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PURIFICATION, CHARACTERIZATION AND APPLICATION OF A YELLOW LACCASE FROM *ABORTIPORUS BIENNIS* MTCC-1176

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ABSTRACT
A laccase secreted by white rot fungus *Abortiporus biennis* MTCC-1176 has been purified and characterized. The simple purification procedure involved concentration by ultrafiltration of culture filtrate and an anion-exchange chromatography on diethylaminoethyl (DEAE) cellulose. The sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gave single protein band indicating that the enzyme preparation was pure and the molecular mass of the enzyme was determined 51.0 kDa. The calculated values of K_m and k_{cat} were 0.4 mM and 58.35 s^{-1} giving K_m /k_{cat} value of 14.6 × $10^4 M^{-1}s^{-1}$ using 2, 6- dimethoxyphenol as the substrate. The pH and the temperature optima of the enzyme were 3.5 and 50^0C, respectively. The purified laccase has yellow colour and does not show absorption band around 610 nm found in blue laccases. It oxidizes veratryl alcohol to veratraldehyde and methyl benzene to benzldehyde in absence of mediator molecules.

KEYWORDS: Copper containing enzymes, yellow laccases, lignolytic enzymes, *Abortiporus biennis* , 2,6- dimethoxy phenol, biotransformation.

INTRODUCTION

Laccases [EC.1.10.2.3] are polyphenol oxidases also called multi copper oxidases due to the presence of copper atoms in catalytic centre [1-6]. Laccase was first reported in exudates of Japanese lacquer tree *Rhus vernicifera*[7] along with other higher plants[8]. White rot fungi, soil saprophytes and edible fungi are the important sources for the laccases[4, 9,10]. Besides these, bacteria [11], insects [12] as well as wasp venom[14] are also laccase producers. Laccases are monomeric, dimeric or tetrameric glycoproteins. They catalyze the four electron reduction of molecular oxygen to water[1-6] per monomer of the enzyme. There are three types of coppers in a lacase monomer presented as type 1 (blue copper centre), type 2 (normal copper centre) and type 3 (coupled binuclear copper centre) which differ in their electron paramagnetic resonance (EPR) signals [6].The catalytic performance of a laccase strongly depends on these three types of coppers. A reaction radical is generated when the organic substrate is oxidized by one electron at the active site of the laccase and this reaction radical further reacts non- enzymatically. Type 1 copper acts as electron recipient due to which electron is received at type1 Cu and is shuttled to the tri nuclear cluster where oxygen is reduced to water.

Ortho and para diphenols, aminophenols, polyphenols, polyamines, lignins, and arylamines and some of the inorganic ions are the substrates for laccases[2-5]. The discovery that in presence of high redox potential substrates of laccases, non phenolic molecules can also be oxidized by laccases, has enhanced the substrate range of the laccases[14]. These high redox potential substrates of laccases have been termed as mediator molecules[15]. The ability of laccase to catalyse the oxidation of various phenolic and non-phenolic compounds coupled to the reduction of molecular oxygen, a conveniently available substrate for various reactions, to water, a non toxic product makes laccases valuable enzymes for commercial applications [5,16]. The application of the laccases spans the food, paper, pulp, textile and cosmetic industries [16]. Laccases have applications in medicinal chemistry, carbohydrate chemistry, hormonal chemistry, polymer chemistry and synthetic organic chemistry [5]. Due to these reasons, laccases are still of current research interests [17-20]. Most of the laccases studied so far are blue laccases characterized by the presence of an absorption band around 610 nm and blue colour. There are reports of other laccases which lack the presence of an absorption band near 610 nm and the blue colour[21-23]. These laccases have been termed as yellow laccases. The studies on yellow laccases are rare and their properties are not well understood. In this communication, the authors report the purification and characterization of a yellow laccase from *Abortiporus biennis* MTCC- 1176. The fungal strain is a white rot isolated by Roy [24] from logs of *Dalbergia sisso* and deposited at Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology Chandigarh , India.

MATERIALS AND METHODS

Materials

Diethyl amino ethyl (DEAE) cellulose was from Sigma Chemical Company, St. Louis (USA) and 2,6-dimethoxy phenol (DMP) was from Fluka, Chemi new Ulm (Switzerland). All other chemicals used in these investigations were either from Hi-media laboratory Ltd. Mumbai (India) or from E. Merck Ltd. Mumbai (India) and were used without further purifications. The chemicals used in the gel electrophoresis of the protein including

molecular weight markers were from Bangalore Geni Pvt. Ltd., Bangalore (India).

