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ISOLATION, PURIFICATION, AND CHARACTERIZATION OF THERMOPHILIC LACCASE FROM THE XEROPHYTE *Cereus pterogonus*

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Three laccase temperature isoforms were isolated and purified to homogeneity from the xerophyte plant species Cereus pterogonus*. This catalytically active protein exhibited an apparent molecular mass of 137 kDa, 90 kDa, and 43 kDa. Under reducing conditions the enzyme yielded a subunit molecular mass of 43 kDa alone, suggesting that the enzyme is a multimer of its subunits. The enzyme exhibited an optimum pH of 10 with 2,6-dimethoxyphenol used as a substrate. The 137 and 90 kDa forms yielded optimum activity at 90°C; whereas the 43 kDa molecular form yielded optimum activity at 60°C. The enzyme kinetic constant Km remained closely similar for all three enzyme forms, whereas Vmax varied by 25% overall. The catalytic activity remained above its* $t_{1/2}$ *value in excess of the 30 min denaturation assay period at 60°C and 90°C. These high-temperature isoforms of the plant laccase enzyme with alkaline pH optima can find great industrial use.*

Keywords: 2,6-dimethoxyphenol, alkaline pH, *Cereus pterogonus*, laccase, xerophyte.

Enzymes that exhibit their catalytic activity at highest temperatures are called thermophilic enzymes. Such enzymes exhibit stabilized conformation with which they carry out their function as biocatalysts. Thermophilicity can be recognized in prokaryotes as well as in eukaryotes. Thermophilic organisms are either classified as extreme thermophiles (60–80°C) or as hyperthermophiles (80–110°C) based on their ability to survive in these temperature ranges.

Laccases (EC 1.10.3.2) are copper-containing metallo-enzymes capable of oxidizing phenols and aromatic amines and in the process reduce molecular oxygen to water. Laccases contain three types of reactive copper centers having different functions: type 1 (blue copper) catalyzed the electron transfer from the substrate while type 2 and type 3 formed a three-member cluster that collectively activated molecular oxygen [1]. They display a wide substrate range, and are known to catalyze the polymerization, depolymerization, methylation and/or demethylation of phenolic compounds [2, 3] and exhibited a role in plant pathogenicity [4] or lignin degradation [5]. Laccases have many industrial and biotechnological applications such as for the decolorization of dyes [6], degradation of xenobiotics [7], biopulping [8], biobleaching [9], in food processing [10], and for the development of biosensors [11]. Alkaline laccase opens up new possibilities for the development of green technology alternatives to existing chemical treatment, as many practical applications in the textile industry for denim bleaching and waste water treatment occur at neutral to alkaline pH [12].

The present study identified for the first time the existence of high-temperature stable (thermophilic) laccase enzyme activity in the xerophytic plant species *Cereus pterogonus*. Isolation, purification, and characterization of this enzyme activity was therefore carried out due to its industrial significance and as an alternate source of thermophilic enzyme that could be indigenously generated employing conventional chromatographic techniques, circumventing the need to procure thermophilic laccase from bacterial sources.

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TABLE 1. Purification Fold Table of Laccase from *Cereus pterogonus*

Step	Total enzyme activity, mol per $min^{-1} \times 10^{-4}$	Protein, mg	Specific activity, IU/mg $\times 10^{-4}$	Purification fold	Yield, %
Crude homogenate	1262	139.2	9.1	1	100
Acetone Precipitation	1230	129.1	9.5	1.05	97.5
Ion exchange chromatography (diethylaminoethyl–Sephadex A-50)					
Active pool fraction I	270	2.0	135.0	14.8	21.4
Active pool fraction II	203	1.9	106.8	11.7	16.1
Gel filtration chromatography (Sephadex G-100)					
137 kDa form	121	0.5	242.0	26.6	9.6
90 kDa form	88.3	0.3	294.3	32.3	6.9
43 kDa form	80.2	0.5	160.4	17.6	6.4

