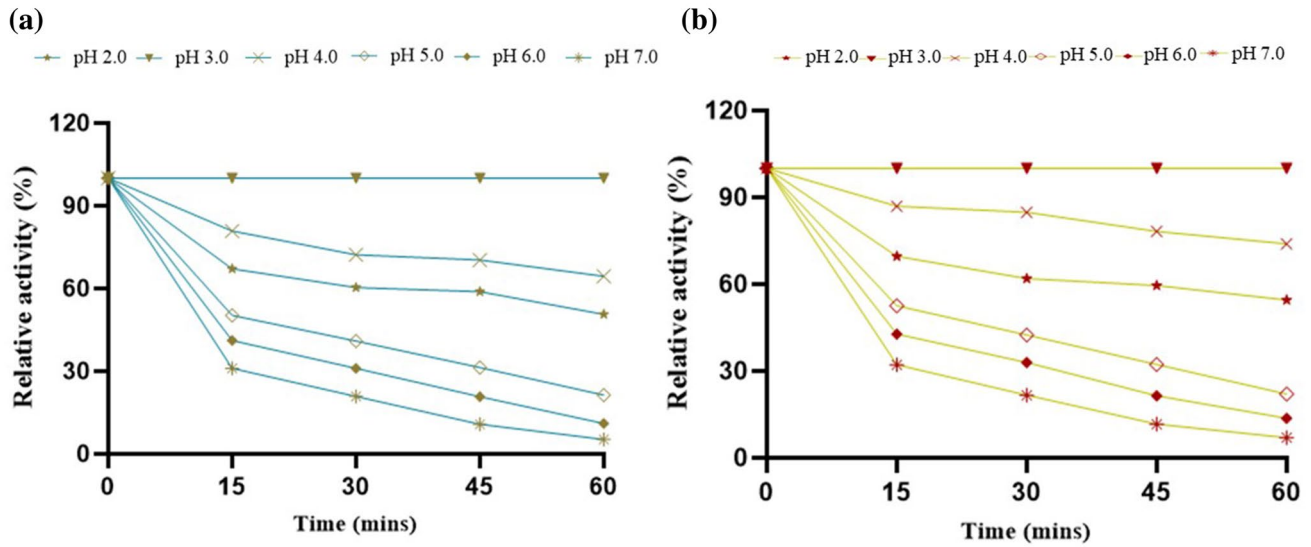

Fig. 6 pH stability of **a** crude laccase **b** partially purified laccase of *Spirulina* CPCC 695. Values are means of three replicates with standard deviation and asterisks (*) indicate level of significant at $p < 0.05$

3.2.3 Optimal Substrate Concentration and Kinetic Constants

The activity of crude and partially purified enzyme increased with increasing substrate concentration and was maximum with 4 mM ABTS ($F = 1.873$, significant at $p < 0.05$) (Fig. 7a). The kinetic parameters Km, Vmax, kcat, and kcat/Km values obtained from Lineweaver- Burk plots and through Michaelis–Menten curve fit, under optimized conditions utilizing ABTS (0–6 mM) were same (Fig. 7b; Table 2). The results suggested that the purified enzyme was more efficient than the crude enzyme.

3.3 Catalytic Properties of Crude and Partially Purified Laccases

3.3.1 Substrate Specificity

Among phenolic (o-cresol, p-cresol, phenol, syringaldazine and guaiacol) and non-phenolic (ABTS) aromatic compounds, the best substrate for *Spirulina* CPCC-695 laccases (crude and partially purified) was ABTS at a concentration of 4 mM in 0.1 M citrate buffer (pH 3.0) under optimal conditions ($F = 1.046$, significant at $p < 0.05$) (Fig. 8a). The order of catalytic efficiency was as follows:

ABTS > o-Cresol > Guaiacol > p-Cresol > Phenol > Syringaldazine

Table 2 Kinetic constants of *Spirulina* CPCC-695 laccases

Laccase form	Km (mM)	Vmax (U/ml)	Kcat/(min)	kcat/Km/(min/mM)
Crude	0.834	494.5	0.0204	0.024
Partially purified	0.585	480.5	0.0198	0.034

3.3.2 Metal Ions Effect

The enzyme activity in presence of barium, calcium, manganese, magnesium, sodium, potassium and iron metals was less than the control, i.e., in absence of metal ions ($F = 1.124$, significant at $p < 0.05$). Maximum inhibition in enzyme activity was observed with iron metal ions (Fig. 8b). The order of enzyme activity inhibition was found as below:

$$BaCl_2 > CaCl_2 > MnSO_4 > MgSO_4 > NaCl > KCl > FeSO_4$$

3.3.3 Inhibitors Effect

The oxidase inhibitor (SDS, Cysteine, Urea, EDTA, DTT, Sodium azide) affected the activity of partially purified enzyme at greater extent than the crude enzyme ($F = 1.029$, significant at $p < 0.05$) (Fig. 8c). The order of inhibition (increasing activity) could be ordered in the following manner:

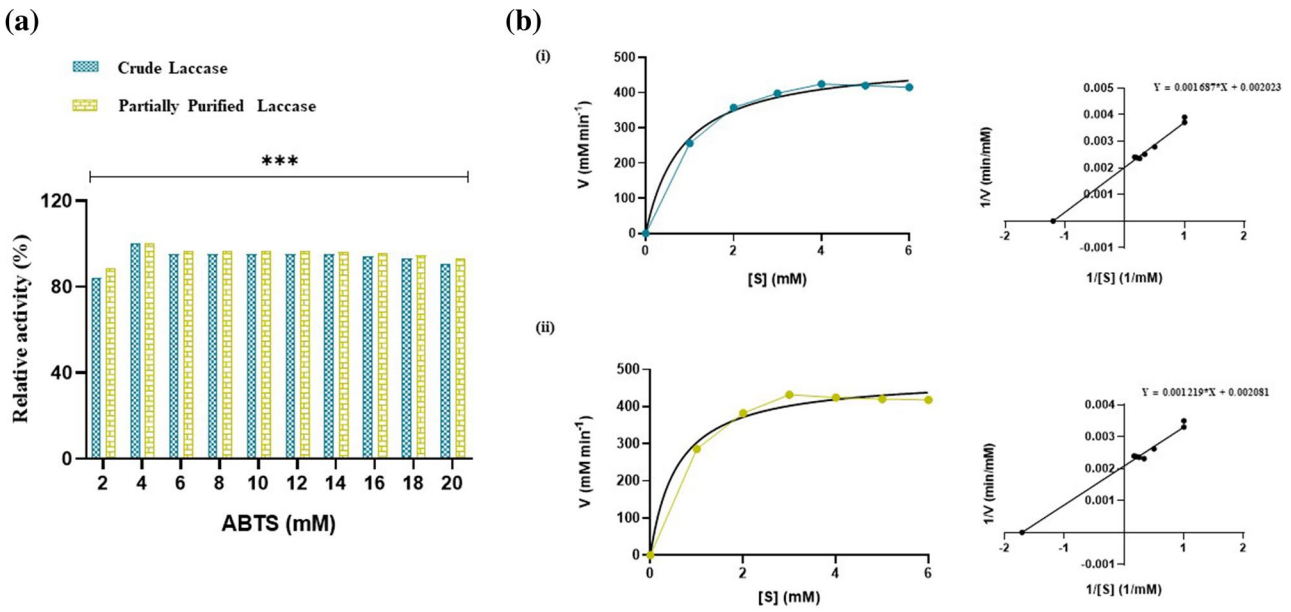

Fig. 7 **a** Effect of substrate concentration on laccase activity of *Spirulina* CPCC 695. Values are means of three replicates with standard deviation and asterisks (*) indicate level of significant at $p < 0.05$. **b** Michaelis–Menten curve fit using GraphPad Prism Software 8.0. and Lineweaver- Burk Plot to calculate kinetic constants for (i) Crude laccase form (ii) Partially purified laccase

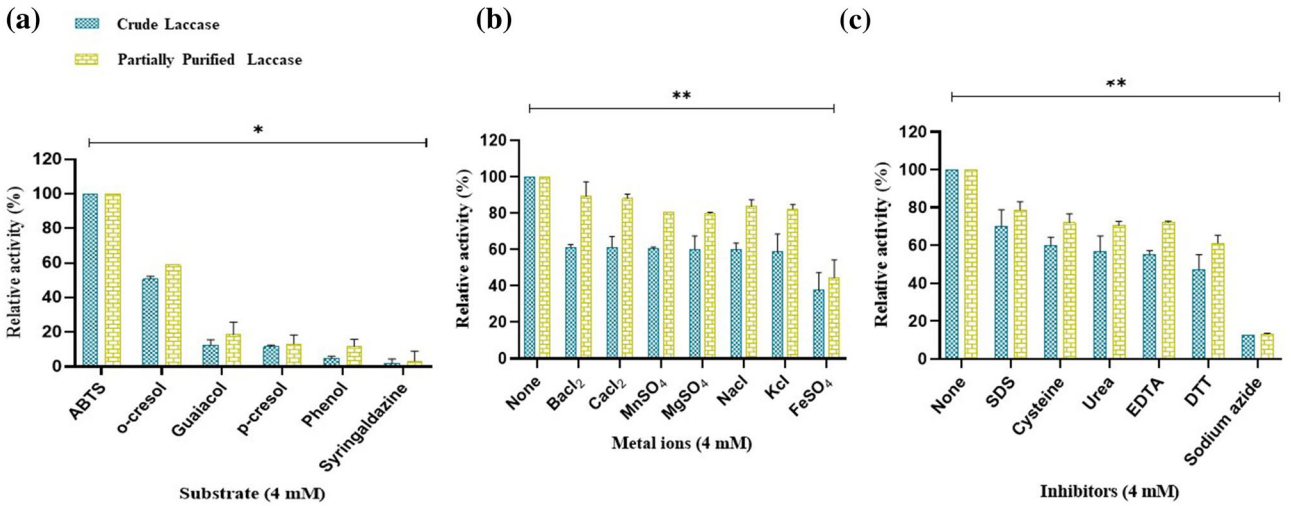

Fig. 8 **a** Substrate specificity of crude and partially purified laccase **b** effect of metal ions **c** effect of inhibitors on the activity of crude and partially purified laccase of *Spirulina* CPCC-695. Values are means of three replicates with standard deviation and asterisks (*) indicate level of significant at $p < 0.05$

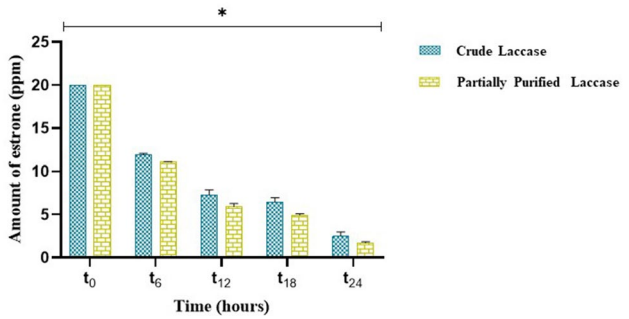

Fig. 9 Estrone degradation ability of the crude laccase form and the partially purified form of *Spirulina* CPCC-695. Values are means of three replicates with standard deviation and asterisks (*) indicate level of significant at $p < 0.05$

Sodium azide > DTT > EDTA > Urea > Cysteine > SDS

3.4 Estrone Degradation by Crude and Partially Purified Laccase

Estrone was treated with both crude and partially purified laccase (200 U/L) at 30 °C. A noticeable reduction (gradual decrease) in estrone concentration was observed with increasing exposure time which was maximum after 24 h of laccase treatment. Degradation efficiency was observed as 87.55% with crude and 91.35% with purified enzyme ($F = 1.135$, significant at $P < 0.05$) (Fig. 9).