The Fungal Strain and Its Growth

The fungal strain was procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh, (India) and was maintained on agar slant as reported in MTCC Catalogue of strains-2000[24]. The growth medium for the fungal strain *A. biennis* MTCC-1176 consisted of yeast extract 5.0g, glucose 10.0 g and agar 15.0g in 1.0 L Milli-Q water. For detection of the extracellular secretion of the laccase by *A. biennis*, the liquid culture growth medium[25] consising of glucose 10.0 g, asparagine 1.0 g, yeast extract 0.5 g, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$, 0.01 g in 1.0 L of Milli-Q water was used. The above liquid culture growth medium containing natural lignin substrates like coir dust, corn cob, wheat straw, saw dust and bagasse particles were separately prepared by adding 500 mg of one of the natural lignin substrates to 25mL of growth medium in 100 mL culture flasks which were sterilized. The sterilized growth media were inoculated with small pieces of mycelia (0.5 cm × 0.5 cm) under aseptic condition and the fungal cultures were grown under stationary culture conditions at 25°C in a biological oxygen demand (BOD) incubator. In order to monitor the production of the laccase in the liquid culture medium, 0.5mL aliquots of the growth medium were withdrawn at the regular intervals of 24 hrs and filtered through sterilized Millipore filter 0.22 μm. The filtered extract was analyzed for the activity of the laccase using DMP as the substrate [25]. Extracellular secretion of the laccase in the liquid culture medium by *A. biennis* MTCC-1176 was determined by plotting the enzyme unit/mL of the growth medium against the number of days after inoculation of the fungal mycelia. Each point on the curve is an average of three measurements. The growth medium for the control experiment has the same composition except that no natural lignolytic substrate has been added. In order to optimize the conditions for maximum production of the laccase by *A. biennis* MTCC-1176 in the liquid culture medium, the amount of the best inducer wheat straw were varied from 100 mg to 1200 mg in 25 mL of the growth medium. The amount of the inducer in the growth medium which gave the maximum height of the enzyme activity peak was taken as the optimal amount of the inducer.

Enzyme Assay

The assay solution (1.0 mL) for DMP as the substrate [25] contained 1.0 mM DMP in 100 mM sodium malonate buffer pH 4.5 at 25 °C. The reaction was monitored by measuring the absorbance change at λ=468 nm and using the molar extinction coefficient value of 49.6 mM^{-1} cm^{-1} [25]. The UV/Vis spectrophotometer Hitachi (Japan) model U-2900 fitted with electronic temperature control unit was used for absorbance measurement. The least count of absorbance measurement was 0.001 absorbance unit. One enzyme unit produced 1 μMole of the product per minute under the specified assay conditions.

Purification of Laccase

For the purification of the laccase, *A. biennis* MTCC-1176 was grown in ten 100mL culture flasks each containing 25mL sterilized growth medium containing optimal amount 1000 mg of the best inducer, wheat straw particles, under stationary culture condition in a BOD incubator at 25°C. The maximum activity of the laccase appeared on 7^{th} day of the inoculation of the fungal mycelia. On the 7^{th} day, all the cultures in the 10 flasks were pooled; mycelia were removed by filtration through four layers of cheese cloth. The culture filtrate was then concentrated using Amicon concentrator cell model-8200. 3.0 mL of concentrated enzyme sample containing 0.5 mg/mL protein after dialysis against 10 mM sod.acetate /acetic acid buffer pH 5.0 was loaded on to the DEAE column (size 0.75 cm x 19.0 cm) which was pre-equilibrated with 10 mM sodium acetate/acetic acid buffer (pH 4.5) and the flow rate was 18 mL/hr. The column was washed with 100 mL of the same buffer. The enzyme was eluted by applying linear gradient of the range 0 to 1 M NaCl in the same buffer (40 mL buffer + 40 mL buffer with 1.0 M NaCl). The fractions of 3.2 mL size were collected and analyzed for the laccase activity[25]. The protein estimation was done by Lowry method[26]. All laccase active fractions were combined and concentrated by Amicon concentrator cell model 8200 and then by model-3 to 4 mL. The enzyme was stored in a refrigerator in 10mM sodium acetate/acetic acid buffer pH 4.5at 4°C. The enzyme does not loose any activity for one month under these conditions.

SDS-PAGE Analysis

The purity of the enzyme preparation was checked by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis[27]. The molecular weight markers were phosphorylase (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa), and were procured from Bangalore Genei Pvt. Ltd. Bangalore (India). Gel was run at a constant current 20 mA. The proteins were stained using Coomassie Brilliant Blue R-250. The molecular weight was determined by Weber and Osborn method [28].

Steady State Enzyme Kinetics

The steady state enzyme kinetics of the purified laccase was studied using DMP, as the substrates following the methods as mentioned in the assay section. K_m and k_{cat} values for the enzyme were determined from the linear regression of double reciprocal plots. The pH and temperature optima of the enzyme were determined by measuring the steady state velocities of the enzyme catalyzed reaction in the solutions of varying pH/temperature keeping the other parameter fixed and drawing graphs of steady state velocity vs pH/temperature of the reaction solution.

UV/Visible spectrum of the purified enzyme and enzymatic reaction products

The UV/Visible of the purified enzyme was recorded using UV/Visible sprectrum Spectrophotometer Hitachi (Japan) model U- 2900. For recording UV/Vis spectra of the reaction products, the same equipment was used.

