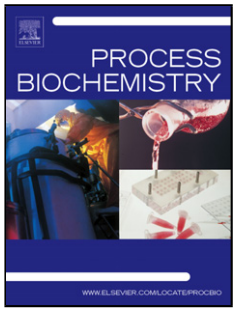

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Purification and characterization of yellow laccase from *Trametes hirsuta* MTCC-1171 and its application in synthesis of aromatic aldehydes

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Highlights

- Purification and characterization of yellow laccase from new fungal strain.
- Use of this purified laccase in syntheses of several aromatic aldehydes.
- No mediator used for such syntheses.
- Identification of products by HPLC and IR.

Abstract A yellow laccase from the culture filtrate of *Trametes hirsuta* MTCC-1171 has been purified. The purification methods involved concentration of the culture filtrate by ammonium sulphate precipitation and an anion exchange chromatography on diethylaminoethyl cellulose. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis gave single protein band indicating that the enzyme preparation was pure. The molecular mass of the enzyme determined from SDS-PAGE analysis was 55.0 kDa. Using 2,6-dimethoxyphenol, 2,2' [Azino-bis-(3-ethylbonzthiazoline-6-sulphonic acid) diammonium salt] and 3,5-Dimethoxy-4-hydroxybenzaldehyde azine as the substrates, the K_m, k_{cat} and k_{cat}/K_m values of the laccase were found to be 420 μM, 13.04 s^{-1}, 3.11×10^4 $M^{-1}s^{-1}$, 225 μM, 13.03 s^{-1}, 1.3×10^5 $M^{-1}s^{-1}$ and 100 μM, 13.04 s^{-1}, 5.8×10^4 $M^{-1}s^{-1}$, respectively. The pH and temperature optima were 4.5 and 60 °C, respectively while pH and temperature stabilities were pH 4.5 and 50 °C. The activation energy for thermal denaturation of the enzyme was 18.6 kJ/mole/K. The purified laccase has yellow colour and does not show absorption band around 610 nm

like blue laccases. The purified laccase transforms toluene, 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene and 3,4-dimethoxytoluene to benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde in the absence of mediator molecules in high yields.

Keywords: 2, 6- dimethoxyphenol, copper containing enzymes, lignolytic enzymes, *Trametes hirsuta*, yellow laccases

Introduction

Laccase [benzenediol: oxygen oxidoreductase; E.C. 1.10.3.2] is a polyphenol oxidase, which belongs to the superfamily of multicopper oxidases and catalyzes the four electron reduction of molecular oxygen to water [1-5]. Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic functions, laccases depend on Cu atoms that are distributed at the three different copper centres viz. type-1 or blue copper centre, type-2 or normal copper centre and type-3 or coupled binuclear copper centres, differing in their characteristics electronic paramagnetic resonance (EPR) signals [6]. The organic substrate is oxidized by one electron at the active site of the laccase generating a reaction radical which further reacts non-enzymatically. The electron is received at type1 Cu and is shuttled to the trinuclear cluster where oxygen is reduced to water.

More than 100 fungal laccases have been purified and characterized. Their different physiochemical properties like temperature optima, pH optima, K_m and molecular masses for different substrates have also been studied [4]. Molecular

masses of fungal lccases have been found in the range of 43-383 kDa [4]. Most of the studies reported so far are on blue laccases. Yellow or white laccases have rarely been studied [7, 8]. Yellow or white laccases differs from blue laccases in two aspects. Blue laccases have the absorption band around 610 nm [7] while yellow or white laccases lack it. Yellow or white laccases oxidize non-phenolic substrates in absence of mediator molecules [8] which are required in case of blue laccases. Thus, yellow or white laccases are better biocatalysts than blue laccases. Ortho and para diphenols, aminophenols, polyphenols, polyamines, lignins, and arylamines and some of the inorganic ions are the substrates for laccases. The ability of laccases to catalyze the oxidation of various phenolic as well as non-phenolic compounds, coupled to the reduction of molecular oxygen to water makes it valuable from the point of view of their different applications [4, **9**-12]. The biotechnological importance of laccases have increased after the discovery that oxidizable reaction substrate range could be further extended in the presence of small readily oxidizable molecules called mediators [13, 14]. During the last two decades, laccases have turned out to be the most promising enzymes for industrial uses [11, 12] having applications in food, pulps, paper, textile and cosmetic industries and in synthetic organic chemistry like selective oxidation of aromatic methyl group, novel penicillins synthesis, coupling of amines, oxidation of amides etc [9, 15-18].

Laccases purified from different sources exhibit different properties and are suitable for different applications. Keeping these points in view, authors have initiated studies on purification, characterization and biotechnological applications of laccases from different sources. It is already reported in the literature that some fungal strains which secrete blue laccases in submerged liquid cultures, secrete yellow laccases in the presence of culture media containing natural lignin substrates [7-8, 11, 19-21].

Moreover, blue laccases transform toluene to benzaldehyde in the presence of mediator molecules [22, 23] whereas some of the yellow laccases perform same reactions in the absence of mediator molecules [24]. Only conversion of toluene to benzaldehyde was done previously [24]. Thus, the objective of this communication was to purify and characterize a novel yellow laccase from *T. hirsuta* MTCC-1171 and to demonstrate the conversion of toluene, 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene and 3,4-dimethoxytoluene to benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde in the absence of mediator molecules.

Materials and methods

Materials

3,5-Dimethoxy-4-hydroxybenzaldehyde azine (syringaldazine), 3-chlorotoluene, 4-chlorotoluene and diethyl amino ethyl (DEAE) cellulose were from Sigma Chemical Company, St. Louis (USA). 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and 2,6-dimethoxy phenol (DMP) were from Fluka, Chemi new Ulm (Switzerland). All other chemicals used in these investigations were either from Himedia 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 samples were from Bangalore Geni Pvt. Ltd., Bangalore (India). DEAE Cellulose column used in purification of enzyme was from Pharmacia, Upssala, Sweden.

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 [25]. The growth medium for the fungal strain *T. hirsuta* MTCC-1171 consisted of malt extract 20.0 g, and agar 20.0 g in 1.0 L Milli-Q water. The pH of the growth medium was adjusted to 6.5.

In order to detect the extracellular secretion of laccase by *T. hirsuta,* the liquid culture growth medium reported by Coll et al. [26] was used. This medium consisted of glucose 10.0 g, asparagine 1.0 g, yeast extract 0.5 g, $MgSO_4 \cdot 7H2O$ and $FeSO_4 \cdot 7H_2O$, 0.01 g in 1.0 L of Milli-Q water. This 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 0.5 g of one of the natural lignin substrates to 25 mL 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 laccase in the liquid culture medium, 0.5 mL 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 laccase using DMP as substrate by the method [26] given in assay section. Extracellular secretion of laccase in the liquid culture medium by *T. hirsuta* MTCC-1171 was determined by plotting 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 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 laccase by *T. hirsuta* MTCC-1171 in the liquid culture medium, the amount of the

best inducer wheat-straw particles were varied from 100 mg to 1000 mg in 25 mL of the growth medium. The amount of 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 using DMP as the substrate [26] contained 1.0 mM DMP in 50 mM sodium malonate buffer (pH 4.5) at 37°C, using ABTS as the substrate [27] contained 0.5 mM ABTS in 0.1 M sodium acetate buffer (pH 5.0) at 25°C and using syringaldazine as the substrate [28] contained 0.1 mM syringaldazine in 50 mM sodium phosphate buffer (pH 6.0) at 50°C. In case of DMP, the reaction was monitored by measuring the absorbance change at λ=468 nm ($\varepsilon = 49.6 \times 10^3$ $M^{-1}cm^{-1}$) while in case of ABTS and syringaldazine, the reactions were monitored by measuring the absorbance change at λ=420 nm ($\varepsilon = 36.0 \times 10^3$ $M^{-1}cm^{-1}$) [27] and λ=530 nm (ε =65.0 mM^{-1} cm^{-1}) [28], respectively. 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 μmol of the product per minute under the specified assay conditions.