4 Discussion

Laccases were first isolated from the Japanese lacquer tree *Rhus vernicifera* [27]. Since then, they have been isolated from plants, fungi and bacteria [28]. However, only few studies are available on algal and cyanobacterial laccase [17, 18, 29, 30]. Laccases have been studied with respect to their biological function, substrate specificity, copper binding structure, and industrial applications [31]. But their role in degradation of endocrine disrupting compounds is very limited including our preliminary studies [14, 19]. So, the present study was planned that involved the purification and characterization of laccases from *Spirulina* CPCC-695 and determination of their estrone degradation potential.

Extracellular partially purified laccase was purified from the culture filtrate of *Spirulina* CPCC-695 through repeated concentration, acetone precipitation and dialysis. Partially purified laccase was colourless (characteristic feature of white/yellow laccase) and exhibited four-fold purity with approximately 46% enzyme yield. However, [17, 18] reported 3.3 fold purification of laccase isolated from *Spirulina platensis* CFTRI with a yield of 51.5%. Mukhopadhyay and Banerjee, [32] purified laccase from *Lentinus squarrosulus* MR 13 and reported 2.77-fold purification with an enzyme yield of 45%. Aslam et al. [33] and More et al. [34] reported laccase of even less purity isolated from *Cladosporium cladosporioides* (1.88) and *Pleurotus sp.* (2.97) but with a higher yield of 65 and 62% respectively.

Spirulina CPCC-695 crude laccase showed maximum absorption at 275 nm which shifted to 255 nm in case of partially purified laccase with a shoulder peak around 330 nm that represents the presence of the T3 binuclear copper pair

[35]. However, no absorption peak corresponding to a T1 blue copper was observed around 600 nm which may be due to the incomplete oxidation of copper, a typical characteristic of white/yellow laccase. Thus, the colourless laccases purified from *Spirulina* CPCC-695 can be grouped as atypical laccase (white/yellow). Fungal laccases isolated from *Trematosphaeria mangrovei* exhibited absorption peak in the range of 260–280 nm [36]. Recently, the colourless protein solution for yellow laccase purified from *Stropharia sp.* ITCC-8422 also showed maximum absorbance around 280 nm but lacked absorption maximum at 605 nm [37]. Moreover, white laccase isolated from *Pleurotus ostreatus* [38], *Panus conchatus* [24], *Myrothecium verrucaria* NF-05 [39], *Myrothecium verrucaria* ITCC 8447 and *Lentinus squarrosulus* MR 13 [32] also lacked the peak around 600 nm but showed absorption in the near UV region.

Laccases (p-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) are the blue multicopper oxidases that are glycoproteins having a molecular mass between 50 and 130 kDa [4, 5, 40]. The partially purified laccase from *Spirulina* CPCC-695 possessed a molecular weight of approximately 80 kDa when analysed by SDS-PAGE. Not much has been reported on cyanobacterial laccase, though, *Spirulina platensis* CFTRI laccase exhibited a low molecular weight of 66 kDa. Laccase isolated from *Chaetomium thermophilium* and *Phlebia tremellosa* showed approximately similar molecular mass of 77 kDa and 75 kDa as seen in the present study [41, 42]. Laccase from other species like basidiomycetous *Coriolus obhirsutus* [43] and *Trametes sp.* [44] possessed molecular mass of 73 kDa and 62 kDa respectively. Many fungal laccases isolated from *Pleurotus ostreatus* 1804 [45], *Lentinus squarrosulus* MR 13, *Pleurotus ostreatus* [32], *Aspergillus niger* [46], and bacteria *Klebsiella pneumoniae* [47] exhibited a molecular mass of 66 kDa. Activity staining in presence of the substrate ABTS resulted in the development of green colour bands, that has been also reported in many fungi like *Pischia pastorii* [48], and cyanobacteria *Phormidium valderianum* [49] and *Spirulina platensis* CFTRI [17, 18].

Optimum pH and temperature are the essential conditions for industrial applications of enzymes. *Spirulina* CPCC-695 laccase exhibited its optimum activity at pH 3.0 with ABTS as substrate, which decreased at neutral and basic conditions. It was observed that the enzymes were completely inactivated at pH-11, which might be due to the binding of a hydroxide anion to the trinuclear coppers of laccase that disturbs the internal electron transfer from T1 to trinuclear centre and ionization of pI [50]. According to Xu [51], both the redox potential difference [$\Delta E0 = E0$ (laccase T1) $- E0$ (substrate, single electron)] between a reducing substrate and the T1 center, and the hydroxyl ions inhibition at the T2/T3 center affects the pH activity profile of a laccase towards phenolic substrates. When the pH increases, the T2/T3 cluster can accept hydroxyl ions, which competes with the $\Delta E0$ contribution, interrupts the trafficking of electrons from T1 copper and decreases laccase activity. Previous studies from our lab, during role of laccase in dye degradation, found the optimum pH of *Spirulina* strain laccase was 3.0 with ABTS as substrate [17, 18]. Fungal laccases also displayed optimum activity in the pH range of 3.0–5.5 (acidic condition) and gets inactivated at neutral and alkaline pH [4, 51–55]. However, fungal laccase isolated from *Lentinus crinitus* and *Psilocybe castanella* exhibited maximum activity at pH 3.5 and pH 4.5 respectively [56].