Biotransformation of Veratryl Alcohol and Methyl Benzene

Biotransformation of veratryl alcohol to veratraldehyde was studied using the reported method[22]. For the biotransformation of methyl benzene to benzaldehyde, 6mL of 100 mM sod. acetate/acid buffer pH 3.5, 4 mL of

20 mM methyl benzene solution in dioxane and 100 µL of enzyme stoke [1.96 IU/mL] were mixed. The spectra of the reaction solution were recorded just after mixing and after 24 hrs of mixing of the enzyme. The veratraldehyde formed in the reaction solution was extracted with ethyl acetate and its absorbance at 310nm were measured and the concentration of veratraldehyde in the extract was calculated using molar extinction coefficient value of 9300 $M^{-1}cm^{-1}$. Similarly benzaldehyde formed in the reaction solution was extracted using n- hexane. The concentration of aldehyde in the extract was determined by measuring absorbance at 240 nm and comparing it by measuring the absorbance of standard solution of benzaldehyde.

Crystallization of the purified enzyme

The purified enzyme was careened for the crystallization using sparse matrix sampling technique available in the literature [29]. The technique used was vapour diffusion in sitting drops. The culture plates with 24 wells were used for setting the various crystallization conditions. One vertical micro bridge was dropped in each well which contained 1.0 mL of the precipitating solution. Drops of 10µL of the protein solution [1 mg/mL] were made in the grooves of micro bridges and 10 µL of the precipitating solution from the wells were added in each of the protein drops. The wells of culture plates were sealed using vacuum grease and cover slips. The drops were observed under microscope at the regular intervals of 15 days.

RESULTS & DISCUSSION

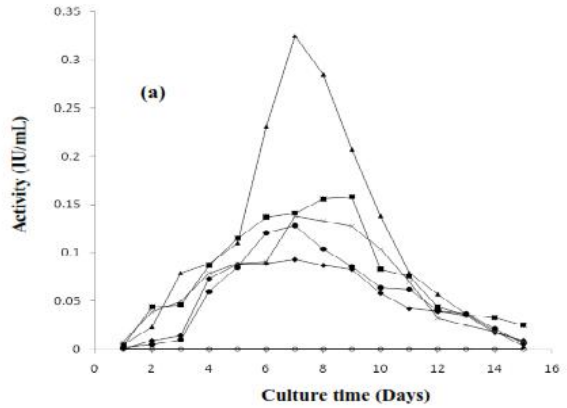
Most of the laccases are characterized by the presence of an absorption band near 610 nm in their UV/Visible spectra and their blue colour[5,6]. These are called blue laccases. These laccases do not oxidize non phenolic compounds alone. However, in presence of high redox potential substrates, the blue laccases can oxidize non phenolic compounds[14]. Such high redox potential substrates of blue laccases are called redox mediators [15]. These mediator molecules extend the substrate range of blue laccases and make them more suitable for applications [5,16]. There are some laccases which lack the absorption band near 610 nm in their UV/Visible spectra and the characteristic blue colour of blue laccases [21-23]. Instead they have yellow colour and are called yellow laccases. The yellow laccases oxidize non phenolic compounds even in the absence of mediator molecules [22]. Thus yellow laccases are better bio catalysts as compared to blue laccases. There are reports in the literature [21-23] that the fungal stains which secrete blue laccases in their liquid culture growth media secrete yellow laccases when grown the media containing solid natural substrates like wheat straw. The fungal strain *A. biennis* MTCC- 1176 grows on log of *Dalbergia sisso*[24] and is expected to secrete a laccase. On the basis of this hypothesis, the authors tested the secretion of laccase by this fungal strain in the liquid culture growth medium supplemented with different natural lignin containing substrates wheat straw, corn cob, bagasse, saw dust and coir dust using only the liquid culture growth medium alone as the control.

TABLE 1: Purification procedure

Steps	Volume	protein (mg/mL)	Activity (IU/mL)	Specific Activity (IU/mg)	Total Protein (mg)	Total Activity (IU)	Purification fold	% Yield
Culture filtrate	250	0.99	0.20	0.20	247.50	50.00	1.00	100.00
Ultrafiltration and dialysis	4.0	2.94	6.13	2.08	11.76	24.52	10.40	49.04
Cellulose Column Chromatography (After concentration)	4.0	0.41	1.96	4.78	1 .64	7.84	23.90	15.68

The results are shown in fig 1(a) which showed that the presence of solid lignin containing natural substrates in the liquid culture growth medium enhanced the level of laccase activity in the medium and the maximum enhancement was in the case of medium supplemented with wheat straw particles. In order to maximize the secretion of laccase by the fungal strain, the effect of varying the amount of wheat straw particles in the liquid culture growth medium, on the secretion of laccases by the fungal strain, was studied. The result is shown in fig 1(b). It was found that 1000 mg of the wheat straw particles /25 ml of the medium gave maximum level of laccase secretion. For the purification of the laccase from the culture filtrate of the fungal strain, it was grown in the medium supplemented by 1000mg/25ml of the medium. The presence of more than 1000mg/25ml of the culture medium of the wheat straw particles lowered the level of laccases secretion in the medium by the fungal strain.

