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Research article

Multicopper oxidase laccases with distinguished spectral properties: A new outlook

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ABSTRACT

Multicopper oxidases (MCOs) has a unique feature of having the presence of four Cu atoms arranged into three (Type I, II and III) spectral classification. MCOs laccase due to its broad range of substrate specificity has numerous biotechnological applications. The two types of laccases include the typical blue and the atypical white, yellow laccases which have been isolated from diverse geographical locations globally. In the present study laccases were identified using Liquid Chromatograph Mass Spectrometer Studies (LCMS) study where blue laccase exhibited homology with *Trametes villosa* Q99044 and Q99046 and white, yellow laccase exhibited homology with *Myrothecium verrucaria* OX = 1859699; Q12737 and Trametes versicolor Q12717 respectively. The spectral comparison between laccases were determined via spectroscopic analysis where UV-spectra of blue laccase from *Trametes versicolor* had a peak at 605 nm (Type I Cu atom) whereas in case of white and yellow laccases the peak was absent and in addition had an absorption peak at 400nm. It was followed by X-Ray Diffraction (XRD) analysis of proteins where α-helix (10°) and β-sheet (22°) structure were observed in case of all the three laccases. However, the intensity of α-helix in white and yellow laccase was stronger as compared to the blue laccase whereas the intensity of β-sheet was stronger in case of blue laccase as compared to other two laccases. Further, Fourier-transform infrared spectroscopy (FTIR) analysis was performed which enabled the analysis of proteins where α-helix (1650–1658 cm^{-1}), β-sheets (1620–1640 cm^{-1}), amide I (1700–1600 cm^{-1}) amide II (bands at under 1400 cm^{-1}) and amide A, B (bands above 3000 cm^{-1}).

1. Introduction

Multicopper oxidases (MCOs) laccase is ubiquitous in nature being found in plants, bacteria, fungi, soil and insects [1]. They have been regarded as a versatile enzyme which has the capability to "catalyze one basic reaction from which all its activities originate" [2]. Laccase are glycosylated monomer or homodimer protein with less monosaccharide compounds (10–25%) in fungal and bacterial laccase as compared to plant laccases [3]. Laccase generally are described as having a UV spectrum at 280 and 600 nm with a shouldering at 330 nm. Typical laccase consists of three Cu centres (Type I, II and III) consisting of four Cu ions. Type I Cu is responsible for intense blue colour and has electro absorption at 605 nm and detectable electro paramagnetic resonance (EPR) spectrum. Type II unlike type I is colourless and EPR detectable. Type III Cu on the other hand has a pair of Cu atom which have weak absorbance at UV spectrum and lacks EPR spectrum. Type II and III together forms the trinuclear cluster of laccases where dioxygen binds and four electron reduction to water takes place. It is due these features that laccase has its application in wide range of industries [4] e.g., clarification of beverage, paper and pulp, textile industry, biosensor, bioremediation and organic synthesis [51]. However, the catalytic ability of enzymes is often hindered in harsh environments like high-acid and high-alkaline solutions due to transformations of enzyme structure which results in low operational stability, difficulties in recovery and reuse [5]. Nonetheless screening of new strains are perquisite which can produce various laccase to tolerate harsh industrial processes (e.g. alkaline tolerant and thermo-stable) and can give maximum enzyme production with minimum energy consumption is the requirement of various biotechnological and environmental sector [4].

Laccase have been studied globally and have been identified with unique characteristic features. Purahong et al [6] has stated that "fungal community structure was similar across different regions, but was nevertheless variable in all regions". Laccase have been identified from various geographical regions e.g. *Ganoderma lucidum* KMK2 (India) [7], *Scytalidium thermophilum* (Tunisia) [8], *P. sanguineus* (Mexico) [9], *Cerrena unicolor* (Poland) [10], *Pycnoporus cinnabarinus* (Australia) [11],

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Pycnoporus sanguineus (Brazil) [12], *Peniophora* species (Netherland) [13], *M verrucaria* (China) [14] and *Stropharia aeruginosa* (Netherland) [15]. Among them atypical white/yellow laccases have been identified which exhibits advantages over typical blue laccase. The blue laccases require the presence of mediator (shuttles in between the substrate and laccase) as compared to yellow/white laccases which function effectively in the absence of mediator [52]. These atypical laccases have also been reported to be stable at acidic pH and high temperature as well (55–60 °C) [14, 17]. White laccase from *M. verrucaria* 24G-4, MD-R-16 and NF-05 were reported by Sulistyaningdyah et al [18], Sun et al [17] and Zhao et al [16]. *M. verrucaria* 24G-4 was obtained from Japan, *M. verrucaria* MD-R-16 was isolated from roots of pigeon pea from China and *M. verrucaria* NF-05 was isolated from soil of China. Similarly, the strain *M verrucaria* ITCC 8447 was isolated from Shreenagar, Rajasthan India during rainy season. Thus, it can be inferred that the strains have been isolated from wide range of climatic and geographical habitats. On the other hand, yellow laccase from *S. aeruginosa* CBS 839.87 was from Netherlands [15] and *Stropharia* sp. ITCC 8422 was isolated from Rajasthan, India during rainy season [19, 20].

The systematic spectral studies have not been reported till date to understand and examine the differences among the spectral properties of various laccases considering the diverse geographical distribution of laccases. Thus, in order to allow better understanding of the spectral characteristic features and differences among the typical and atypical nature of laccases the present study was designed where the sequence homology using LCMS and detailed spectral analysis was performed using two multicopper oxidase laccases producing strains which were isolated from western province of Rajasthan, India. These strains *M. verrucaria* ITCC 8447 and *Stropharia* sp. ITCC 8422 were deposited at Indian Type Culture Collection [19, 20, 21]. In order to examine distinguished spectral properties, blue laccase from *Trametes versicolor* was used as standard (control) along with two isolated strains (*M. verrucaria* ITCC 8447 and *Stropharia* sp. ITCC 8422) which are efficient producers of white and yellow laccase. The sequence homology of white, yellow and blue laccase was performed using LCMS studies. Further all the three laccases were primarily analyzed using UV visible spectra, followed by detailed spectroscopic analysis consisting of X-Ray Diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR) for determining the typical and atypical nature of laccase.

2. Materials and methods

2.1. Chemicals and reagents

The blue laccase from *Trametes versicolor* (38429-1G) and ABTS were purchased from Sigma. All other chemicals were of analytical grade and purchased from Merck, Hi-media, India.