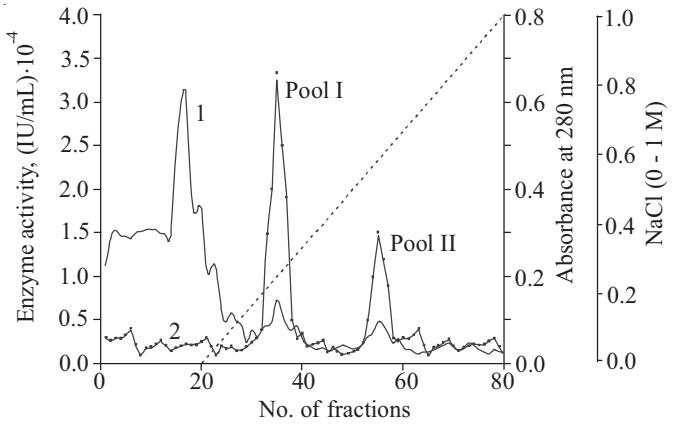

Fig. 1. Diethylaminoethyl–Sephadex A-50 anion exchange chromatography of acetone-precipitated protein. Dotted line starting at the 20th fraction onwards represents the NaCl gradient in the range 0 to 1 M. Absorbance at 280 nm (1); enzyme activity (2).

The laccase enzyme activity in the 20% (w/v) cladode homogenate supernatant was found to be 5.7×10^{-4} IU/mL, and the specific activity was 9.1×10^{-4} IU/mL. In plants this enzyme is found to do lignification. We were able to isolate and purify alkaliphilic isoforms of laccase enzyme from the xerophyte *Cereus pterogonus*. The enzyme showed highest activity at basic pH. Only very few alkaliphilic isoforms for this enzyme have been reported till now [13]. The *Cereus pterogonus* laccase enzyme activity was purified as shown in Table 1.

The substrate, 2,6-dimethoxyphenol, selected for use in the assay has a phenolic structure and wide stability in the pH range 3–12 [12]. In our study, the enzyme was found to be inactive when precipitated with ammonium sulfate and sodium sulfate, in comparison to earlier reports [1] on laccase enzyme precipitation using these chemical reagents that suggested of sulfate inhibition of the plant laccase enzyme activity. However, precipitation of plant laccase with acetone yielded more than 90% of enzyme activity.

Diethylaminoethyl–Sephadex A-50 anion exchange chromatography of the acetone-precipitated enzyme yielded two sets of enzyme-active fractions (Fig. 1). Of these, the first set of enzyme-active fractions was collected employing NaCl at 0.25 M in the eluting buffer, while the second set of fractions was collected at 0.6 M NaCl concentration. Pool I was purified 14.8-fold with a yield of 21.4%, and pool II was purified 11.7-fold with a yield of 16.1%. Each pool was taken in a dialysis bag for concentration using diatomaceous earth (Celite).