The results of the purification of the enzyme from the culture filtrate of the fungal strain are summarized in table 1 and the elution profile of the enzyme from the anion exchange column of DEAE cellulose in shown in fig 2. The enzyme bound to the DEAE cellulose equilibrated to 10 mM sodium acetate/acetic acid buffer pH 4.5 and was eluted by the linear gradient of NaCl in the same buffer in the range 0.1-0.8 molar NaCl. Nearly 24 fold purification with 15.68% recovery of the enzyme activity was achieved using a simpler procedure of concentration of culture filtrate by ultra filtration and anion exchange chromato graphy of DEAE cellulose.



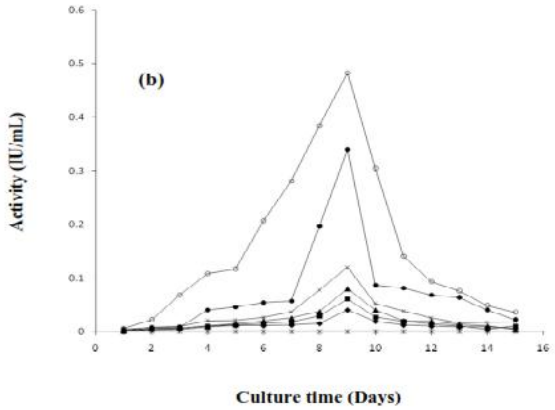


Figure 1(a) Secretion of laccase by *A. biennis* MTCC-1176 in the liquid culture medium supplemented with different natural lignin containing substrates: wheat straw(▲), corn cob(●), bagasse(×), saw dust(♦), coir dust(■), control(o).Each data point is an average of triplicate measurements and the standard deviation is less than 10%.

(b) Optimization of laccase secretion by *A. biennis* MTCC- 1176 in liquid culture medium supplemented with different amount of wheat straw: 100 mg (♦), 200 mg (■), 400 mg (▲), 600 mg (×), 800 mg (●), 1000 mg (o). Above 1000mg, the level of enzyme secretion decreased. Each data point is an average of triplicate measurements and the standard deviation is less than 10%.

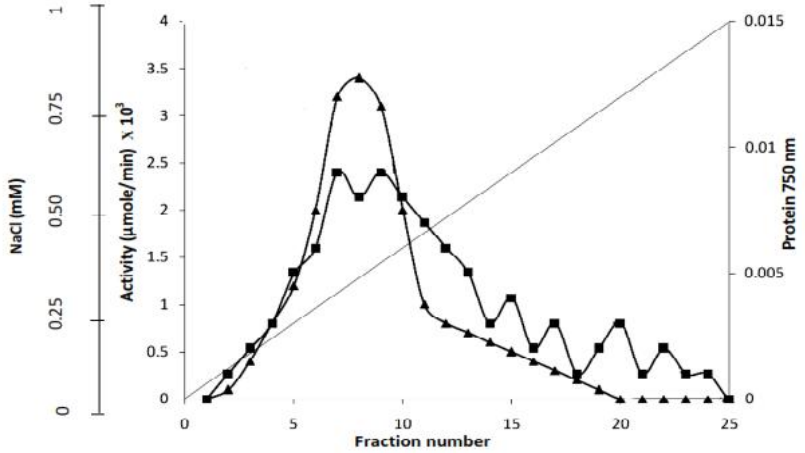


FIGURE 2.0 Elution profile of the enzyme from the DEAE cellulose column: Activity (▲), Protein (■), and NaCl gradiant (---).

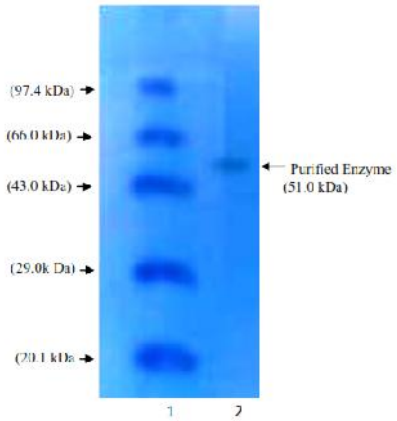


FIGURE 3.0 SDS-PAGE of the purified laccase. Lane 1 contains molecular weight markers and lane 2 contains 20µg of the purified enzyme.

The results of sodium dodesyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the enzyme shown in fig 3. in which lane 2 contains the purified enzyme and in lane 1 are molecular weight markers. The single protein band in lane 2 of fig. 3 confirms the purity of the purified enzyme. The determination of molecular weight of the purified enzyme from SDS-PAGE analysis was 51.0 kDa.