Purification of laccase

For the purification of laccase, *T. hirsuta* MTCC-1171 was grown in ten 100 mL culture flasks each containing 25 mL sterilized growth medium with optimal amount 600 mg of the inducer, wheat-straw particles, under stationary culture condition in a BOD incubator at 30°C. The maximum activity of laccase appeared on 7^{th} day of the inoculation of fungal mycelia. On 7^{th} day, all the cultures in 10 flasks were pooled, mycelia were removed by filtration through four layers of cheese cloth. The culture

filtrate was saturated up to 30% with ammonium sulphate and centrifuged using refrigerated centrifuge Sigma (Germany) model 3K-30 at 5480×g for 20 min. at 4°C. The precipitate was discarded and the supernatant was saturated up to 90% by further addition of ammonium sulphate. The resulting suspension was centrifuged by repeating the same process of centrifugation and the supernatant was discarded. The precipitate was dissolved in 100 mM sodium acetate buffer pH 4.5 and dialyzed against 10 mM sodium acetate buffer pH 4.5 in volume ratio 1:1000 with three changes at the intervals of 8 hrs. The dialyzed enzyme sample 15 mL containing 1.4 mg/mL protein was loaded on to DEAE column (size 0.75 cm x 18.0 cm) which was pre-equilibrated with 10 mM sodium acetate/acetic acid buffer (pH 4.5) and the flow rate was 15 mL/hr. Column was washed with 100 mL of the same buffer. The enzyme was eluted by applying linear gradient of NaCl in the range of 0.23 to 0.84 M in same buffer (35 mL buffer and 35 mL buffer). The fractions of 5.0 mL size were collected and analyzed for laccase activity [26]. The protein estimation was done by Lowry method [29]. All laccase active fractions were combined and concentrated by Amicon concentrator cell model 8200 and then by model-3 to 3 mL. The enzyme was stored in refrigerator in 10 mM sodium acetate/acetic acid buffer pH 4.0. The enzyme does not loose any activity for one month under these conditions.

Copper content analysis

This is based on the study of complex formation of 2, 2'-biquinoline with copper present in protein, spectrophotometrically, at 546 nm [30, 31]. Purified protein was first diluted with 0.1 M phosphate buffer (pH 6.0) to 2mL and then this solution was diluted to 5 mL with 2, 2'-biquinoline solution in glacial acetic acid. A blank solution was also prepared using similar method with 2 ml of buffer. Absorbances were

measured at 546 nm and the copper content was estimated by reported method [30, 31].

SDS-PAGE analysis

Purity of the enzyme preparation was checked by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis [32]. The molecular mass 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 Polyacrylamide Gel Electrophoresis [33]. The molecular mass was determined by Weber and Osborn method [34].

Native-PAGE and zymogram analysis

The native polyacrylamide gel electrophoresis of the purified enzyme was done using the reported method [35]. The composition of resolving and stacking gel was similar to that used in SDS-PAGE except SDS was absent. Native molecular mass protein markers used were similar to SDS-PAGE analysis. Two sets of native gel were done. One set was strained with Coomassie Brilliant Blue R-250 and the other set was used for zymogram [36] preparation. For the preparation of zymogram 100 mM DMP solution was made in 10 mM sodium tartrate buffer pH 5.0. The native gel was dipped in zymogram solution for five minutes and a brown band appeared. The zymogram was removed from DMP solution and was washed thrice with 10 mM sodium tartrate buffer pH 5.0 at the interval of five minutes.

Steady state enzyme kinetics

The steady state enzyme kinetics of the purified laccase was studied using DMP, ABTS and syringaldazine as the substrates following the methods as mentioned in

assay section and kinetic parameters were calculated for different substrates from Lineweaver-Burk plots by incorporating the data obtained during enzymatic assay. K_m and k_{cat} values for the enzyme were determined from linear regression of double reciprocal plots. The pH and temperature optima of enzyme were determined by measuring the steady state velocities of enzyme catalyzed reaction in the solutions of varying pH/temperature keeping the other parameter fixed and drawing graphs of steady state velocities versus the variable parameter.

Thermal and pH stabilities

Thermal stability of the enzyme was tested by incubating an enzyme aliquot at particular temperature ranges from 30-70 $^{\circ}$C for 60 minutes, assaying its residual activity and plotting the residual activity against temperature. For thermal denaturation of enzyme, the rate constants for denaturation of enzyme at different temperatures were determined by keeping the enzyme aliquots at different fixed temperatures and assaying the enzyme activity at regular intervals of time and plotting residual activity against time. The rate constants were calculated from $t_{1/2}$ values using the equation $k = 0.693/t_{1/2}$. Energy of activation for thermal denaturation was calculated from Arrhenius plot.

pH stability of enzyme was tested by incubating an enzyme aliquot at particular pH value ranges from 3.0-5.0 for 60 minutes, assaying its residual activity and plotting the residual activity against pH.

Bioconversions in the absence of mediators

The bioconversions of toluene to benzaldehyde [22-24] was done in 15 mL of 100 mM sodium acetate buffer pH 4.5 containing 20 mM toluene in 20 mL of dioxane and 60 µg of purified laccase kept in a 100 mL conical flask which was stirred vigorously for 30 minutes. The reaction solution was extracted thrice with 40 mL of

n-hexane. 20 µL of the n-hexane extract was injected in Waters HPLC Model 600E using spherisorb C_{18} 5 UV, 4.5×250 mm column at 25°C. The eluant phase was methanol at the flow rate of 0.5 mL/min. The detection was made using Waters UV detector model 2487 at λ=254 nm.

The bioconversions of 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene and 3,4-dimethoxytoluene to 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde, respectively were also studied using the same method described above except time of stirring the reaction solutions were 90, 90, 120, 120 and 90 minutes, respectively.

Since only small amounts of chemical auxiliaries are applied which remain in the aqueous phase after extraction of the aldehydes with a suitable organic solvent (hexane/ethyl acetate), very pure compounds are obtained requiring no further purification. During these oxidations, no side reactions occur because of the high specificity of laccase as biocatalyst. Thus, authors have used hexane/ethyl acetate as organic solvent for the extraction of products and found the almost pure benzaldehydes and substituted benzaldehydes in high yields (average yield was 96%).

All the synthesized products were identified and characterized by HPLC and IR techniques.