Laccase generally possesses the temperature optima between 30 and 50 °C and rapidly loses activity at temperatures more than 60 °C [40, 57]. The optimum temperature for *Spirulina* CPCC-695 laccase was found to be 40 °C. The temperature increase from 20–40 °C enhanced the enzyme activity. According to Irshad et al. [58], this possibly occurs by enhancing the kinetic energy of the molecules and increasing the interaction between enzyme active site and interacting groups of the substrate. Laccase isolated from *Spirulina platensis* CFTRI showed maximum activity at 30 °C and retained almost 97% activity at 40 °C [16]. This may be due to the different cultural conditions, utilization of different nutrients for its metabolic activity, stress mediated enzyme production and variation in gene expression. Laccase from *Amorphophallus campanulatus* also showed optimal activity at 40 °C that gets inhibited at higher temperatures [59]. Fungal laccase isolated from *Schyzophylum commune* [58], *Fomitiporia mediterranea* [60], *Trematosphaeria mangrovei* [36] and the bacterial laccases isolated from *Aquisalibacillus elongates* [61] and *Bacillus subtilis* MTCC 2414 [62] have also shown a temperature optima of 40 °C.

At optimal temperature (40 °C), laccase exhibited no change in its activity with increasing exposure time upto one hour. The enzyme retained approximately 57% of its activity after being heated at 60 °C for 60 min. However, the extent of decrease was more pronounced at higher temperature, i.e., 70 and 80 °C. This suggested that at optimal temperature the laccase enzyme was stable against thermal denaturation. It is known that the source of the microorganism affects the thermal stability patterns of laccase. Atalla et al. [36] also observed that *Trematosphaeria mangrovei* laccase showed maximum stability at optimum temperature (40 °C) even after 60 min exposure. Above and below this temperature there occurred loss in enzyme activity. Laccase isolated from *Trametes trogii* S0301 [63], *Trametes versicolor* [64] and *Trametes* sp. MA-X01 [65] also showed similar thermal stability trend, i.e., maximum thermal stability at its optimum temperature and the stability was less at higher temperatures, i.e., 70 and 80 °C. The presence of hydrophobic or charged residues that increases enzyme rigidity and restrict

conformational changes during substrate binding influence enzymes thermal stability [66].

Spirulina CPCC-695 exhibited maximum stability at the optimum pH (3.0) and showed 100% relative activity which decreased at neutral pH. Crude enzyme retained almost 64% activity and the purified form retained almost 74% activity at pH 4.0. The enzyme was unstable at neutral and extreme acidic pH (2.0). pH stability in the acidic range is rare among laccases. *Spirulina platensis* CFTRI, however, retained 100% activity at pH 8.0 [17, 18]. Laccase isolated from *Trematosphaeria mangrovei* also showed maximum stability at its optimum pH value that decreased when moved away from optima [36].

To measure the kinetic properties of laccase enzyme reacting with ABTS, Km (the substrate concentration at which the velocity rate is half maximal), Vmax (the rate of reaction when the enzyme is saturated with substrate), kcat (the number of substrate molecules turned over per enzyme per molecule per second), and the ratio kcat/Km, called as specificity constant were determined using GraphPad Prism and compared to the values obtained through Lineweaver–Burk Plot. Both the methods gave the same values for the kinetic parameters showing that the fit to the Michaelis–Menten equation using the software was perfect. Partially purified laccase exhibited a low Km value (0.60 mM), higher kcat (24.02 min^{-1}), and higher kcat/Km ratio (41.06) suggesting that it is more efficient and possess a higher affinity for ABTS than the crude enzyme, which showed higher Km values (0.83 mM), low kcat (17.98) and a lower kcat/Km ratio (21.56) reflecting its lesser efficiency. Algae and cyanobacterial laccase enzyme kinetics have not been studied so well till date. Higher Km values of 1.42 and 2.5 mM has been reported in laccase purified from *Trematosphaeria mangrovei* and *Trametes atroviride* [36, 67]. *Trametes versicolor* laccase exhibited Km values in the range of 0.01–45 mM (brenda-enzymes.org). Km values similar to the present study has been reported in laccase isolated from *Chalara (syn. Thielaviopsis) paradoxa* CH 32 (0.77 mM) [68]. However, much lower Km values have been reported in laccase isolated from *Bacillus subtilis* (0.106 mM) [69],*Pleurotus sp.* (0.25 mM) [34] and the Lac 1 isoform of *Pleurotus nebrodensis* (0.16 mM) [55], *Cerrena maxima* (0.06 mM) [70] and *Schyzophylum commune* IBL-06 (0.025 mM) [58].