The UV/Visible spectrum of the purified enzyme with reference to the buffer in which the enzyme was purified is shown in fig. 4(a). The lack of absorption band near 610 nm in the UV/Visible spectrum of the purified enzyme

shows that the purified enzyme is not a blue laccase. The colour of the purified enzyme is yellow showing that it is a yellow laccase (fig. 4, b). Yellow laccases oxidize veratryl alcohol to veratraldehyde in absence of mediator molecules. The results of the studies of oxidation of veratryl alcohol to veratraldehyde by the enzyme in the absence of mediator molecule are shown in figure 5 (a) in which spectrum (I) of the reaction solution containing veratryl alcohol, the purified laccase in 100mM sodium acetate/ acetic acid buffer pH 3.5 was recorded just after mixing the reagents and spectrum (II) was recorded after 24 hrs of mixing the reagents. The results of the studies of the biotransformation of methyl benzene to benzaldehyde by the purified laccase in absence of a mediator molecule is shown in fig. 5 (b) in which spectrum (I)was recorded just after mixing the reagents, the spectra marked as II were recorded after 10 min and 20 min of mixing of reagent and spectrum III was recorded after 24 hrs of mixing the reagents. The increase in absorbance around 240nm clearly shows that methyl benzene is converted to benzaldehyde by the purified laccase in absence of any mediator molecule.

The result of steady state kinetic studies of the purified enzyme using 2,6-dimethoxy phenol [DMP] as the variable substrate are shown in fig. 6 in the form of Michelis-Menton curve and double reciprocal plot. The calculated k_m and k_{cat} values of the enzyme for DMP are 0.4 mM and 58.35s^{-1} giving k_{cat}/k_m value of 14.6×10^4 $M^{-1}s^{-1}$.

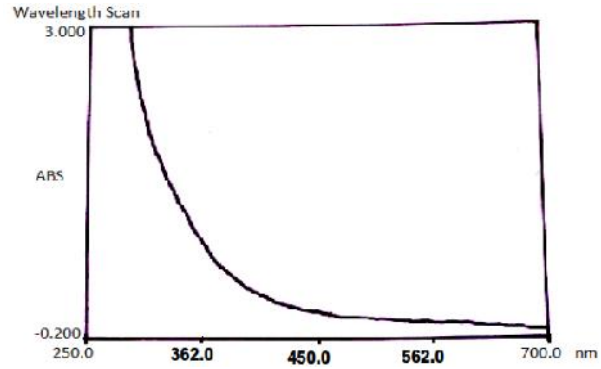


FIGURE 4.0 (a) UV/Visible spectrum of the purified enzyme (8.04 µmolar) with reference to10 mM sod. acetate/ acetic acid buffer pH 4.5

(b) 8.04 µmolar enzyme in 10 mM sod.acetate/ acetic acid buffer pH 4.5.

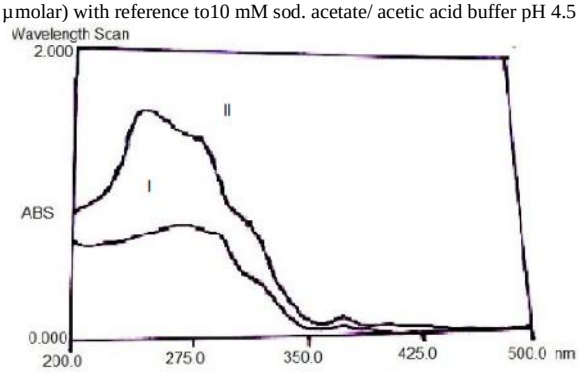
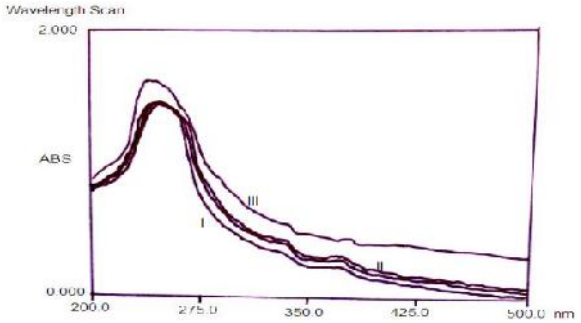


FIGURE 5.0 (a) UV/Visible spectrum of biotransformation of veratryl alcohol to veratraldehyde using purified yellow laccase in the absence of mediator molecule (details in the text).Spectrum I is just after mixing the reagents and the spectrum II is after 24 hrs of mixing the reagents.



(b) UV/Visible spectrum of biotransformation of methyl benzene to benzaldehyde using purified yellow laccase in the absence of mediator molecule (details in the text). Spectrum I is just after mixing the reagents and the closed two spectra marked as II are after 10 mint and 20 min of mixing the reagents. Spectrum III is after 24 hrs of mixing the reagents

The conversion of veratryl alcohol to veratraldehyde is accompanied by the increase in absorbance at 310nm. The recorded spectra clearly established the conversion of veratryl alcohol to veratraldehyde in absence of a mediator molecule. Veratraldehyde was extracted with ethyl acetate and the absorbance of the extracted solution was measured at 310 nm. The calculated concentration of veratraldehyde in the extracted solution was in the order of 0.7 mM using molar extinction coefficient value of 9300$M^{-1}cm^{-1}$.