High performance liquid chromatography (HPLC)

Retention time for reactants toluene, 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene, 3,4-dimethoxytoluene were 2.2, 3.0, 4.2, 7.3, 3.3 and 6.6 minutes, respectively while retention time for standard of products benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde were 3.2, 3.3, 3.6, 6.2, 3.7 and

5.9 minutes, respectively. Retention time for synthesized products benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3, 4-dimethoxybenzaldehyde were approximately same as for standard: 3.2, 3.3, 3.6, 6.2, 3.7 and 5.9 minutes, respectively.

Infrared (IR) spectroscopy

Major bands obtained from spectral analysis for different enzymatically synthesized compounds were: For benzaldehyde: ~3064, ~2819, ~2737, ~1918, ~1702, ~1391, ~1287, ~746, ~714 cm^{-1}. For 4-nitrobenzaldehyde and 3-nitrobenzaldehyde: ~3087, ~2858, ~2759, ~2045, ~1694, ~1572, ~1384, ~815, ~701 cm^{-1}. For 3-chlorobenzaldehyde and 4-chlorobenzaldehyde: ~3075, ~2840, ~2740, ~2016, ~1697, ~1315, ~833, ~689, ~602 cm^{-1}. For 3,4-dimethoxybenzaldehyde: ~3021, ~2981, ~1701, ~1376, ~1223, ~751 cm^{-1}.

Results and discussion

Fig. 1(a) shows the secretion of laccase in the liquid culture growth medium amended with various lignin containing natural substrates like corn cob, coir dust, saw dust, wheat straw and bagasse particles and inoculated with *T. hirsuta* MTCC-1171. The control experiment has similar medium composition except that the natural lignin containing substrate was absent. It is obvious from the figure that lignin containing natural substrates when present in the liquid culture growth medium enhanced the secretion of laccase. This enhancement of extracellular secretion of laccase is maximum in case of the growth medium containing wheat-straw particles. In order to optimize the secretion of laccase in the presence of wheat-straw particles, secretion of laccase in presence of different amounts of wheat-straw particles were studied. The results are shown in fig. 1(b). The maximum level of laccase was secreted in the

liquid culture medium containing 600 mg of the wheat-straw particles per 25 mL of the culture medium.

The purification procedure of laccase from the liquid culture filtrate of *T. hirsuta* MTCC-1171 is summarized in table-1. The enzyme bound to DEAE cellulose in 10 mM sodium acetate/acetic acid buffer pH 4.5 and was eluted by the linear gradient of NaCl in the range 0.23 to 0.84 M. The fraction numbers 4-8 were combined and concenterated to 3 mL by using the Amicon concentrator cells. The method gave 10.42 fold purification with 12.57% recovery of the enzyme activity. The concentrated enzyme sample was analyzed by SDS-PAGE, native PAGE and zymogram preparation. The results of SDS-PAGE, native PAGE and zymogram preparation are shown in Fig. 2(a), (b) and (c), respectively. In Fig. 2(a), reported lane 1 contains molecular mass markers and lane 2 contains the purified enzyme. The appearance of single protein band in SDS-PAGE indicated that the enzyme sample was pure. In Fig. 2(b), lane 1 contains native molecular mass markers similar to SDS-PAGE and lane 2 contains the purified enzyme. The appearance of single protein band in native-PAGE (lane 2), also indicated that the enzyme sample was pure. The position of purified enzyme in the zymogram Fig. 2(c) of native PAGE coincides with the position of purified laccase in the native PAGE. The molecular mass of purified laccase determined from the analysis [31] of SDS-PAGE was 55.0 kDa. Same molecular mass was also obtained from native-PAGE analysis. The molecular mass of purified laccase is well compared to the molecular masses of laccases reported in the literature which were in the range 43-383 kDa [4, 37].

Since laccases are the copper containing enzymes, it is common to determine the copper contents in the newly purified laccase. The copper content of the newly purified laccase was determined spectrophotometrically by 2, 2'-biquinoline at 546

nm [30, 31] which was found to be 2.1 copper ions per enzyme. The uncertainties in determining copper concentration and protein concentration could affect the results. The standard error for each measurement was estimated to be less than 5%.

The Michaelis-Menten and double reciprocal plots for purified enzyme using DMP as the variable substrate are shown in Fig. 3. Similar types of Michaelis-Menten and double reciprocal plots were obtained in case of syringaldazine and ABTS, hence have not been given here. The $K_{m,}$ k_{cat} and k_{cat}/K_m values determined from the double reciprocal plot using DMP, syringaldazine and ABTS as the substrates of the enzyme with standard error were given in table 2. The range of K_m values reported [4] for fungal laccases using DMP, ABTS, and syringaldazine as the substrates are 8-14720 μM, 4-770 μM and 3-3400 μM, respectively. However, the K_m value for this laccase for DMP compares well with the K_m of laccase purified from *Trametes gallica* Lac-1 [4].The K_m value for ABTS compares well with the K_m of laccase purified from *Pleurotus sajor-caju* IV and *Marasmius quercophilus* [4]. The K_m value for syringaldazine compares well with the K_m of laccase purified from *Pleurotus ostreatus* POXA 1b [4]. Low values of K_m and high values of K_{cat} and K_{cat}/k_m (table 2) of purified laccase from *T. hirsuta* MTCC-1171 for different substrates like DMP, ABTS and syringaldazine, clearly demonstrate that this purified enzyme has good catalytic efficiency and can be utilized as effective biocatalyst.

The results of the variation of the activity of the purified laccase with the variation of pH of the reaction solution are shown in Fig. 4 by dotted line. The determined pH optimum of enzyme was 4.5. The pH optima reported in the literature [4] for laccases using DMP as the substrate are in the range 3.0-8.0 pH units. The pH optimum of the purified laccase compares well with pH optima of laccases of *Chalara paradoxa, Gaeumannomyces graminis, Pleurotus ostreatus* POXA 1b, *Pleurotus*

ostreatus POXA 1W, *Pleurotus ostreatus* POXC and *Volvarielia volvacea* [4]. The results of the studies on the pH stability of laccase are shown in Fig. 4 by solid line where the residual activities of enzyme have been plotted against the different pHs for which enzyme has been exposed for 1 h. It follows that the enzyme has maximum stability at pH 4.5.

The results of variation of the activity of the purified laccase as a result of variation of the temperature of the enzyme catalyzed reaction solution are shown in Fig. 5 by dotted line. The temperature optimum was 60 °C. The range of temperature optima reported for other laccases are 25-80 °C. The purified laccases of *Chaetomium termophilum* and *Coniothyriem minitans* also have temperature optima at 60 °C [4]. The result of the studies on the thermal stability of the purified laccase is shown in Fig. 5 by solid line where the residual activities of enzyme have been plotted against the different temperatures at which enzyme has been exposed for 1 h.

The purified laccase does not possess blue colour as visually observed in cases of blue laccases. Instead, it has yellow colour. The purified laccase lacked absorption band around 610 nm generally observed in cases of blue laccases, which indicated that it was a yellow laccase. It has also been reported that yellow laccase [7, 8] oxidizes non-phenolic substrates in the absence of electron transfer mediators which is essential for the oxidation of non-phenolic substrates by blue laccases. The purified laccase oxidized veratryl alcohol to veratraldehyde in the absence of mediator molecules confirming that it is a yellow laccase [7, 8].