2.2. Liquid chromatograph mass spectrometer studies of various laccases

The sequence of blue laccase and isolated white and yellow laccase were determined using LCMS studies. The white and yellow laccase were produced as per previous study Agrawal et al [21] and Agrawal and Verma [19, 20]. The extracellular proteins were concentrated and considered for further analysis. The concentrated yellow and blue laccase were alkylated using urea (6M) and tris buffer (100 mM, pH 7.8) and DTT (200 mM in tris buffer) and vortex for 60 min at 28 ± 2 °C followed by adding iodoacetamide (200 mM in tris buffer) as an alkylating agent and again vortex for 60 min at 28 ± 2 °C. After incubation reducing agent was added to remove the alkylating agent and incubated for 60 min. The sample was diluted to reduce concentration of urea to 0.6 M and then 20 μg chymotrypsin was added and samples were digested overnight at 37 °C. Then the pH was reduced to less than 6 and analyzed using Advance Biopeptide Column 2.1 × 100 mm, 2.7 micron using QTOF-LCMS at Agilent Technologies, Centre of Excellence Manesar, Gurugram India.

The concentrated white laccase was dissolved in tris buffer (pH 7), digested, desalted followed by analysis using HR-LCMS Orbitrap at IIT Bombay SAIF by analytical column: PepMap RSLC C18 2um, 100A x 50 cm, Pre-column: Acclaim PepMap 100, 100um x 2cm nanoviper, with mobile phase as solvent A: 0.1% FA in milliq water, solvent B: 80:20 (ACN: milliq water) + 0.1% FA. The data was analyzed using Thermo Proteome Discoverer 2.2.

2.3. 3D ribbon representation of various laccases

The LCMS study enabled the identification of the sequence similarity of laccase from existing database. The sequence obtained (https://www.uniprot.org/) was used for the prediction of the 3D ribbon representation of multicopper oxidases from SWISS-MODEL Server or Repository (https://swissmodel.expasy.org/) [39, 40, 41, 42, 43].

2.4. Purification and the spectral studies of various laccases

The production and extraction of laccases were performed from isolated strains as per previous reports [19, 20, 21] and unpublished data for spectral studies. The cell free supernatant was concentrated using ammonium sulphate (80 %) precipitation and dialyzed using dialysis membrane-110. Further the dialysed sample was purified using ion exchange chromatography, DEAE-Sephadex column and equilibrated with acetate buffer (0.1 M pH 5). The sodium chloride gradient (0–1 M) was applied at a flow rate of 0.5 mL/min. The collected fractions were analyzed for laccase activity and protein content [21]. The purified white and yellow laccase along with the blue laccase was considered for further spectral studies.

2.4.1. UV-absorption of various laccases for spectral analysis

The blue laccase are typical laccases and have been studied extensively for the past few years. On the other hand, white/yellow laccase also known as atypical laccase are less studied and scattered information is available. Atypical laccase differs from typical laccase in UV-absorption spectra at 605 nm. Except for the lack of absorbance all other features of atypical laccase are similar to the typical laccase [22]. The UV visible spectra of all three laccases were determined using UV-visible spectrophotometer (Halo- DB, Dynamica Asia Limited Hong Kong) at a wavelength range of 200–800 nm followed by a comparative spectral analysis [21].

2.4.2. Spectral assessment of various laccases using XRD

The XRD studies were performed for detecting the presence of α helix and β sheets. Purified white and yellow laccase as mentioned under section 2.4 were lyophilized using lyophilizer (Allied Freezer, New Delhi, India). The blue laccase and lyophilized proteins of white and yellow laccases were scanned at 2θ range of 10–25° [21] by PANalytical EMPYREAN, Netherlands using Cu as the anode material at Department of Physics, Central University of Rajasthan.

2.4.3. Spectral evaluation of various laccases using FTIR

The spectral studied of typical and a typical laccase were further extended to FTIR studies. It has been stated that the FTIR has advantages over other techniques as they can be used for obtaining spectrum of protein at a wide range of environments, requiring less time as well as sample. Thus, FTIR analysis was performed for the blue laccase and purified white and yellow laccase at a wavenumber range of 500–4000 cm^{-1} [21] at IIT Bombay, Sophisticated Analytical Instrument Facility (SAIF), India.

2.5. SDS-PAGE and silver staining of various laccases

The blue laccase from *Trametes versicolor* and white laccase from *M verrucaria* ITCC 8447 was determined using SDS-PAGE as per Laemmli [46]. The bands were confirmed by Commassie Brilliant Blue R-250

staining method. The yellow laccase from *Stropharia* sp. ITCC 8422 was determined using silver staining protocol, where the gel was fixed using fixing solution (methanol 50%, acetic acid 10%, formaldehyde 50μL in 100 mL) for 60 min, washed with 50% ethanol (3 times) for 20min each, followed by sensitizing the gel with sodium thiosulfate (20mg/100mL) for 60 s. The gel was washed followed by treatment with silver nitrite solution (200mg/100mL) for 30 min and washing. Later developing solution (sodium carbonate 6g, hypo solution 2mL, formaldehyde 50μL) was added and incubated till the bands appeared followed by the addition of 5% acetic acid to stop the reaction.

3. Result and discussion

3.1. Liquid chromatograph mass spectrometer studies of various laccases

M verrucaria ITCC 8447 has been reported to produce 1549.7 U/L of laccase after the optimization of various nutritional and physiological parameters [21]. On the other hand, yellow laccase by *Stropharia* sp. ITCC 8422 has been reported to produce 164.4 U/L on 18th day [19, 20]. The extracellular white/yellow laccases were concentrated and analyzed using LCMS for the confirmation of the production of laccase by these two strains as well as to identify its sequence homology with the existing database.

The LCMS analysis of blue laccase from *Trametes versicolor* exhibited homology with laccase-1, laccase-2 from *Trametes villosa* (White-rot fungus) with the accession number Q99044 and Q99046 respectively. The white laccase from *M. verrucaria* ITCC-8447 exhibited identity with MCOs bilirubin oxidase from *Myrothecium verrucaria* OX = 1859699 with accession Q12737. In the study by Sulistyaningdyah et al [18] NH_2-terminal amino acid sequence of laccase from *Myrothecium verrucaria* 24G-4 exhibited homology with bilirubin oxidase from *M. verrucaria* MT-1 which was further validated using experimental analysis. Similarly, *M verrucaria* ITCC-8447 via experimental analysis proved the production of laccase [21]. The laccase of *Stropharia* sp. ITCC-8422 exhibited homology with laccase-5 from Trametes versicolor (White-rot fungus; *Coriolus versicolor*) with accession number Q12717. This confirms the production of laccase by *Stropharia* sp. ITCC-8422.