Spirulina CPCC-695 laccase exhibited broad substrate specificity (ABTS, o-cresol, p-cresol, phenol, guaiacol and syringaldazine) and showed highest affinity towards ABTS. Laccases isolated from other organism like green algae *Tetracystis aeria* [29] and many fungi including *Trametes trogii* [71], *Pycnoporus sanguineus* [72], *Galerina sp.* [73], *Fomitella fraxinea* [74], *Sporothrix carnis* CPF-05 [75] and *Trametes polyzona* WRF03 [76] have also shown maximum activity with ABTS. According to Johannes and Majcherezyk [77], laccase oxidizes ABTS to a cation radical, i.e., ABTSazine ($ABTS^{+}$) and subsequently to $ABTS^{2+}$ which results in formation of blue green colour. The preference of laccase towards various phenolic substrates depends on the type and relative positions of the substituent groups attached to their respective benzene ring [78]. Generally, laccase activity increases from meta- through para- to ortho-substituted phenols [4]. Laccase showed higher activities against the ortho-substituted substrates (o-cresol, guaiacol) as compared to the p-cresol in the present study. Further, the number and position of the hydroxyl groups on the aromatic ring, redox potential of phenolic substrates also affect the oxidation of the ortho-substituted phenols. In case of phenolic substrates, transfer of one electron takes place first from the substituent group to the type 1 copper atom at the active site of the enzyme and produces highly unstable aryloxy radicals that undergoes further reduction or polymerization to generate resonating structures that form coloured products [79]. However, it showed a limited specificity towards syringaldazine (a dimer of two molecules of 2, 6 dimethoxyphenol linkedby an azide bridge), these findings were in congruence with the result obtained by Niladevi et al. [80] and Abdelgalil et al. [81] in case of laccase isolated from *Streptomyces psammoticus* and *Alcaligenes faecalis* respectively.

Metal ions (Ba^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, K^{+}) showed no effect on laccase activity and maximum inhibition was elucidated by Fe^{2+} ions. Sadhasivam et al. [82] also reported that K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ba^{2+} and Ca^{2+} had no significant effect over *Trichoderma harzianum* WL1 laccase activity. More et al. [34] also observed that metal ions Cu^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Hg^{2+} does not stimulated the laccase enzyme activity. Thermostable laccase isoenzyme (Lac 37 II) produced by *Trametes trogii* S0301 also showed no change in its enzyme activity in presence of Na^{+}, Mn^{2+}, Mg^{2+}, Co^{2+}, and Cu^{2+} [63]. The inhibitory effect of Fe^{2+} on laccase activity has been also observed in laccase isolated from *Alcaligenes faecalis* [81], *Trematosphaeria mangrovei* [36], *Pleurotus sp.* [34], *Trametes versicolor* [83], *Trametes trogii* S0301 [63]. Fe^{2+} ion acts as a reducing agent and competes with laccase to oxidize ABTS to ABTS cations, thus inhibiting the laccase activity [84].

Laccases isolated from *Spirulina* CPCC-695 were completely inhibited by 1 mM sodium azide and DTT as expected. Fungal laccases isolated from *Trematosphaeria mangrovei* [36], *Trichoderma atroviride* [67], *Pleurotus sp.* [34] *and Fomitella fraxinea* [53] also got inhibited by sodium azide and DTT. According to Ryan et al. [85], sodium azide binds to the type 2 and 3 copper site, affects internal electron transfer and inhibits laccase activity. *Spirulina* CPCC-695 laccase retained almost 75% of its activity in presence of SDS which was almost similar to *Trichoderma harzianum* WL1 laccase that retained almost 69.04%

of its activity. Further, EDTA (15%) showed less inhibition on laccase activity, similar to *Trichoderma harzianum* laccase which was mildly inhibited by the metal chelator EDTA (16.8% inhibition) [82].

The high biodegradation percentage of estrone by laccase can be explained by the presence of electron donating functional groups, i.e., the hydroxyl group at position 3 in the ring-A which induces the electrophilic attack by laccase [86, 87]. Estrone was degraded by 88 and 91% after 24 h from crude and partially purified laccase isolated from *Spirulina* CPCC-695. Sei et al. [26] and Kim et al. [42] confirmed estrone biodegradation potential of laccase isolated from non-photosynthetic organism like white rot fungus and *Phlebia tremellosa* respectively.

5 Conclusions

The present study showed that laccases from *Spirulina* CPCC-695 are capable to degrade EDCs like estrone. The results obtained from visual observation, and spectral analysis suggest that *Spirulina* CPCC-695 produces atypical laccase. Comparative observations on crude and partially purified laccase for studied parameters including (temperature, pH and substrate specificity) have shown enhanced ability of partially purified enzyme. However, there was not much difference in the estrone degradation efficiency of crude and partially purified enzyme. Thus, in order to make use of laccase more cost-effective, the crude enzyme can be used preferentially as it will save cost and time involved in purification.

Acknowledgements Authors are thankful to Culture Collection Centres of India, University of Madras, for providing the *Spirulina* CPCC-695 species.

Author Contributions NS and TF designed the experiment, BA helped in the protein purification, DY helped in data curation. NS carried out the lab work and wrote the initial manuscript that was corrected and finalised by TF.

Declarations

Competing interest The authors declare that they have no known competing interests.