One of the applications of the laccases in organic synthesis is selective oxidation of the aromatic methyl groups to the corresponding aldehydes. The chemical routes of this conversion are inconvenient because the reaction has to be stopped at the aldehyde stage so that carboxylic acid could not be formed. Moreover, they require

drastic reaction conditions which pollute the environment. The conversion done with laccase occurs under milder conditions, yield is > 95% and the process is ecofriendly. Laccases are the preferred enzymes because they cycle on oxygen and generate no side products except water. The use of blue laccases for this purpose has been studied but they require mediator molecules like ABTS [22] and 1-hydroxy-1H-benzotrizole (HBT) [23]. But the laccase purified by authors has strong capability to oxidize toluene as well as different substituted toluenes to respective aromatic aldehydes selectively without using any mediator molecules. This was tested using toluene, 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene and 3,4-dimethoxytoluene as the substrates, successfully. All the reactions are ecofriendly and good examples of green chemistry. They always occur at room temperature and pressure without any side reactions. In all reactions, water is only the byproduct. Since, there is no need of mediators, drastic reaction conditions, costly reagents etc use of this laccase as biocatalyst may be more valuable in the synthesis of these compounds economically as well as environmentally. This laccase may be used in the industrial process for future large scale production of these substituted benzaldehydes by purifying laccase and enhancing the amount of substrate according to requirements using same method as given in experimental section.

In order to demonstrate the advantage of this purified laccase over other fungal laccases, comparative catalytic experiments have been done by authors. Authors have found that laccase from *T. hirsuta* MTCC-1171 selectively oxidizes the several substituted toluenes to corresponding benzaldehydes within <2 hrs in the absence of mediator while several other laccases do not oxidize the same substrates in the absence of mediator molecules. Laccase from *Trametes hirsuta* MTCC-11397 has poor oxidizing capability and takes very long time in the absence of mediator

399 molecule but causes rapid oxidation of the same substrates in presence of ABTS as
400 mediator molecule within 2 hrs. Other laccase from *T. versicolor*, *Coriolus versicolor*
401 MTCC-138 [38], *Pleurotus ostreatus* MTCC-1803 [39], *Xylaria polymorpha* MTCC-
402 1100 [40, 41] and *Phellinus linteus* MTCC-1175 [42] failed to oxidize the above
403 described substrates in the absence of mediator molecule. In all the above
404 comparative experiments, laccases used were obtained from liquid culture growth
405 media of respective fungal sources and reactions were studied spectrophotometrically.
406 These catalytic comparative experiments have been given in table 3.

407 In above enzyme catalyzed biotransformations, all products formed are easily
408 available and simple. HPLC technique has been used to confirm the actual product
409 formation by comparing the HPLC chromatograms of standard aldehyde compounds
410 with the enzymatically transformed compounds. The retention time of the standard
411 samples of toluene, 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene,
412 3,4-dimethoxytoluene, benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde,
413 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde were
414 2.2, 3.0, 4.2, 4.3, 3.3, 6.6, 3.2, 3.3, 3.6, 6.2, 3.7 and 5.9, respectively. Thus, the
415 retention time of the products of the enzyme catalyzed reactions (3.2, 3.3, 3.6, 6.2,
416 3.7, and 5.9) revealed that the products of the enzyme catalyzed reactions were
417 benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde,
418 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde (table 4). Chromatograms for
419 the conversion of toluene to benzaldehyde are given in Fig. 6.

420 In IR-spectra, bands between 2819-2858 cm^{-1} were due to aldehydic C-H
421 stretching while bands between 1695-1705 cm^{-1} were due to conjugated aldehydic
422 C=O stretching which prove the conversion of aromatic methyl group into aromatic
423 aldehydic group. One IR-spectrum for benzaldehyde and one for substituted

benzaldehyde i.e. 3-nitrobenzaldehyde are given in Fig. 7 and 8, respectively for proof.

Conclusions

In this communication, a novel yellow laccase from a new fungal strain *T. hirsuta* MTCC-1171 has been purified and characterized. The use of this enzyme in synthesis of aromatic aldehydes from toluene and different substituted toluenes in the absence of mediator molecules has been demonstrated. Thus, this purified laccase is a better biocatalyst than blue laccases in which the use of mediator molecule is necessary. All the laccase catalyzed reactions in this communication are good examples of green chemistry.

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Legends to the figures:

Fig. 1 (a) Secretion of laccase by *T. hirsuta* MTCC-1171 in the liquid culture medium supplemented with different natural lignin containing substrates: wheat straw(♦), corn cob(■), bagasse(*), saw dust(×), coir dust(▲), control(●)

(b) Optimization of laccase secretion by *T. hirsuta* MTCC-1171 in liquid culture medium supplemented with different amount of wheat straw : 100 mg (●), 200 mg(■), 400 mg (▲) 500mg(×), 600 mg (*), 800 mg (Δ) , 1000 mg (♦).

Fig. 2 SDS-PAGE **(a)**, native-PAGE **(b)** and zymogram **(c)** of the purified laccase.

595 **(a)** Molecular mass markers (lane 1), Purified laccase (lane 2).

596 **(b)** Molecular mass markers (lane 1), Purified laccase (2).

597 **(c)** Pure enzyme stained with DMP.

598 **Fig. 3** Michaelis-Menten curve (solid line curve) and double reciprocal plot (dotted
599 line as insert figure) for the laccase of *T. hirsuta* MTCC-1171 using DMP as
600 the variable substrate.

601 In both cases, 1 mL reaction solution contained 0.0-1.4 mM DMP, 50 µL of
602 the enzyme in 100 mM sodium malonate buffer pH 4.5 at 37 ^{0}C.

603 **Fig. 4** Determination of pH optimum (dotted line) and pH stability (solid line) of the
604 purified enzyme.

605 1 mL of reaction solution contained 1.0 mM DMP in 100 mM sodium
606 malonate buffer with varying pH from 3.0 to 5.5 in case of pH optimum while
607 3.0 to 5.0 in case of pH stability.

608 **Fig. 5** Determination of temperature optimum (dotted line) and thermal stability (solid
609 line) of the purified enzyme.

610 1 mL of reaction solution contained 1.0 mM DMP in 100 mM sodium
611 malonate buffer pH 4.5 with varying temperature from 20 to 90 $^{\circ}$C in case of
612 temperature optimum and 30 to 70 $^{\circ}$C in case of thermal stability.

613 **Fig. 6** Transformation of toluene to benzaldehyde by the laccase of *T. hirsuta* MTCC-1171 in
614 the absence of mediator.

615 **(a)** The chromatogram of the pure toluene.

616 **(b)** The chromatogram of the pure benzaldehyde.

617 **(c)** The chromatogram of the product of enzymatic reaction with Toluene.

618 The reaction solution contained 20 mM of methylbenzene, 20 ml dioxane and 15
619 mL sodium acetate buffer (pH 4.5) at room temperature was stirred for 30 minutes
620 and extracted thrice with ethyl acetate and 20 µL was injected in HPLC.

621 **Fig. 7** IR-spectrum for enzymatically synthesized product benzaldehyde.

622 **Fig. 8** IR-spectrum for enzymatically synthesized product 3-nitrobenzaldehyde.