3.2. 3D ribbon representation of various laccases

The sequence of MCOs were identified after LCMS for blue laccase and the sequence obtained were ADGPAFINQCPISSGHSF and AEDVADVKAANPVPKAW for laccase 1 and 2 from *Trametes villosa* Q99044 and Q99046. On the other hand, for white and yellow laccases sequences were AAFDGWAEDITEPGSFK and RLVSISCDPNFTF from *Myrothecium verrucaria* OX = 1859699; Q12737 and Trametes versicolor (White-rot fungus; *Coriolus versicolor*) Q12717 respectively. The LCMS studies enabled the prediction of the 3D ribbon representation which has been represented in Figure 1 (a–d). It was also observed that all the laccase has the presence of 4 x Copper (II) Ion and ranged from 519-572 amino acid residues.

3.2.1. UV-absorption of various laccases for spectral analysis

Blue laccase from *Trametes versicolor* had the presence of peak at 605 nm (Figure 2b) which represents the presence Type I Cu atom, responsible for the intense blue colour of typical blue laccase [11]. The purified white laccase is different from blue laccase, where peak at 605 nm was absent which is typically present in type I of blue laccase (Figure 2c) and has an absorption peak at 400nm (Palmieri et al 1997). Additionally, shouldering at 330 nm was absent which corresponds to T3 binuclear copper. The absence of peak at 605 nm can be due to the incomplete oxidation of copper which is typical feature of white laccase and it is in

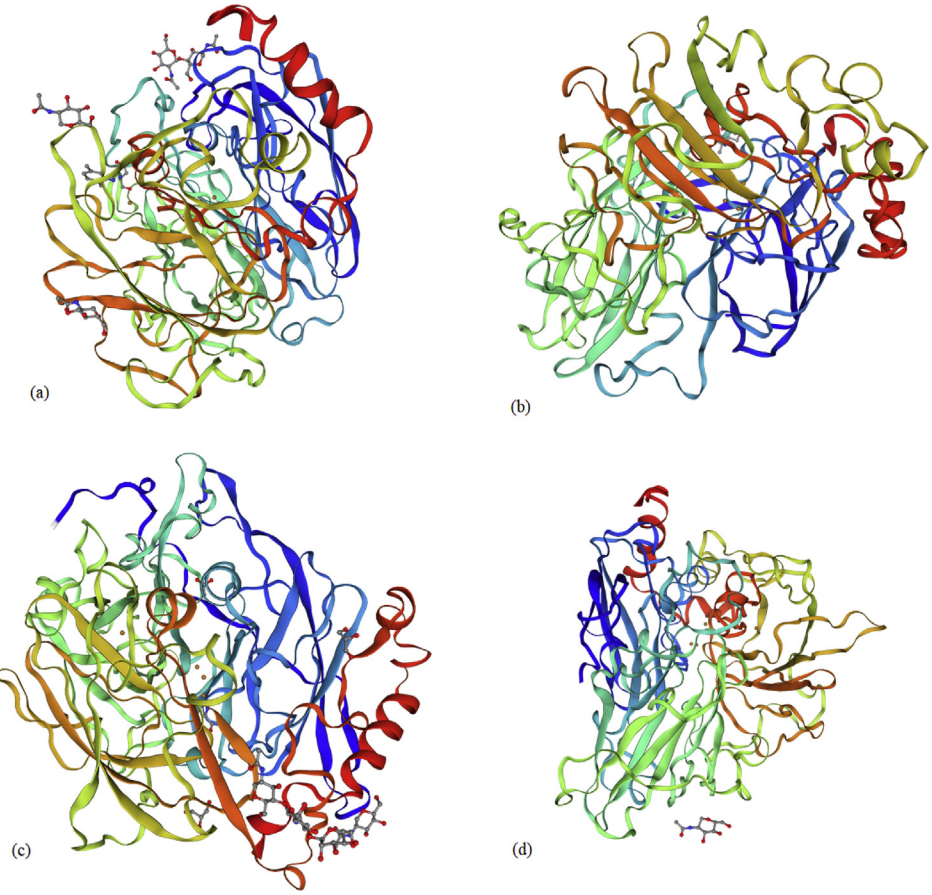

Figure 1. 3D ribbon representation of various laccases as obtained after LCMS study using the sequence homology (a–b) blue; (c) white; (d) yellow.

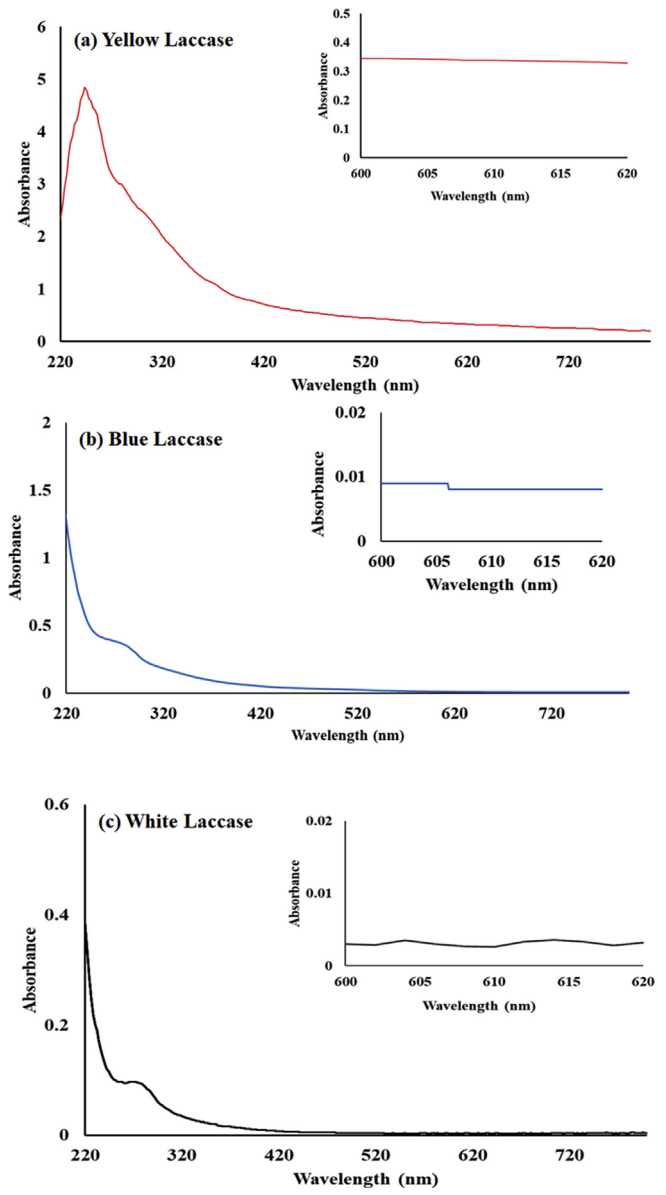

Figure 2. The UV-visible spectra of various laccases from (a) *Stropharia* sp. ITCC 8442 (b) *T versicolor* and (c) *M verrucaria* ITCC 8447 (Figure 2a has been reproduced/adapted from Agrawal and Verma [19] with permission from Elsevier; Figure 2c has been reproduced/adapted from Agrawal et al [21] with permission from Elsevier).