It is worth mentioning here that the catalytic performances of laccases span several orders of magnitude for different substrates and are characteristics for specific protein [4]. The optimal functional pH and temperature of the purified enzyme were also determined. The results are shown in fig 7(a) and (b), respectively. In fig 7 (a), the activity of enzyme has been plotted against the pH of the enzymatic reaction solution using DMP as the substrate. The calculated pH optimum of the enzyme was 3.5 pH units. In fig 7(b), the activity of the purified enzyme, has been plotted as a function of temperature of the enzymatic reaction solution. The calculated optimal temperature is 50.0°C.Thus the optimal function pH and temperature of the purified enzyme are 3.5 pH unit and 50.0°C. These values for optimal pH and temperature are not unusual in view of reported studies of other fungal laccases [4].

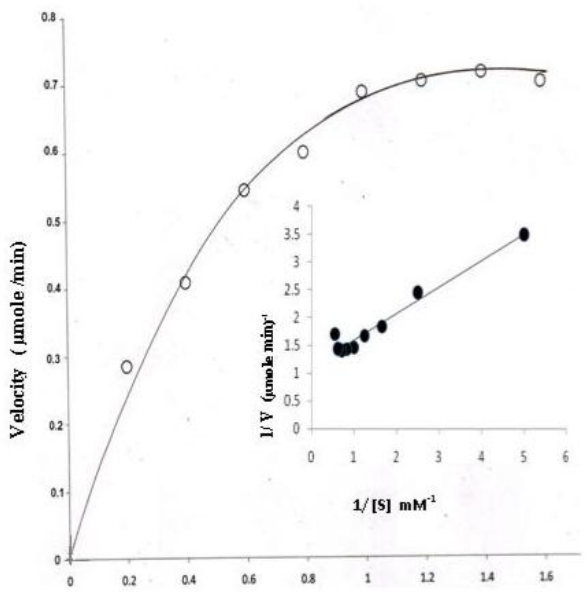


FIGURE 6.0 Michaelis-Menten curve **(a)** and double reciprocal plot **(b)** for the laccase of *A. biennis* MTCC-1176 using DMP as the variable substrate. In (a) and (b) 1 mL reaction solution contained 0.0-2.0 mM DMP, 50 µL of the enzyme stock (8μM) in sodium malonate buffer pH 4.5 at 25 ^{0}C.

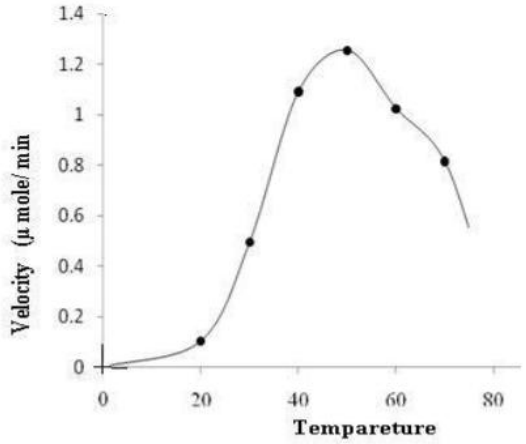


FIGURE 7.0 (b) Determination of temperature optimum of the purified enzyme. In this case 1 mL reaction solution contained 1.0 mM DMP in 100 mM sodium malonate buffer pH 4.5 with varying temperature from 20 to 100 ^{0}C. 50 µL of the enzyme stock (8μM) was used.

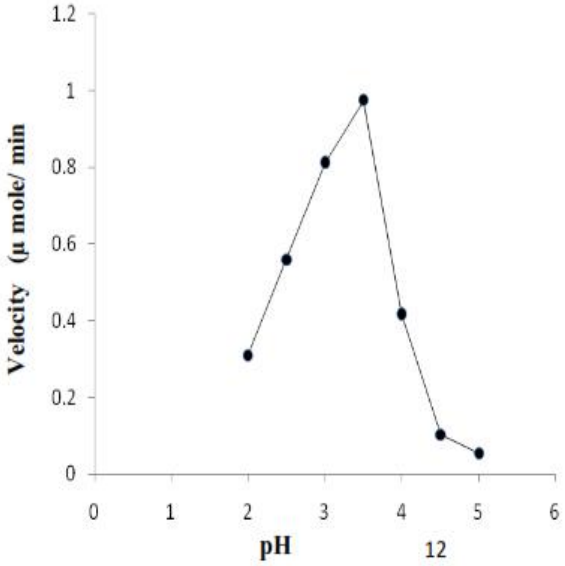


FIGURE 7.0 (a) Determination of pH optimum of the purified enzyme. In this case 1 mL reaction solution contained 1.0 mM DMP in 100 mM sodium malonate buffer with varying pH from 2.0 to 5.0 and temperature was fixed at 25 ^{0}C . 50 µL of the enzyme stock (8μM) was used.