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Table 1 Different steps involved in purification procedure of laccase from the liquid culture filtrate of *T. hirsuta* MTCC-1171

Steps	Volume (mL)	Specific activity (IU/mg)	Total protein (mg)	Total activity (IU)*	Purification fold	Yield %
Culture filtrate	100	0.36	100.0	35.80	1.00	100.00
Ammonium sulphate precipitation	10	1.28	14.0	17.90	3.56	50.00
Dialysis with 10 mM Sodium acetate buffer	15	1.33	13.5	18.00	3.70	50.28
DEAE cellulose column chromatography	3	3.75	1.2	4.50	10.42	12.57

*IU is international unit/enzyme unit which is unit for the amount of enzyme that catalyzes the conversion of one μmole of the substrate per minute under the specified assay conditions

Table 2 K_m, k_{cat}, k_{cat}/K_m and standard error values determined from the double reciprocal plots using DMP, ABTS and syringaldazine as the substrates.

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^4$ $M^{-1}s^{-1}$)
DMP	420 ±2.55	13.04 ±0.02	3.11 ±0.03
ABTS	225 ±3.06	13.03 ±0.03	13.00 ±0.03
Syringaldazine	100.00 ±3.00	13.04 ±0.04	5.80 ±0.04

Table 3 A comparative chart showing the selective oxidations of toluene and substituted toluenes to corresponding benzaldehydes by laccases obtained from various fungal sources.

Laccases from different fungal sources.	Selective oxidation in the absence of mediator	Selective oxidation in the presence of mediator	References
Trametes hirsuta MTCC-1171	Excellent (0.5-2.0 hrs)	-	This work
Trametes hirsuta MTCC-11397	Poor (>24 hrs)	Excellent (0.5-2.0 h)	*
Trametes versicolor	No	Excellent (0.5-1.0 h)	*
Coriolus versicolor MTCC-138	No	Excellent (0.5-2.0 h)	37
Pleurotus ostreatus MTCC-1803	No	Excellent (0.5-1.5 h)	38
Xylaria polymorpha MTCC-1100	No	Excellent (0.5-1.5 h)	39, 40
Phellinus linteus MTCC-1175	No	Excellent (0.5-1.5 h)	41

* The selective oxidation by these species is not reported and authors have done it for the purpose of comparing its catalytic efficiency with laccase of *T. hirsuta* MTCC-1171. Thus, this is a part of the manuscript.

Table 4 Retention times (min) for reactant, standard benzaldehydes/substituted benzaldehydes and respective synthesized benzaldehyde/substituted benzaldehydes with percentage yield of products.

Toluene (Reactant)	Benzaldehyde (Standard)	Benzaldehyde (Product)	Yield (%)
2.2	3.2	3.2	96
3-Nitrotoluene (Reactant)	3-Nitrobenzaldehyde (Standard)	3-Nitrobenzaldehyde (Product)	
3.0	3.3	3.3	100
4-Nitrotoluene (Reactant)	4-Nitrobenzaldehyde (Standard)	4-Nitrobenzaldehyde (Product)	
4.2	3.6	3.6	92
3-Chlorotoluene (Reactant)	3-chlorobenzaldehyde (Standard)	3-Chlorobenzaldehyde (Product)	
7.3	6.2	6.2	97
4-Chlorotoluene (Reactant)	4-Chlobenzaldehyde (Standard)	4-Chlorobenzaldehyde (Product)	
3.3	3.7	3.7	95
3,4-Dimethoxytoluene (Reactant)	3,4-Dimethoxybenzaldehyde (Standard)	3,4-Dimethoxybenzaldehyde (Product)	
6.6	5.9	5.9	100