accordance with the study reported by Plameiri et al. [23]; Zhou et al. [25]; Zhao et al. [14]. Likewise, for yellow laccase as well it did not have absorption maximum at 605 nm (Figure 2a) thereby, confirming the production of yellow laccase by *Stropharia* sp. ITCC-8422. The yellow laccase feature is in accordance with the study by Mukhopadhyay and Banerjee [24] and Daroch et al. [15].

Thus, the UV-visible spectra of blue laccase represent its typical nature whereas the absence of absorbance in white/yellow laccase represents its atypical nature. Similar data has been reported by Schliephake et al [11] where at 605 nm an increase in absorption was observed and for white laccase the study was similar to Plameiri et al. [23]; Zhou et al. [25]; Zhao et al. [14] and for yellow laccase the study was similar to Daroch et al [15].

3.2.2. Spectral assessment of various laccases using XRD

The XRD analysis was performed to analyze the structure of blue, white/yellow laccases. The XRD analysis of proteins at an angle of 2θ and approximately 10°and 22° represents the α-helix and β-sheet structure [26, 27]. The XRD of blue laccase had the presence of α-helix at 10° along with β-sheet at 22°. Similar observation was observed in case of white [21] and yellow laccase (Table 1). However, the intensity of white and yellow laccase α-helix was stronger as compared to the blue laccase. On the other hand, β-sheet structure was stronger in case of blue laccase as compared to white and yellow laccase (Figure 3).

3.2.3. Spectral evaluation of various laccases using FTIR

FTIR analysis enables the analysis of proteins and polypeptides with nine specific IR absorption bands i.e., amide A, B and I to VII of which the amide bands I and II exhibits very distinctive vibrational bands of the protein backbone [28, 29, 30]. The amide I band of protein is the most sensitive region to the spectra 1700–1600 cm^{-1} which is contributed due to the C=O stretch vibrations in the peptide linkages which is approximately 80%. Amide I band is related to the secondary structure of protein whereas amide II is due to the in-plane NH bending which constitutes 40–60% of the potential energy and CN stretching vibrations as well which constitutes 18–40%. It is due to these factors that amide I is more sensitive than amide II bands in the FTIR spectra of proteins [28, 31]. While obtaining the FTIR spectra of protein it has to be taken into consideration that in case water is present in the sample three peaks can be detected at 3400 cm^{-1}, 2125 cm^{-1} and 1645 cm^{-1} representing O–H stretching, H_2O association and H–O–H association. In all the cases absorbance at 1645 cm^{-1} was observed which could be due to the amide I band overlapping with H_2O band which thereby is also responsible for higher absorbance [31].

The blue laccase had the presence of α-helix and β-sheets at 1650–1658 cm^{-1} and 1620–1640 cm^{-1} and the band detecting the presence of amides III (bands under 1400 cm^{-1}) and amides A, B (bands above 3000 cm^{-1}) were also visible distinctly in the FTIR spectrum (Figure 4). White laccase had peaks at 1650–1658 cm^{-1} and 1620–1640 cm^{-1} representing α-helix and β-sheets respectively whereas the bands at under 1400 cm^{-1} and above 3000 cm^{-1} signifies the presence of amides II and amides A, B [32] which are very clearly present in case of white laccase. The band present in 1600–1700 cm^{-1} represents the amide I and II. The amide I band due to the presence of overlap broad underlying components i.e., α helix, β sheet and random structures are featureless (Figure 4). In case of yellow laccase, the FTIR spectra was similar to that of white laccase where the peaks at 1650–1658 cm^{-1} and 1620–1640 cm^{-1} where present however it was slightly more intense as compared to white laccase. The bands below 1400 cm^{-1} (amides II) and the band below 3000 cm^{-1} (amides A and B) were similar to that of white laccase. The secondary structure band assignment also consists of random and coil structure at 1642-1657 cm^{-1} and 1662-1686 cm^{-1} respectively present in white laccase however was less or negligible in case of yellow and blue laccase (Figure 4). Thus, it can be inferred from the FTIR that in case of blue laccase the intensity of α-helix was more as compared to white and yellow laccase.

3.3. Comparative spectral assessment and application of various laccases

The UV visible spectra of white laccase exhibited an absorption peak at 400nm and absence of peak at 605 nm which represents Type I Cu [23]. White laccase lacks typical blue colour which can be due to the change in the valence state of Cu^{2+} and lack of the EPR signal due to the Fe^{2+} which has low spin electronic configuration [14]. The replacement of Cu^{2+} can be inferred but the lack of absorption can also be due to the incomplete oxidation of the copper which has an occupied electron configuration of d10 and no d-d transition [14]. It has to be noted that white laccase has the preference of neural pH as compared to other laccase where the optimal pH ranges from 3-5 [21]. The other characteristic feature is the presence of anomalous metal content which contributes towards its unique feature. White laccase was identified from *Pleurotus ostreatus* where blue spectra were lacking and consisted of one Cu, one Zn and two Fe atoms. On the other hand, yellow laccase was

Table 1. The peaks detected in the XRD analysis of the blue, white and yellow laccases.