References

1. Ting YF, Praveena SM (2017) Sources, mechanisms, and fate of steroid estrogens in wastewater treatment plants: a mini review. Environ Monit Assess 189(4):178
2. Reichert G, Hilgert S, Fuchs S, Azevedo JCR (2019) Emerging contaminants and antibiotic resistance in the different environmental matrices of Latin America. Environ Pollut 255:113140
3. Liu ZH, Kanjo Y, Mizutani S (2009) Removal mechanisms for endocrine disrupting compounds (EDCs) in wastewater treatment—physical means, biodegradation, and chemical advanced oxidation: a review. Sci Total Environ 407(2):731–748
4. Baldrian P (2006) Fungal laccases–occurrence and properties. FEMS Microbiol Rev 30(2):215–242
5. Morozova OV, Shumakovich GP, Gorbacheva MA, Shleev SV, Yaropolov AI (2007) "Blue" laccases. Biochem Mosc 72(10):1136–1150. https://doi.org/10.1134/s00062979071001 12
6. Kudanga T, Nemadziva B, Le Roes-Hill M (2017) Laccase catalysis for the synthesis of bioactive compounds. Appl Microbiol Biotechnol 101(1):13–33
7. Hakulinen N, Rouvinen J (2015) Three-dimensional structures of laccases. Cell Mol Life Sci 72(5):857–868
8. Jones SM, Solomon EI (2015) Electron transfer and reaction mechanism of laccases. Cell Mol Life Sci 72(5):869–883
9. Scheiblbrandner S, Breslmayr E, Csarman F, Paukner R, Führer J, Herzog PL, Shleev SV, Osipov EM, Tikhonova TV, Popov VO, Haltrich D (2017) Evolving stability and pH-dependent activity of the high redox potential *Botrytis aclada* laccase for enzymatic fuel cells. Sci Rep 7(1):1–13
10. Claus H (2003) Laccases and their occurrence in prokaryotes. Arch Microbiol 179(3):145–150
11. Lu L, Zeng G, Fan C, Ren X, Wang C, Zhao Q, Zhang J, Chen M, Chen A, Jiang M (2013) Characterization of a laccase-like multicopper oxidase from newly isolated *Streptomyces sp.* C1 in agricultural waste compost and enzymatic decolorization of azo dyes. Biochem Eng J 72:70–76
12. Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. Phytochemistry 60(6):551–565
13. Palanisami S, Saha SK, Lakshmanan U (2010) Laccase and polyphenol oxidase activities of marine cyanobacteria: a study with poly R-478 decolourization. World J Microbiol Biotechnol 26(1):63–69
14. Sami N, Fatma T (2019) Studies on estrone biodegradation potential of cyanobacterial species. Biocatal Agric Biotechnol 17:576–582
15. Chen H, Pan SS (2005) Bioremediation potential of spirulina: toxicity and biosorption studies of lead. J Zhejiang Univ Sci B 6(3):171–174
16. Afreen S, Anwer R, Singh RK, Fatma T (2018) Extracellular laccase production and its optimization from *Arthrospira maxima* catalyzed decolorization of synthetic dyes. Saudi J Biol Sci 25(7):1446–1453
17. Afreen S, Bano F, Ahmad N, Fatma T (2017) Screening and optimization of laccase from cyanobacteria with its potential in decolorization of anthraquinonic dye Remazol Brilliant Blue R. Biocatal Agric Biotechnol 10:403–410
18. Afreen S, Shamsi TN, Baig MA, Ahmad N, Fatima S, Qureshi MI, Hassan MI, Fatma T (2017) A novel multicopper oxidase (laccase) from cyanobacteria: purification, characterization with potential in the decolorization of anthraquinonic dye. PLoS ONE 12(4):e0175144
19. Sami N, Ansari S, Yasin D, Fatma T (2020) Estrone degrading enzymes of *Spirulina* CPCC-695 and synthesis of bioplastic precursor as a by-product. Biotechnol Rep 26:e00464
20. Madkour FF, Kamil AE-W, Nasr HS (2012) Production and nutritive value of *Spirulina platensis* in reduced cost media. Egypt J Aquat Res 38:51–57
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
22. Bourbonnais R, Leech D, Paice MG (1998) Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. Biochim Biophys Acta (BBA) 1379(3):381–390

23. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685
24. Zhao D, Zhang X, Cui D, Zhao M (2012) Characterisation of a novel white laccase from the deuteromycete fungus *Myrothecium verrucaria* NF-05 and its decolourisation of dyes. PLoS ONE 7(6):e38817
25. Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56(3):658–666
26. Sei K, Takeda T, Soda SO, Fujita M, Ike M (2007) Removal characteristics of endocrine-disrupting chemicals by laccase from white-rot fungi. J Environ Sci Health Part A 43(1):53–60
27. Yoshida H (1883) LXIII.—chemistry of lacquer (Urushi): part I— communication from the chemical society of Tokio. J Chem Soc Trans 43:472–486
28. Chandra R, Chowdhary P (2015) Properties of bacterial laccases and their application in bioremediation of industrial wastes. Environ Sci Process Impacts 17(2):326–342
29. Otto B, Schlosser D (2014) First laccase in green algae: purification and characterization of an extracellular phenol oxidase from *Tetracystis aeria*. Planta 240(6):1225–1236
30. Otto B, Beuchel C, Liers C, Reisser W, Harms H, Schlosser D (2015) Laccase-like enzyme activities from chlorophycean green algae with potential for bioconversion of phenolic pollutants. FEMS Microbiol Lett. https://doi.org/10.1093/femsle/fnv072
31. Xu F, Kulys JJ, Duke K, Li K, Krikstopaitis K, Deussen HJW, Abbate E, Galinyte V, Schneider P (2000) Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compounds. Appl Environ Microbiol 66(5):2052–2056
32. Mukhopadhyay M, Banerjee R (2015) Purification and biochemical characterization of a newly produced yellow laccase from *Lentinus squarrosulus* MR13. 3 Biotech 5(3):227–236
33. Aslam MS, Aishy A, Samra ZQ, Gull I, Athar MA (2012) Identification, purification and characterization of a novel extracellular laccase from *Cladosporium cladosporioides*. Biotechnol Biotechnol Equip 26(6):3345–3350
34. More SS, Renuka PS, Pruthvi K, Swetha M, Malini S, Veena SM (2011) Isolation, purification, and characterization of fungal laccase from *Pleurotus sp*. Enzyme Res 2011:1–7
35. Reinhammar B (1984) Copper proteins and copper enzymes, vol 3. CRC Press, Boca Raton
36. Atalla MM, Zeinab HK, Eman RH, Amani AY, Abeer AAEA (2013) Characterization and kinetic properties of the purified *Trematosphaeria mangrovei* laccase enzyme. Saudi J Biol Sci 20(4):373–381
37. Agrawal K, Verma P (2020) Multicopper oxidase laccases with distinguished spectral properties: a new outlook. Heliyon 6(5):e03972
38. Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G (1997) A novel white laccase from *Pleurotus ostreatus*. J Biol Chem 272(50):31301–31307
39. Zhou P, Fu C, Fu S, Zhan H (2014) Purification and characterization of white laccase from the white-rot fungus *Panus conchatus*. BioResources 9(2):1964–1976
40. Xu F (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35(23):7608–7614
41. Chefetz B, Chen Y, Hadar Y (1998) Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. Appl Environ Microbiol 64(9):3175–3179
42. Kim Y, Yeo S, Kim MK, Choi HT (2008) Removal of estrogenic activity from endocrine-disrupting chemicals by purified laccase of *Phlebia tremellosa*. FEMS Microbiol Lett 284(2):172–175
43. Shin EH, Choi HT, Song HG (2007) Biodegradation of endocrine-disrupting bisphenol A by white rot fungus *Irpex lacteus*. J Microbiol Biotechnol 17(7):1147–1151
44. Xiao Y, Tu X, Wang J, Zhang M, Cheng Q, Zeng W, Shi Y (2003) Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes sp.* strain AH28–2. Appl Microbiol Biotechnol 60(6):700–707
45. Prasad KK, Mohan SV, Bhaskar YV, Ramanaiah SV, Babu VL, Pati BR, Sarma PN (2005) Laccase production using *Pleurotus ostreatus* 1804 immobilized on PUF cubes in batch and packed bed reactors: influence of culture conditions. J Microbiol 43(3):301–307
46. Ferraroni M, Westphal AH, Borsari M, Tamayo-Ramos JA, Briganti F, de Graaff LH, van Berkel WJ (2017) Structure and function of *Aspergillus niger* laccase McoG. Biocatalysis 3(1):1–21
47. Gaur N, Narasimhulu K, Setty YP (2018) Extraction of ligninolytic enzymes from novel *Klebsiella pneumoniae* strains and its application in wastewater treatment. Appl Water Sci 8(4):1–17
48. Garg N, Bieler N, Kenzom T, Chhabra M, Ansorge-Schumacher M, Mishra S (2012) Cloning, sequence analysis, expression of *Cyathus bulleri* laccase in *Pichia pastoris* and characterization of recombinant laccase. BMC Biotechnol 12(1):1–12
49. Palanisami S, Lakshmanan U (2011) Role of copper in poly R-478 decolorization by the marine cyanobacterium *Phormidium valderianum* BDU140441. World J Microbiol Biotechnol 27(3):669–677
50. Munoz C, Guillen F, Martinez AT, Martinez MJ (1997) Induction and characterization of laccase in the ligninolytic fungus *Pleurotus eryngii*. Curr Microbiol 34(1):1–5
51. Xu F (1997) Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. J Biol Chem 272(2):924–928
52. Karamyshev AV, Shleev SV, Koroleva OV, Yaropolov AI, Sakharov IY (2003) Laccase-catalyzed synthesis of conducting polyaniline. Enzyme Microb Technol 33(5):556–564
53. Park KM, Park SS (2008) Purification and characterization of laccase from basidiomycete *Fomitella fraxinea*. J Microbiol Biotechnol 18(4):670–675
54. Shin KS, Lee YJ (2000) Purification and characterization of a new member of the laccase family from the white-rot basidiomycete *Coriolus hirsutus*. Arch Biochem Biophys 384(1):109–115
55. Yuan X, Tian G, Zhao Y, Zhao L, Wang H, Ng TB (2016) Biochemical characteristics of three laccase isoforms from the basidiomycete *Pleurotus nebrodensis*. Molecules 21(2):203
56. Moreira-Neto SL, Matheus DR, Machado KMG (2009) Influence of pH on the growth, laccase activity and RBBR decolorization by tropical basidiomycetes. Braz Arch Biol Technol 52:1075–1082
57. Palonen H, Saloheimo M, Viikari L, Kruus K (2003) Purification, characterization and sequence analysis of a laccase from the ascomycete Mauginiella sp. Enzyme Microb Technol 33(6):854–862
58. Irshad M, Asgher M, Sheikh MA, Nawaz H (2011) Purification and characterization of laccase produced by *Schyzophylum commune* IBL-06 in solid state culture of banana stalks. BioResources 6(3):2861–2873
59. Paranjpe PS, Karve MS, Padhye SB (2003) Characterization of tyrosinase and accompanying laccase from *Amorphophallus campanulatus*. Indian J Biochem Biophys 40(1):40–45
60. Abou-Mansour E, Polier J, Pezet R, Tabacchi R (2009) Purification and partial characterisation of a 60 KDa laccase from *Fomitiporia mediterranea*. Phytopathol Mediterr 48(3):447–453
61. Rezaei S, Shahverdi AR, Faramarzi MA (2017) Isolation, one-step affinity purification, and characterization of a polyextremotolerant laccase from the halophilic bacterium *Aquisalibacillus elongatus* and its application in the delignification of sugar beet pulp. Biores Technol 230:67–75
62. Muthukumarasamy NP, Jackson B, Joseph Raj A, Sevanan M (2015) Production of extracellular laccase from *Bacillus subtilis* MTCC 2414 using agroresidues as a potential substrate. Biochem Res Int 2015:1–9