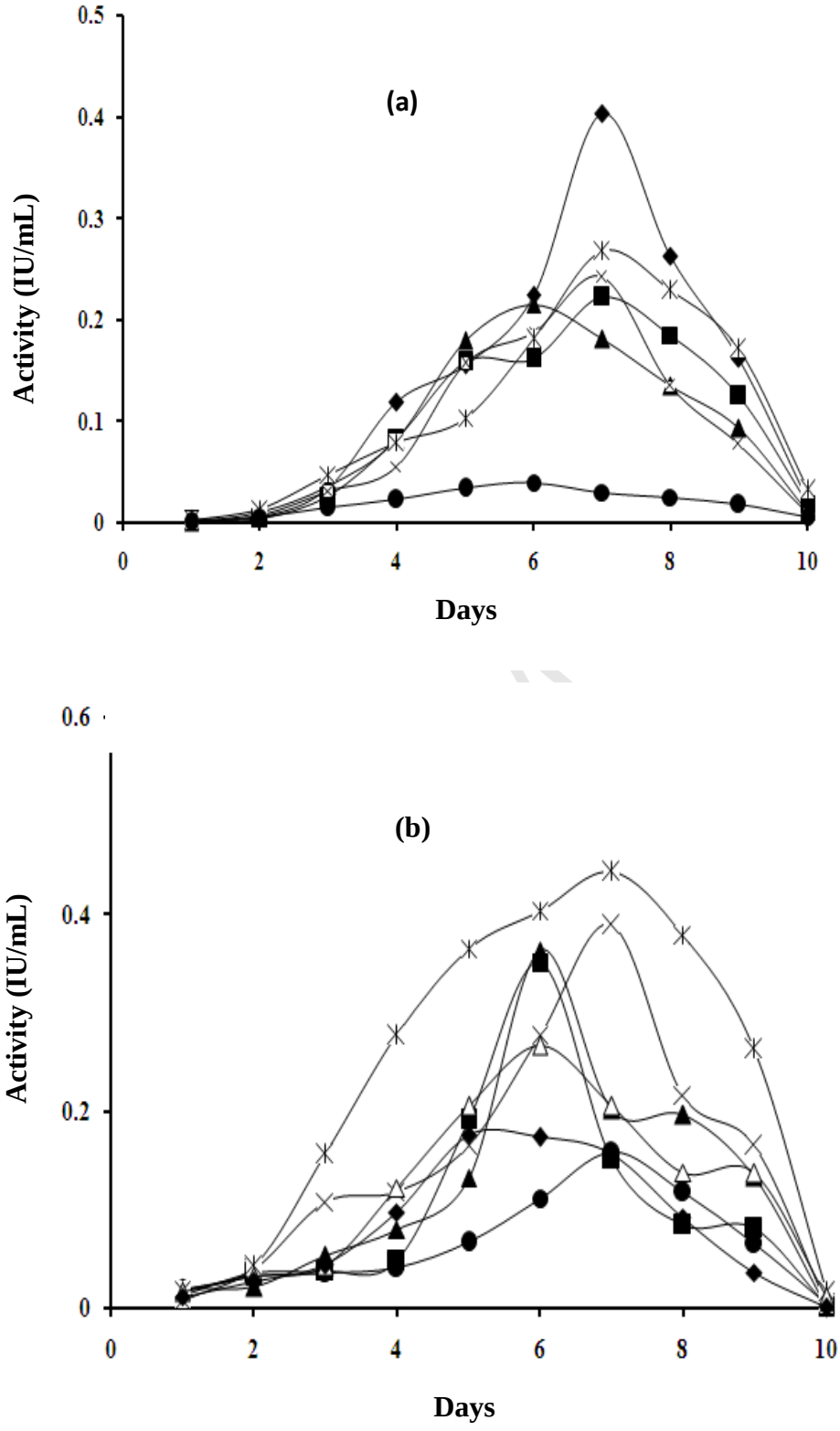


Fig. 1

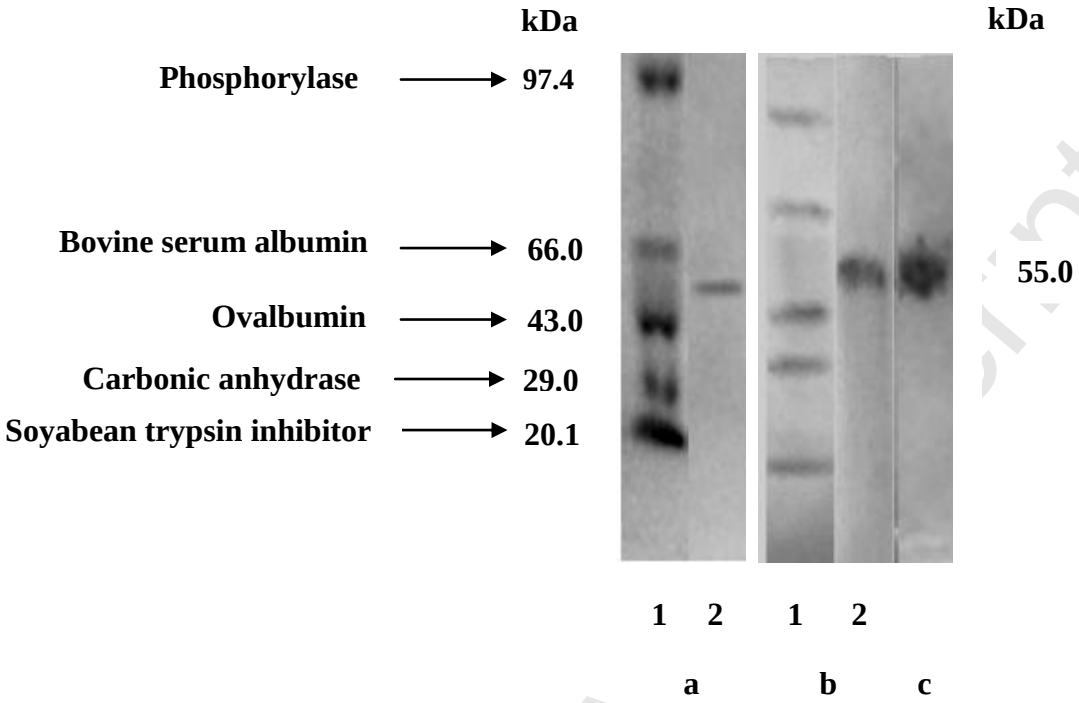


Fig. 2

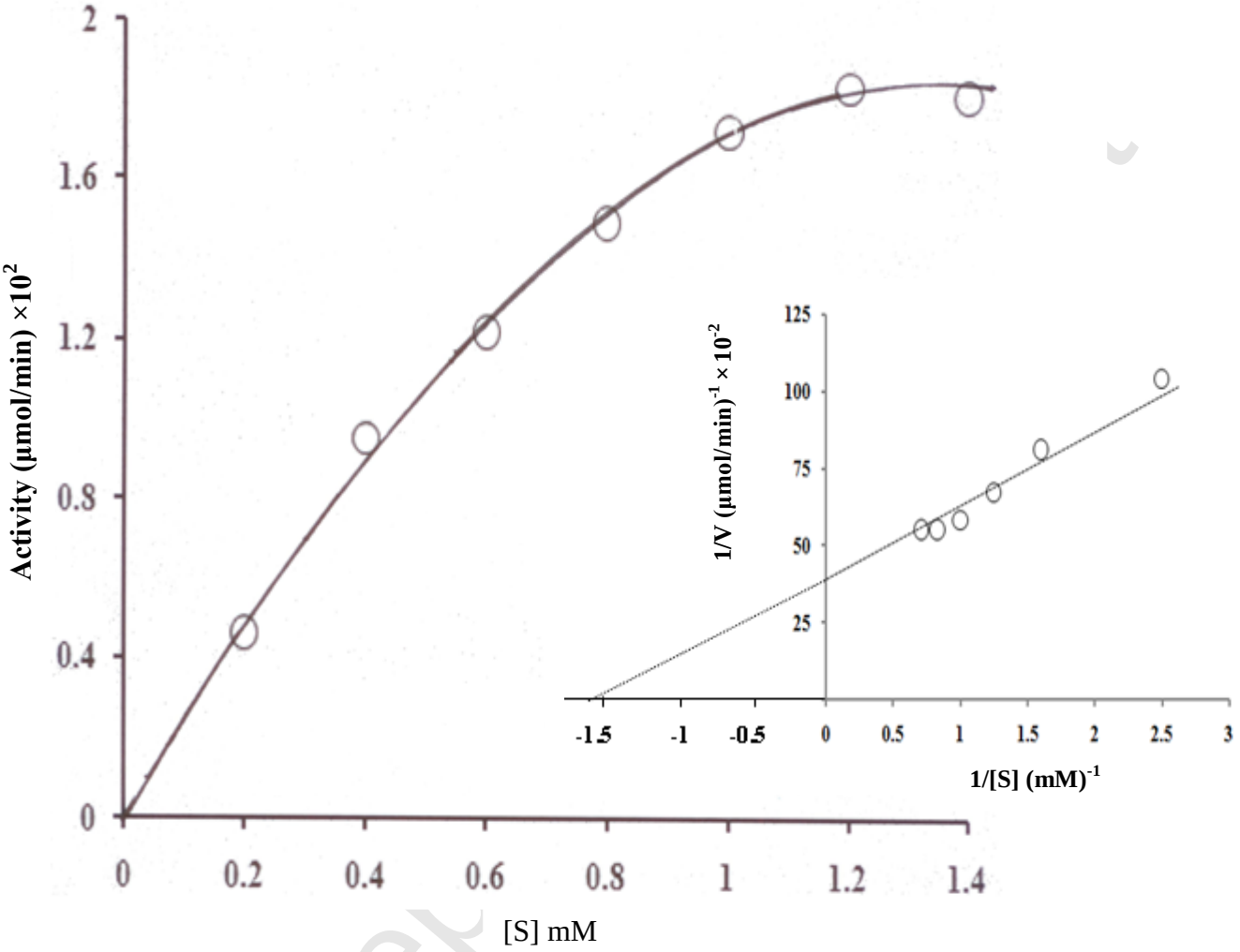
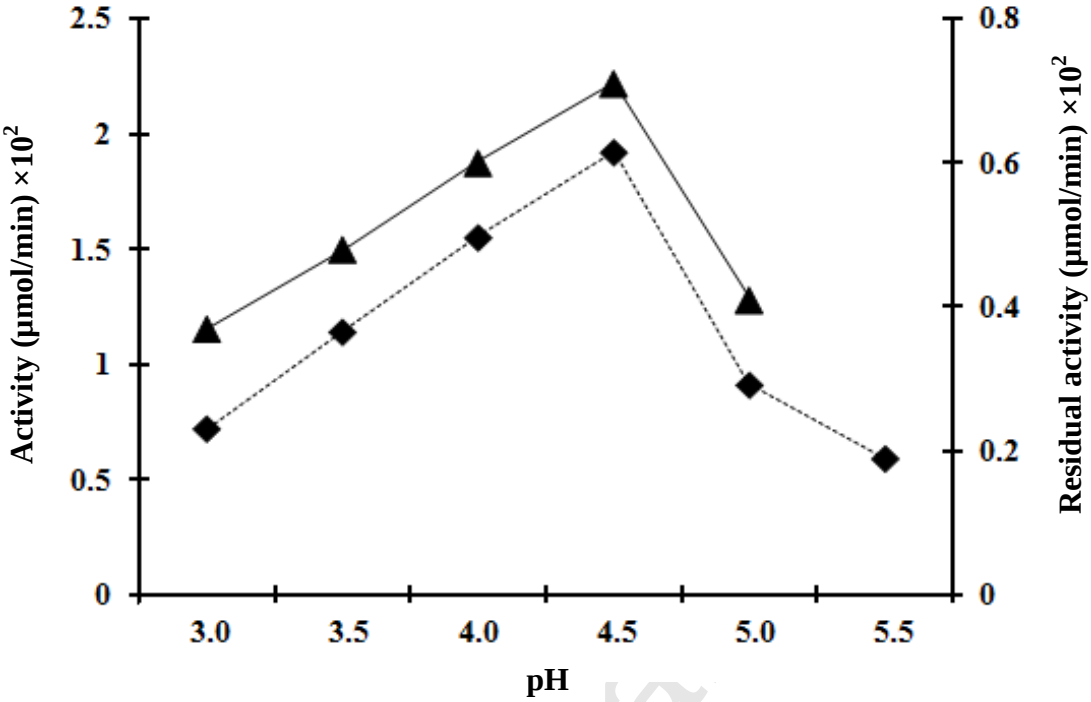