Blue Laccase	**α helix** 10.03, 10.14, 10.15, 10.23, 10.24, 10.26, 10.32, 10.44, 10.54, 10.60, 10.66, 10.68, 10.70, 10.72, 10.73, 10.75, 10.86, 10.87, 10.90, 10.95 and 10.97,
	β sheet 22.01, 22.02, 22.05 to 22.10, 22.24, 22.31 to 22.34, 22.38, 22.40, 22.60, 22.76, 22.81and 22.94
White Laccase	**α helix** 10.02°, 10.71°, 11.40°, 11.43° and 11.48°
	β sheet 22.55° and 22.69°
Yellow Laccase	**α helix** 10.02, 10.06, 10.08, 10.16, 10.19, 10.31 and 10.35°, 10.54° 10.61°, 10.84°, 10.98°
	β sheet 22.07, 22.10, 22.22, 22.91, 22.92, 22.98 and 22.99

produced as a result of solid-state fermentation where yellow brown laccase lacks the typical blue spectra and had an atypical EPR spectrum [1].

The yellow laccase on the other hand are artificially reduced blue laccase which also lacks absorption spectra at 605 nm and an EPR spectrum [33, 34]. The yellow laccase occurs by the reduction of Type I Cu site by aromatic product of lignin degradation or the binding of specific amino acid of the polypeptide of enzyme to a molecule of the modified product produced by lignin degradation. The other factor which is responsible for the production of yellow laccase could be due to heterogeneity induced by glycosylation [15]. A "yellow" laccase with "blue" spectroscopic features was identified from *Sclerotinia sclerotiorum* (Moţ et al 2012). The modified molecule of the apoenzyme (yellow laccase) performs electron transfer mediator similar to the role of mediator e.g., ABTS as in case of blue laccase [33, 34]. Thus, enabling yellow laccase to act effectively in the absence of mediator to oxidise non-phenolic compounds in contrast to blue laccase where mediator is required, thereby increasing its biotechnological and industrial potential [19, 20, 35]. Yellow laccases and blue laccase have four copper atoms like the blue laccase whereas white laccases contain one copper, two zinc ions and one iron ion per protein molecule [23].

The substrate affinity of laccase has been reported and it was observed that ABTS was the most effective substrate for blue, white (*M verrucaria* ITCC 8447) [47,53] and yellow laccase (data not shown). The

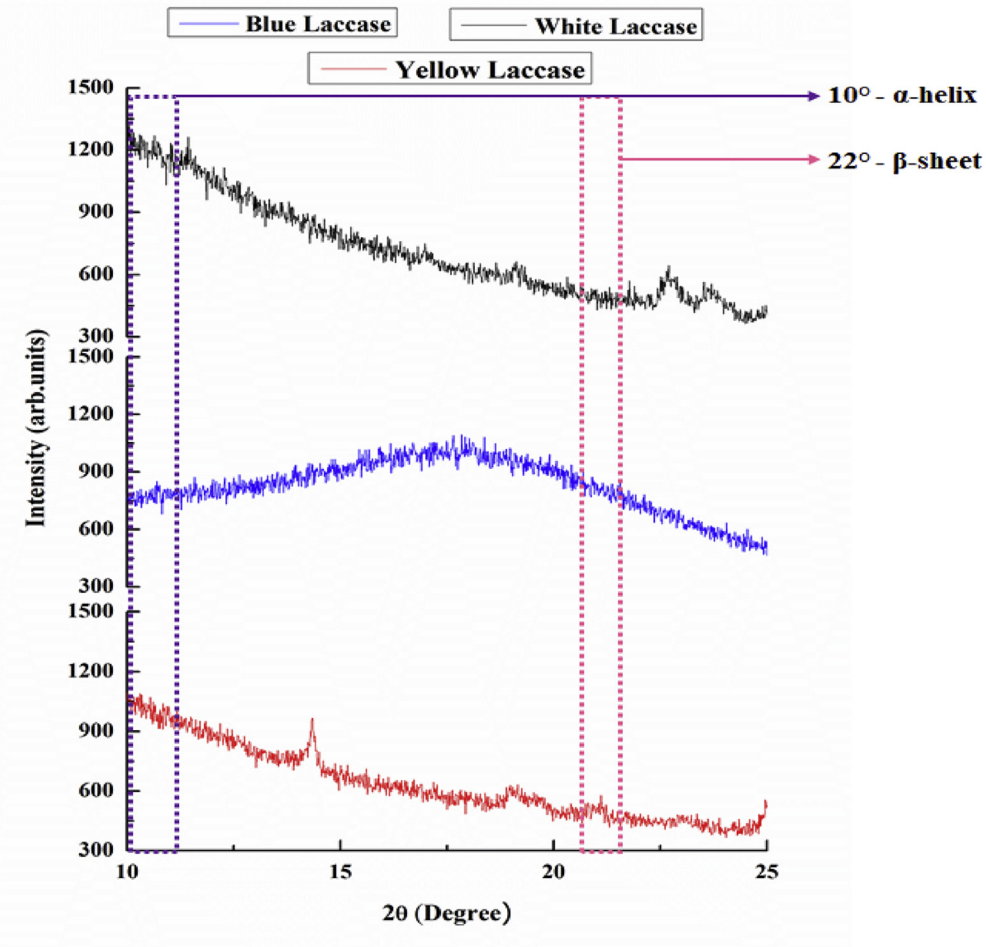

Figure 3. The XRD of various laccases from *T versicolor*, *M verrucaria* ITCC 8447 and *Stropharia* sp. ITCC 8442. (The XRD of white laccase has been reproduced/ adapted from Agrawal et al [21] with permission from Elsevier).