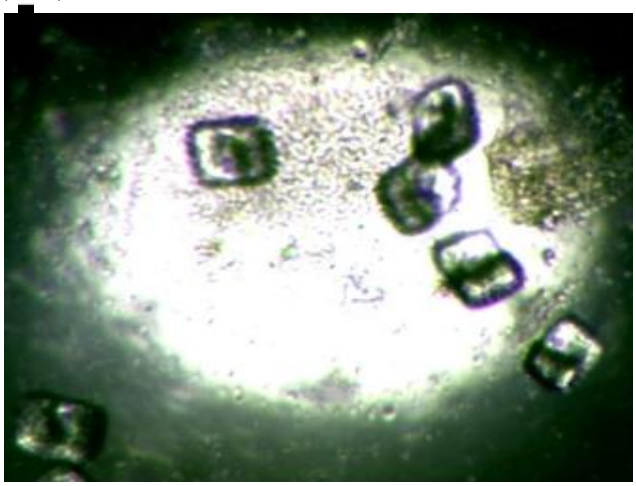


FIGURE 8.0 Enzyme crystals in 0.05 molar potassium phosphate and 20% polyethylene glycol 8000 in 20 µL droplet containing enzyme 0.5 mg/mL.

There are a few relatively recent studies on yellow laccases[30-32] but the basic questions related to their enzymology are still unanswered. The lack of the presence of absorption band near 610 nm in the UV/Visible spectra of yellow laccases and the absence of blue colour are related to the change in the environment of type 1 copper. This has been confirmed by the distinct EPR signal of type 1 copper in yellow laccases as compared to blue laccases [21]. However, at least, one yellow laccase has been reported[23] which lacked the presence of absorption band near 610 nm in its UV/Visible spectrum but its EPR signal resembled the EPR signal of blue laccases. On the basis of circular dichroism studies of this yellow laccase, it has been concluded that the overall structure of this laccase is similar to other laccases and the peculiar property of this laccase is due to type 1 copper center environment. That there is no basic deference between the structure of active site of blue laccases and yellow laccases have also been concluded by Ike *et al.* [31] on the basis of molecular modeling of a yellow laccase from *Leucoagaricus gongylophorus*. The proposed explanation for the difference in the properties of yellow laccases as compared to blue laccases is the binding of low molecular mass phenolic molecules arising from lignin degradation and modifying the type 1 copper center of the laccase [21]. But no experimental proof of this proposal has been given in the litrature [21,31].

The crystal structure of none of the yellow laccases is available [31]. One possible way to resolve the mystery of the yellow laccases is to crystallize at least one yellow laccase in the form of suitable protein crystals for x-rays crystallographic studies. The authors have initiated the studies on the crystallization of the purified yellow laccase. The screening test for the crystallization for the

purified yellow laccase has been done using the procedure given in the literature [29]. The result achieved is shown in fig.8. The purified yellow laccase has shown the tendency to crystallize. However more efforts are needed to improve the quality of crystals and make them suitable for x- rays crystallographic studies.

Concluding remarks

In conclusion, this communication reports a yellow laccase from a new fungal strain which can be purified conveniently and can be used for different bio transformation reactions. Further studies to resolve the mysteries yellow laccases are under way in our laboratory.

ACKNOWLEDGEMENTS

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REFERENCES

[1] *Multi-Copper Oxidases* (Messerschmidt A., ed), World Scientific Publishing: Singapore 1997.

[2] Yadav, M. and Yadav, K.D.S., Structural and functional aspects of lignolytic enzymes. Lignocellulose Biotechnology: Future Prospects (Kuhad RC & Singh IK, eds), International Publishing House, Pvt. Ltd., New Delhi 2007, pp. 63-88.

[3] Riva, S., Laccases: blue enzymes for green chemistry , *Biotechnol.* 2006, 24, 219-226.

[4] Baldrian, P.,Fungal laccases-occurrence and properties, .FEMS Microbiol Rev. 2006 30(2),215-42.

[5] Dwivedi, U.N., Singh, P., Pandey, V.P., and Kumar, A., Structure–function relationship among bacterial, fungal and plant laccases, *J. Mol. Cat. B: Enzymatic*, 2011, 68, 117-128.

[6] Quintanar, L., Yoon, J., Aznar, C. P., Palmer, A.E., Andersson, K.K., Britt, R.D. and Solomon, E.I., Spectroscopic and Electronic Structure Studies of the Trinuclear Cu Cluster Active Site of the Multicopper Oxidase Laccase: Nature of Its Coordination Unsaturation J. Am. Chem. Soc. 2005, 127, 13832-13845.

[7] Yoshida, H., Chemistry of lacquer (Urushi) Part I. Communication from the Chemical Society of Tokio *J. Chem. Soc., Trans.*1883, 43, 472-486.

[8] Francisco, J. E., David, M., Lígia, O. M., Rosa, G.,Adriano, O. H.,Peter,F. L.,Maria, A C., Substrate and Dioxygen Binding to the Endospore Coat Laccase from *Bacillus subtilis*, J. Biol. Chem.,2004,. 279(22), 23472–23476.