Fig. 3



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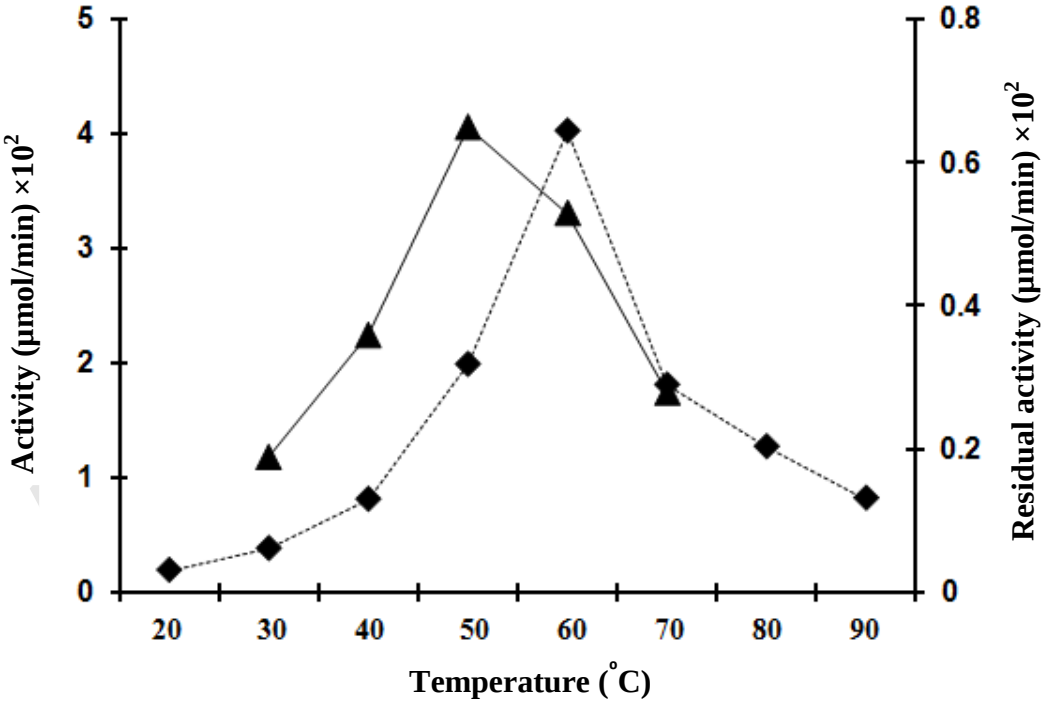

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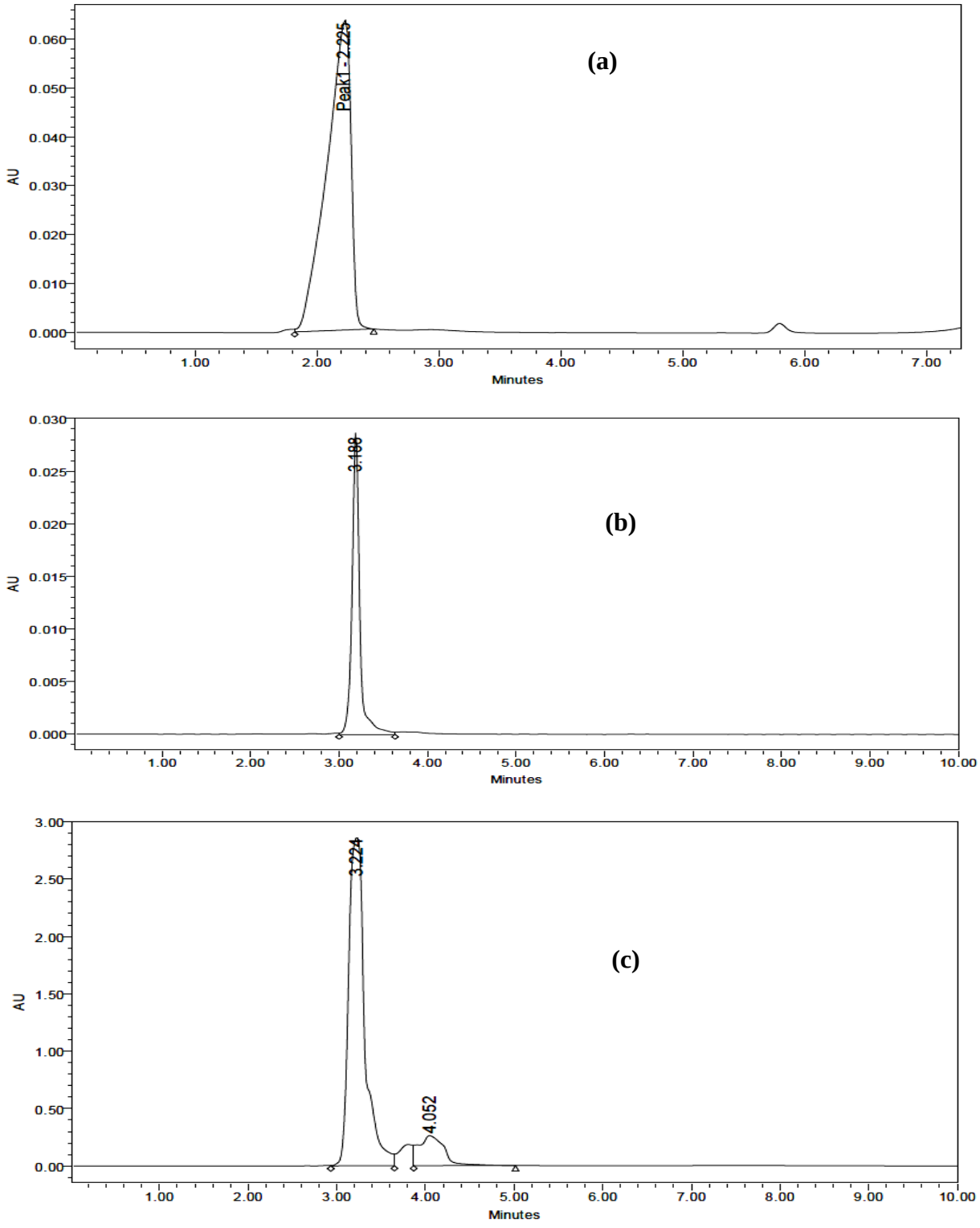