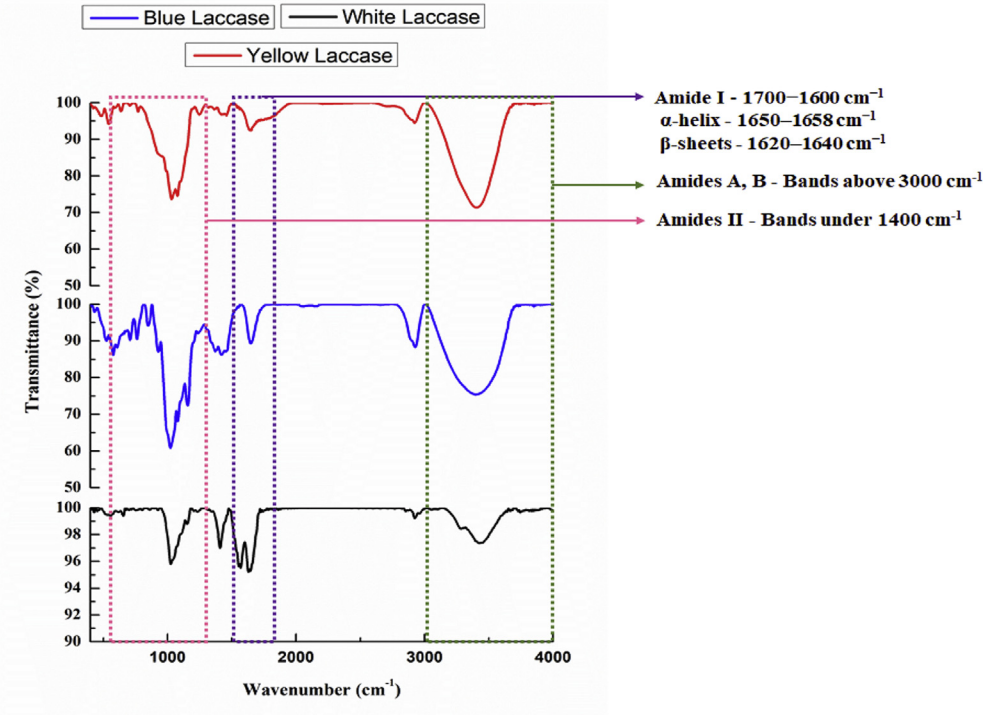

Figure 4. The FTIR of various laccases from *T versicolor*, *M verrucaria* ITCC 8447 and *Stropharia* sp. ITCC 8442 (The FTIR of white laccase has been reproduced/ adapted from Agrawal et al [21] with permission from Elsevier).

Km and Vmax values of the purified white laccase has been reported as 2.5 mM and1818.2 μmol/min/L. Similarly, for yellow laccase from *Pleurotus ostreatus* D1 it was observed that the Km and Vmax for syringaldazine, 2,6-dimethoxyphenol, and ABTS were higher than the corresponding values for yellow laccase from *Panus tigrinus* 8/18, and the values were close to the blue laccase from *Pleurotus ostreatus* strain. Thus, it has been observed that the Km and Vmax varies from strain to strain however maximum affinity for laccase has been reported towards ABTS [33, 34, 35, 48]. Laccases have also been used for its effective bioremediation potential and they have effectively removed various structural different dyes e.g. azo, anthraquinone [14, 19, 20, 50]. In addition, blue laccase from *Trametes versicolor* [49] white laccase from *M. verrucaria* ITCC 8447 [21] and yellow laccase from *Stropharia* sp. ITCC 8422 also has high potential in the delignification of agricultural biomass. The white and yellow laccase has more advantage over blue laccase they can oxidizes non-phenolic compounds in the absence of mediators [33, 34], thereby further broadening its application in various biotechnological sectors.

3.4. Distinguish features of atypical laccases

The white laccases from *M. verrucaria* 24G-4, MD-R-16, NF-05 and ITCC 8447 were stable at a pH 8–11.5, 4.6–6.5, 2–7 and 7–9 [16-18,21]. The stability of the enzyme over a wide range of acidic to alkaline pH (2–11) and the thermal stability ranged from 20-60 °C. The molecular weight of white laccase from *M verrucaria* ITCC 8447 ranged from 63-75 kDa (Figure 5d). On the other hand, yellow laccase from *Stropharia aeruginosa* is stable over 4–12 pH range for Yel 3p (5–9) and Yel 1p (4–12) with the optimal temperature being 40 °C with molecular weight being reported as 55 kDa due to its monomeric nature Daroch et al [15]. In the present study the molecular weight of blue and glycosylated yellow laccase from *Trametes versicolor*, *Stropharia* sp. ITCC 8422 was 63–75 and 100–135 kDa respectively (Figure 5b, f). The molecular weight of laccase ranges from 63 to 100 kDa which is contributed due to glycosylation (10–50 %) (Table 2). However, the molecular weight can be as high as 320 kDa [36, 37, 38]. Glycosylation (is a reaction where a glycosyl donor attaches to hydroxyl or other functional group of another molecule which acts as a glycosyl acceptor) in laccases is responsible for secretion, thermal stability, proteolytic susceptibility, activity and copper [1, 44].

Table 2. The distinguished features of various multicopper oxidase laccases.

White Laccase	pH	Temperature (°C)	Molecular weight (kDa)	Reference
M. verrucaria 24G-4	8–11.5	30–50	62	[18]
M. verrucaria MD-R-16	4.6–6.5	35–55	-	[17]
M. verrucaria NF-05	2–7	20–60	66	[16]
M. verrucaria ITCC 8447	7–9	30–40	~63–75	[21]
Yellow Laccase				
Stropharia aeruginosa				
Yel 1p	4–12	Up to 40	55	[15]
Yel 3p	5–9			
Blue Laccase				
Trametes versicolor IBL-04	5–8	25–40	63	[45]
Trametes versicolor	2.5–4	0–50	97	[16]

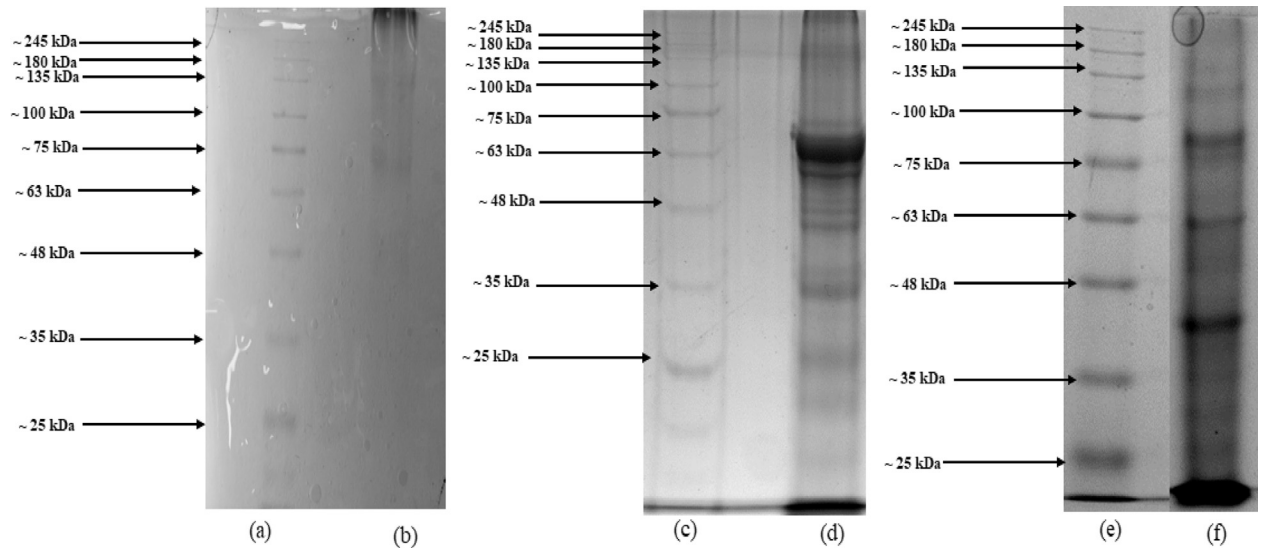

Figure 5. The SDS-PAGE of (b) blue laccase (d) white laccase and silver staining of (f) yellow laccase where (a, c, e) represents the protein ladder (See Supplementary Figure 1 for full image).