[9] Thurston, C., The structure and function of Yellow Laccases. Microbiol. 1994, 140,19-26.

[10] Gochev, V.K and Krastanov A.I. Fungal Laccases(a review) Bulgarian J. of Agr Science 13, 75-83 (2007).

[11] Thomas, B.R., Yonekura, M., Morgan, T.D., Czapla, T.H., Hopins, T.L. and Kramer,K.J., Laccase: Microbial Sources, Production, Purification, and Potential Biotechnological Applications, *Biochem.* 19, 611-622.

[12] Parkinson, N.; Smith, I.; Weaver, R.; Edwards, J.P. A new form of arthropod phenoloxidase is abundant in the parasitoid wasp *Pimpla hypo chondriaca*. *Insect Biochem. Mol. Biol.* 2001, *31*, 57–63.

[13] Bourbonnais, R., and Paice, M.G. Oxidation of non Phenolic substrates; An extended role for laccase in lignin biodegradation FEBS 267(1), 99-102 (1990).

[14] Morozova, O.V., Shumakovich, G.P., Gorbacheva, M.A., Shleev, S.V., Yaropolov, A.I., Review: "Blue" Laccases, *Appl Biochem and Biotechnol*. 2007, 72(10), 1136-1150.

[15] Couto, S.R., and Harrera, J.L.T., Industrial and biotechnological applications of laccases: a review *Biotechnol. Advan.*, 2006, 24, 500-513.

[16] Sumathi, T. Viswnath, B., Lakshmi,A.S., SaiGopal, D.V.R, Production of laccase by Cochlibolus sp. Isolated from plastic Dumped soil and their ability to degrade Low molecular weight PVC , Biochem. Res. Intern., 2016, Article ID 9519527, 10 pages.

[17] Monsel, R.A. Abd El. Hassan, EA, Ramodan E.M., Production of laccase enzyme for their potential Application to decolorize fungal pigments on aging paper and parchment. Annal. Agri. Sci.2016, 61(1), 145-154.

[18] Munk L, Punt A.M., Kabel, M.A., and Meywr, A.S., Laccase catalysed grafting of –N-OH type mediators to lignin via redical coupling. RSC Adv.2017, 7, 3358-3368.

[19] Jia, W., Wang, P., Laccase- mediated in situ oxidation of dopa for bio-inspired coloration of silk fibric, RSC Adv. 2017, 7, 12977-12983.

[20] Leontievsky, A.A., Vares, T., Lankinen, P., Shergill, J.K., Pozdnyakova, N., Myasoedova, N.M., Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiol. Lett.* 1997, 156, 9-14.

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

[22] Mot., A.C., Parvn, M., Domian, G., Irimie, F. D., Darula, Z., Medziihradszky, K.F., Brem B., Silaghi-Dumitrescu, R.M., Yellow laccase with blue spectroscopic feature from *Sclerotinia sclerotisrum*. Process Biochem. 2012, 47, 966-975.

[23] Catalogue of strains (Fifth ed), Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology, Chandigarh, India, 2000, p.45.

[24] Coll, M.P., Fernandez-Abalos, J.M., Villanueva, J.R., Santamaria, R. and Perez, P., "Purification and characterization of a phenoloxidase (laccase) from the lignin-deg rading basidiomyc ete PM1 (CE C T 2971), Appl. Environ.Microbiol., 1993, 59, 2607-2613.

[25] Lowry, O.H., Rosebrough, N.J., Farrand, A.L., and Randall, R.J., Protein measurement with the Folin-phenol reasent. *J. Biolog. Chem.*, 1951, vol. 193, pp. 265-275.

[26] Polyacrylamide Gel Electrophoresis: Laboratory Techniques, Pharmacia, Laboratory Separation Division, Uppsala Sweden, 1984.
[27] Weber, K., and Osborn, M., The reliability of molecular weight determination by dodecyl sulphat polyacrylamide gel electrophoresis, *J. Biol. Chem.*, 1969, 244, 4406-4412.
[28] Jancarik, J. and Kim, Sung-Hov. Sparse matrix sampling: a screening msthod for crystallization of protein. J. Appli. Crystal. 1999, 24, 409-411.
[29] Mukhopdhyay, M. and Banerjee, R. Purification and Biochemical Characterization of a newly produced yellow laccase from *Lentinus squarrosulus* MR_{13}, Biotech. 2015, 5, 227-236.
[30] Ike, P.T.L., Moveira, A.C., De Almeida, F.G., Ferreira, D., Birolli, W.G., Porto, A.L.M. and Souza, D.H.F., Functional characterized of a yellow laccase from *Lcoagaricus gongylophurus*, Sprmgerplus , 2015, 4, 654. Doi 10, 1186/540064-015-1464-y.
[31] Ademakinwa, A.N. and kinetic studies on purified yellow laccase from newly isolated *Aureobasidium pullutans* NAC8 obtains from soil containing decayed plant matter, J. of Genetic Engin. Biotechnol.2016, 14(1), 143-151.