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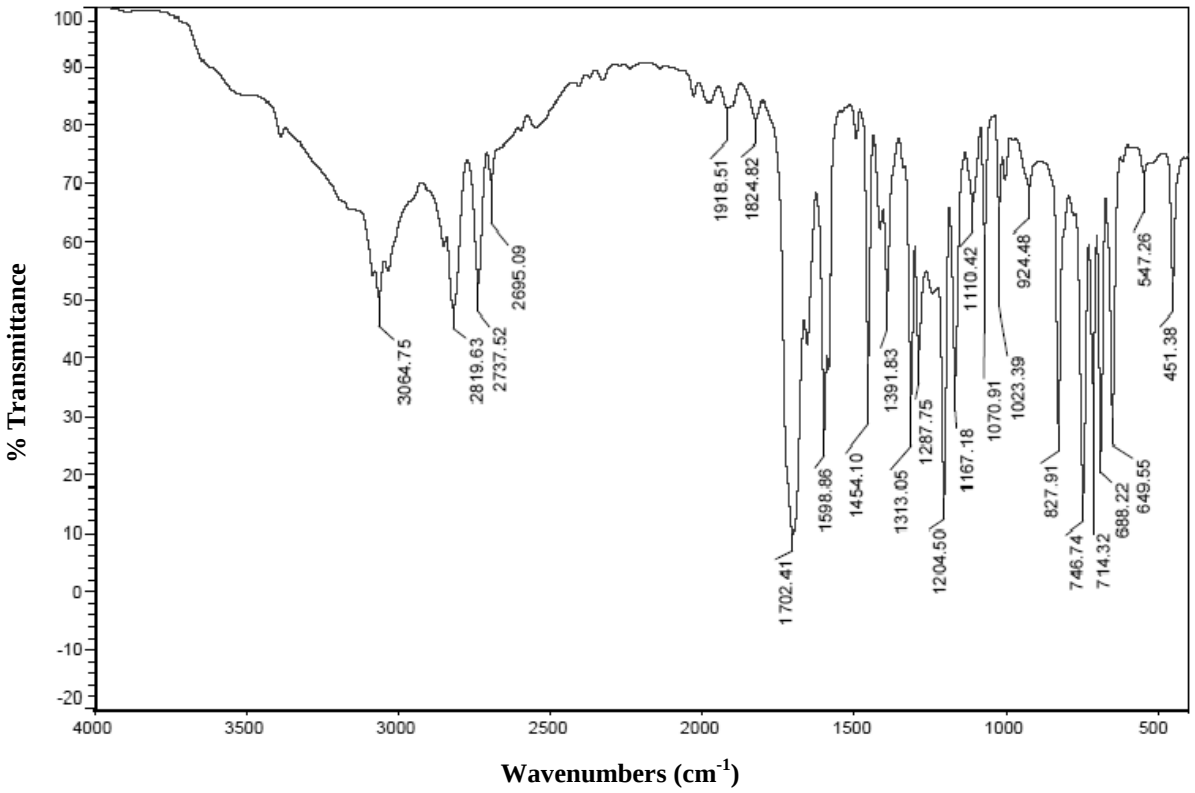

Fig. 7

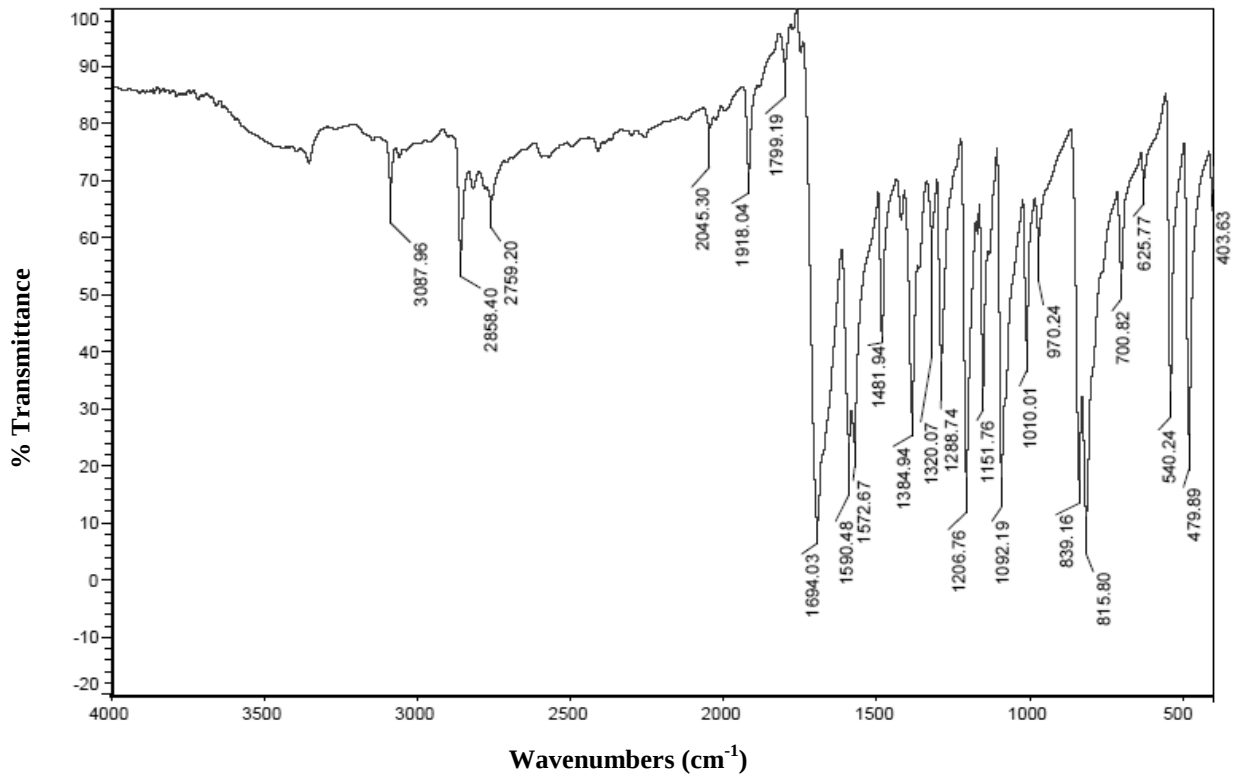

Fig. 8