4. Conclusion

The sequence of white/yellow and blue laccase exhibited homology with *Trametes villosa, Myrothecium verrucaria* OX = 1859699, Trametes versicolor after LCMS study. The present study enabled better understanding of the typical and atypical laccases. Various spectral analysis i.e., UV-spectra, XRD and FTIR, of blue laccase exhibited different features as compared to other two atypical laccases. The presence of peak at 605 nm in the UV visible spectra, was observed in case of blue laccase whereas it was absent in other two laccases. The XRD data showed that the α-helix intensity was stronger in white/yellow laccase as compared to the blue laccase, whereas the β-sheet structure was stronger in case of blue laccase. Similarly, the FTIR data showed that the β-sheets were more intense with blue laccase as compared to white and yellow laccase. The spectral analysis help infer that the blue laccases are different as compared to white and yellow laccase which is in correlation with the previous study [15, 19, 20, 23].

Declarations

Author contribution statement

K. Agrawal: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

P. Verma: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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References

[1] V. Madhavi, S.S. Lele, Laccase: properties and applications, BioResources 4 (2009) 1694–1717.
[2] A.M. Mayer, R.C. Staples, Laccase: new functions for an old enzyme, Phytochemistry 60 (2002) 551–565.
[3] J. Rogalski, M. Wojtas-Wasilewska, R. Apalovič, A. Leonowicz, Affinity chromatography as a rapid and convenient method for purification of fungal laccases, Biotechnol. Bioeng. 37 (1991) 770–777.
[4] K. Agrawal, V. Chaturvedi, P. Verma, Fungal laccase discovered but yet undiscovered, Bioresour. Bioprocess. 5 (2018) 4.
[5] S. Pang, Y. Wu, X. Zhang, B. Li, J. Ouyang, M. Ding, Immobilization of laccase via adsorption onto bimodal mesoporous Zr-MOF, Process Biochem. 51 (2016) 229–239.
[6] W. Purahong, T. Arnstadt, T. Kahl, J. Bauhus, H. Kellner, M. Hofrichter, D. Krüger, F. Buscot, B. Hoppe, Are correlations between deadwood fungal community structure, wood physico-chemical properties and lignin-modifying enzymes stable across different geographical regions? Fungal Ecol. 22 (2016) 98–105.

[7] K. Murugesan, I.H. Nam, Y.M. Kim, Y.S. Chang, Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture, Enzym. Microb. Technol. 40 (2007) 1662–1672.

[8] S.B. Younes, S. Sayadi, Purification and characterization of a novel trimeric and thermotolerant laccase produced from the ascomycete *Scytalidium thermophilum* strain, J. Mol. Catal. B Enzym. 73 (2011) 35–42.

[9] E. Dantán-González, O. Vite-Vallejo, C. Martínez-Anaya, M. Méndez-Sánchez, M.C. González, L.A. Palomares, J. Folch-Mallol, Production of two novel laccase isoforms by a thermotolerant strain of *Pycnoporus sanguineus* isolated from an oil-polluted tropical habitat, Int. Microbiol. 11 (2008) 163–169.

[10] A. Michniewicz, S. Ledakowicz, R. Ullrich, M. Hofrichter, Kinetics of the enzymatic decolorization of textile dyes by laccase from *Cerrena unicolor*, Dyes Pigments 77 (2008) 295–302.

[11] K. Schliephake, D.E. Mainwaring, G.T. Lonergan, I.K. Jones, W.L. Baker, Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnoporus cinnabarinus*, Enzym. Microb. Technol. 27 (2000) 100–107.

[12] T.A. Garcia, M.F. Santiago, C.J. Ulhoa, Properties of laccases produced by *Pycnoporus sanguineus* induced by 2, 5-xylidine, Biotechnol. Lett. 28 (2006) 633–636.

[13] M.L. Niku-Paavola, R. Fagerström, K. Kruus, L. Viikari, Thermostable laccases produced by a white-rot fungus from *Peniophora species*, Enzym. Microb. Technol. 35 (2004) 100–102.

[14] D. Zhao, X. Zhang, D. Cui, M. Zhao, Characterisation of a novel white laccase from the deuteromycete fungus *Myrothecium verrucaria* NF-05 and its decolourisation of dyes, PloS One 7 (2012).

[15] M. Daroch, C.A. Houghton, J.K. Moore, M.C. Wilkinson, A.J. Carnell, A.D. Bates, L.A. Iwanejko, Glycosylated yellow laccases of the basidiomycete *Stropharia aeruginosa*, Enzym. Microb. Technol. 58 (2014) 1–7.

[16] M.J. Han, H.T. Choi, H.G. Song, Purification and characterization of laccase from the white rot fungus *Trametes versicolor*, J. Microbiol. 43 (2005) 555–560.

[17] J. Sun, N. Guo, L.L. Niu, Q.F. Wang, Y.P. Zang, Y.G. Zu, Y.J. Fu, Production of laccase by a new *Myrothecium verrucaria* MD-R-16 isolated from Pigeon Pea [*Cajanus cajan* (L.) Millsp.] and its application on dye decolorization, Molecules 22 (2017) 673.

[18] W.T. Sulistyaningdyah, J. Ogawa, H. Tanaka, C. Maeda, S. Shimizu, Characterization of alkaliphilic laccase activity in the culture supernatant of *Myrothecium verrucaria* 24G-4 in comparison with bilirubin oxidase, FEMS Microbiol. Lett. 230 (2004) 209–214.

[19] K. Agrawal, P. Verma, Biodegradation of synthetic dye Alizarin Cyanine Green by yellow laccase producing strain *Stropharia* sp. ITCC-8422, Biocatalysis Agric. Biotechnol. 21 (2019) 101291.

[20] K. Agrawal, P. Verma, Column bioreactor of immobilized *Stropharia* sp. ITCC 8422 on natural biomass support of *L. cylindrica* for biodegradation of anthraquinone violet R, Bioresour. Technol. Rep. 8 (2019) 100345.