63. Yang X, Wu Y, Zhang Y, Yang E, Qu Y, Xu H, Chen Y, Irbis C, Yan J (2020) A thermo-active laccase isoenzyme from *Trametes trogii* and its potential for dye decolorization at high temperature. Front Microbiol 11:241
64. Zhang Y, Rochefort D (2011) Activity, conformation and thermal stability of laccase and glucose oxidase in poly (ethyleneimine) microcapsules for immobilization in paper. Process Biochem 46(4):993–1000
65. Wang Q, Ding L, Zhu C (2018) Characterization of laccase from a novel isolated white-rot fungi *Trametes sp*. MA-X01 and its potential application in dye decolorization. Biotechnol Biotechnol Equip 32(6):1477–1485
66. Karshikoff A, Nilsson L, Ladenstein R (2015) Rigidity versus flexibility: the dilemma of understanding protein thermal stability. FEBS J 282(20):3899–3917
67. Chakroun H, Mechichi T, Martinez MJ, Dhouib A, Sayadi S (2010) Purification and characterization of a novel laccase from the ascomycete *Trichoderma atroviride*: application on bioremediation of phenolic compounds. Process Biochem 45(4):507–513
68. Robles A, Lucas R, Martínez-Cañamero M, Omar NB, Pérez R, Gálvez A (2002) Characterisation of laccase activity produced by the hyphomycete Chalara (syn *Thielaviopsis*) *paradoxa* CH32. Enzyme Microb Technol 31(4):516–522
69. Martins LO, Soares CM, Pereira MM, Teixeira M, Costa T, Jones GH, Henriques AO (2002) Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J Biol Chem 277(21):18849–18859
70. Koroleva OV, Gavrilova VP, Stepanova EV, Lebedeva VI, Sverdlova NI, Landesman EO, Yavmetdinov IS, Yaropolov AI (2002) Production of lignin modifying enzymes by co-cultivated white-rot fungi *Cerrena maxima* and *Coriolus hirsutus* and characterization of laccase from Cerrena maxima. Enzyme Microb Technol 30(4):573–580
71. Zouari-Mechichi H, Mechichi T, Dhouib A, Sayadi S, Martinez AT, Martinez MJ (2006) Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: decolorization of textile dyes by the purified enzyme. Enzyme Microb Technol 39(1):141–148
72. Dantán-González E, Vite-Vallejo O, Martínez-Anaya C, Méndez-Sánchez M, González MC, Palomares LA, Folch-Mallol J (2008) Production of two novel laccase isoforms by a thermotolerant strain of *Pycnoporus sanguineus* isolated from an oil-polluted tropical habitat. Int Microbiol 11(3):163–169
73. Ibrahim V, Mendoza L, Mamo G, Hatti-Kaul R (2011) Blue laccase from *Galerina* sp: properties and potential for Kraft lignin demethylation. Process Biochem 46(1):379–384
74. Park N, Park SS (2014) Purification and characterization of a novel laccase from *Fomitopsis pinicola* mycelia. Int J Biol Macromol 70:583–589
75. Olajuyigbe FM, Fatokun CO (2017) Biochemical characterization of an extremely stable pH-versatile laccase from *Sporothrix carnis* CPF-05. Int J Biol Macromol 94:535–543
76. Ezike TC, Udeh JO, Joshua PE, Ezugwu AL, Isiwu CV, Eze SO, Chilaka FC (2021) Substrate specificity of a new laccase from *Trametes polyzona* WRF03. Heliyon 7(1):e06080
77. Johannes C, Majcherczyk A (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol 66(2):524–528
78. D'annibale A, Celletti D, Felici M, Di Mattia E, Giovannozzi-Sermanni G (1996) Substrate specificity of laccase from *Lentinus edodes*. Acta Biotechnol 16(4):257–270
79. Kersten PJ, Kalyanaraman B, Hammel KE, Reinhammar B, Kirk TK (1990) Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem J 268(2):475–480
80. Niladevi KN, Jacob N, Prema P (2008) Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: purification and characterization. Process Biochem 43(6):654–660
81. Abdelgalil SA, Attia AR, Reyed RM, Soliman NA (2020) Partial purification and biochemical characterization of a new highly acidic NYSO laccase from *Alcaligenes faecalis*. J Genet Eng Biotechnol 18(1):1–11
82. Sadhasivam S, Savitha S, Swaminathan K, Lin FH (2008) Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. Process Biochem 43(7):736–742
83. Zhou C, Dong A, Wang Q, Yu Y, Fan X, Cao Y, Li T (2017) Effect of common metal ions and anions on laccase catalysis of guaiacol and lignocellulosic fiber. BioResources 12(3):5102–5117
84. Wang X, Hu J, Liang Y, Zhan H (2011) Effects of metal ions on laccase activity. Asian J Chem 23(12):5422
85. Ryan S, Schnitzhofer W, Tzanov T, Cavaco-Paulo A, Gübitz GM (2003) An acid-stable laccase from *Sclerotium rolfsii* with potential for wool dye decolourization. Enzyme Microb Technol 33(6):766–774
86. Tadkaew N, Hai FI, McDonald JA et al (2011) Removal of trace organics by MBR treatment: the role of molecular properties. Water Res 45:2439–2451
87. Yang S, Hai FI, Nghiem LD et al (2013) Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. Biores Technol 141:97–108
88. https://brenda-enzymes.org/enzyme.php?ecno=1.10.3.2&Suchword=&reference=&UniProtAcc=&organism%5B%5D=Trametes+versicolor&show_tm=0