[21] K. Agrawal, N. Bhardwaj, B. Kumar, V. Chaturvedi, P. Verma, Process optimization, purification and characterization of alkaline stable white laccase from *Myrothecium verrucaria* ITCC-8447 and its application in delignification of agroresidues, Int. J. Biol. Macromol. 125 (2019) 1042–1055.

[22] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, G. Sannia, Laccases: a never-ending story, Cell. Mol. Life Sci. 67 (2010) 369–385.

[23] G. Palmieri, P. Giardina, C. Bianco, A. Scaloni, A. Capasso, G. Sannia, A novel white laccase from *Pleurotus ostreatus*, J. Biol. Chem. 272 (1997) 31301–31307.

[24] M. Mukhopadhyay, R. Banerjee, Purification and biochemical characterization of a newly produced yellow laccase from *Lentinus squarrosulus* MR13, 3 Biotech 5 (2015) 227–236.

[25] P. Zhou, C. Fu, S. Fu, H. Zhan, Purification and characterization of white laccase from the white-rot fungus *Panus conchatus*, BioResources 9 (2014) 1964–1976.

[26] E.G. Bendit, A quantitative X-ray diffraction study of the alpha–beta transformation in wool keratin, Textil. Res. J. 30 (1960) 547–555.

[27] J. Chen, X. Chen, Q. Zhu, F. Chen, X. Zhao, Q. Ao, Determination of the domain structure of the 7S and 11S globulins from soy proteins by XRD and FTIR, J. Sci. Food Agric. 93 (2013) 1687–1691.

[28] S. Krimm, J. Bandekar, Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins, Adv. Protein Chem. 38 (1986) 181–364.

[29] H. Susi, D.M. Byler, Resolution-enhanced fourier transform infrared spectroscopy of enzymes, Methods Enzymol. 130 (1986) 290–311.

[30] W.K. Surewicz, H.H. Mantsch, New insight into protein secondary structure from resolution-enhanced infrared spectra, Biochim. Biophys. Acta 952 (1988) 115–130. http://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=7678532.

[31] J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, Acta Biochim. Biophys. Sin. 39 (2007) 549–559.

[32] E. Goormaghtigh, J.M. Ruysschaert, V. Raussens, Evaluation of the information content in infrared spectra for protein secondary structure determination, Biophys. J. 90 (2006) 2946–2957.

[33] A. Leontievsky, N. Myasoedova, N. Pozdnyakova, L. Golovleva, Yellow' laccase of *Panus tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators, FEBS Lett. 413 (1997) 446–448.

[34] A.A. Leontievsky, T. Vares, P. Lankinen, J.K. Shergill, N.N. Pozdnyakova, N.M. Myasoedova, N. Kalkkinen, L.A. Golovleva, R. Cammack, C.F. Thurston, A. Hatakka, Blue and yellow laccases of ligninolytic fungi, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 156 (1997) 9–14.

[35] N.N. Pozdnyakova, J. Rodakiewicz-Nowak, O.V. Turkovskaya, J. Haber, Oxidative degradation of polyaromatic hydrocarbons and their derivatives catalyzed directly by the yellow laccase from *Pleurotus ostreatus* D1, J. Mol. Catal. B Enzym. 41 (2006) 8–15.

[36] D.A. Wood, Production, purification and properties of extracellular laccase of *Agaricus bisporus*, Microbiology 117 (1980) 327–338.

[37] D.A. Wood, Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*, Microbiology 117 (1980) 339–345.

[38] B. Reinhammar, Copper Proteins and Copper Enzymes, vol. 3, CRC Boca Raton FL, 1984, pp. 1–35.

[39] P. Benkert, M. Biasini, T. Schwede, Toward the estimation of the absolute quality of individual protein structure models, Bioinformatics 27 (2011) 343–350.

[40] M. Bertoni, F. Kiefer, M. Biasini, L. Bordoli, T. Schwede, Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology, Sci. Rep. 7 (2017).

[41] S. Bienert, A. Waterhouse, T.A.P. de Beer, G. Tauriello, G. Studer, L. Bordoli, T. Schwede, The SWISS-MODEL Repository - new features and functionality, Nucleic Acids Res. 45 (2017) D313–D319.

[42] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F.T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede, SWISS-MODEL: homology modelling of protein structures and complexes, Nucleic Acids Res. 46 (2018) W296–W303.

[43] N. Guex, M.C. Peitsch, T. Schwede, Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective, Electrophoresis 30 (2009) S162–S173.

[44] F. Xu, in: M.C. Flickinger, S.W. Drew (Eds.), Laccase in: Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and bioseparation, Wiley, New York, 1999, pp. 1545–1554.

[45] M. Asgher, H.M.N. Iqbal, M.J. Asad, Kinetic characterization of purified laccase produced from *Trametes versicolor* IBL-04 in solid state bio-processing of corncobs, BioResources 7 (2012) 1171–1188.

[46] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[47] K. Agrawal, P. Verma, Potential removal of hazardous wastes using white laccase purified by ATPS–PEG–salt system: an operational study, Environ. Technol. Innovat. 17 (2020) 100556.

[48] J. Rodakiewicz-Nowak, J. Haber, N. Pozdnyakova, A. Leontievsky, L.A. Golovleva, Effect of ethanol on enzymatic activity of fungal laccases, Biosci. Rep. 19 (1999) 589–600.

[49] M. Kolb, V. Sieber, M. Amann, M. Faulstich, D. Schieder, Removal of monomer delignification products by laccase from *Trametes versicolor*, Bioresour. Technol. 104 (2012) 298–304.

[50] S.B. Pointing, L.L.P. Vrijmoed, Decolorization of azo and triphenylmethane dyes by *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase, World J. Microbiol. Biotechnol. 16 (2000) 317–318.

[51] S. Rodríguez-Couto, Fungal laccase: a versatile enzyme for biotechnological applications, in: Recent Advancement in White Biotechnology through Fungi, Springer, Cham, 2019, pp. 429–457.

[52] A.N. Ademakinwa, F.K. Agboola, Biochemical characterization and kinetic studies on a purified yellow laccase from newly isolated *Aureobasidium pullulans* NAC8 obtained from soil containing decayed plant matter, J. Genet. Eng. Biotechnol. 14 (2016) 143–151.

[53] K. Agrawal, P. Verma, Laccase: addressing the ambivalence associated with the calculation of enzyme activity, 3 Biotech 9 (2019) 365.