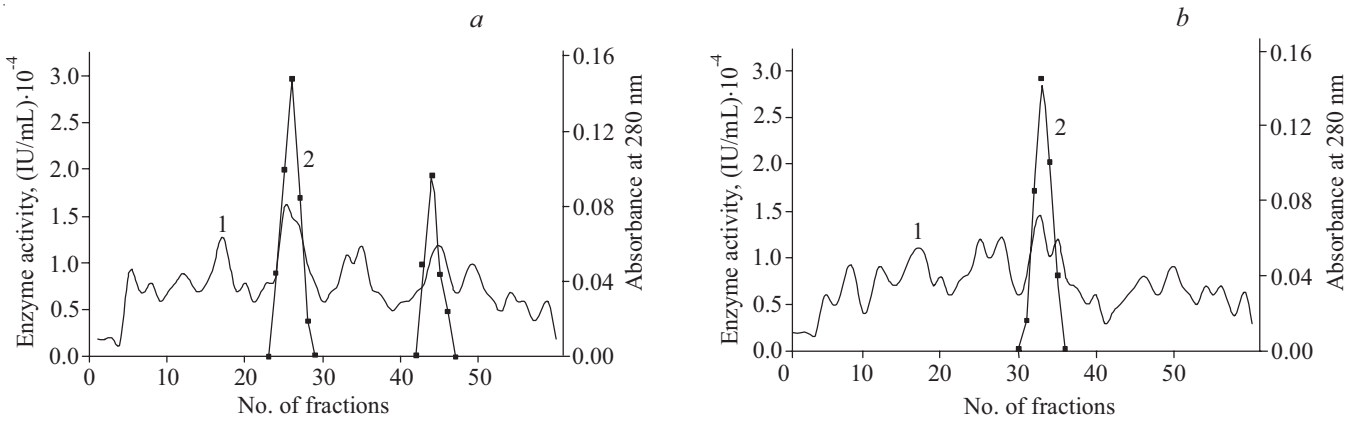

Fig. 2. Sephadex G-100 gel filtration chromatography of anion exchange pool fraction I (*a*) and Sephadex G-100 gel filtration chromatography of anion exchange pool fraction II (*b*). Absorbance at 280 nm (1); enzyme activity (2).

The concentrated diethylaminoethyl–Sephadex A-50 eluted pools subjected to gel permeation chromatography employing Sephadex G-100 yielded three distinctly different enzyme active pools. Pool I from diethylaminoethyl–Sephadex A-50 chromatography yielded two sets of enzyme-active fractions corresponding to the molecular weights of 137 kDa and 43 kDa, following gel permeation chromatography (Fig. 2,*a*). Pool II from diethylaminoethyl – Sephadex A-50 chromatography yielded enzyme-active fractions corresponding to a molecular weight of 90 kDa following gel permeation chromatography (Fig. 2,*b*). Their purification levels were determined to be 26.6-, 32.3-, and 17.6-fold with a yield of 9.6, 6.9, and 6.4% respectively. These results suggest the existence of laccase enzyme in trimeric, dimeric, and monomeric forms.

This observation was further confirmed based on results of the sodium dodecyl sulfate – polyacrylamide gel electrophoresis analysis of the three enzyme-active pools employing 10% polyacrylamide gel electrophoresis under reducing conditions. The identification of a distinct polypeptide band at 43 kDa was suggestive of the generation of dimeric and trimeric forms of laccase enzyme as a multiple of the 43-kDa form. It was further noted that the 137-kDa form remained as a single band under nonreducing conditions during zymogram analysis. However, when heated at 80°C for 3 min, the 137-kDa form additionally yielded 90-kDa and 43-kDa forms, confirming the existence of three different molecular forms of laccase enzyme. Quantitative estimate of each of these forms could be made by heating a sample of the 137-kDa enzyme form (50 μg at 80°C for 3 min) and determining the yield of the 137-kDa (10 μg), 90-kDa (15 μg), and 43-kDa (20 μg) forms on a microgram basis using densitometry. While the three molecular forms cited could be detected under these conditions, heating the 137-kDa form at 80°C for 5 min converted it completely to 90- and 43-kDa forms, indicating the heat-driven dissociation of the 137-kDa molecular form to its dimeric and monomeric components. It also suggests the possibility of having undetectable amounts of the 137-kDa form after 5 min heating. The existence of multiple forms of laccase had been reported earlier [14] in bacterial sources.

Temperature optima of these three forms of laccases were determined and are as shown in Fig. 3. Three temperature-dependent enzyme forms were found to exist, one at 60°C (43-kDa form) and a couple at 90°C (137-kDa and 90-kDa forms). Salt bridge formation, hydrophobicity, protein conformation, etc. confer thermostability in a protein. The trimeric 137-kDa form was found to be more stable at the higher temperature compared to the monomeric 43-kDa form, indicative of the conformational orientation of the 137-kDa form to accommodate the substrate and exhibit its activity at the higher temperature. The pH optima of all three enzyme forms were found to be pH 10. The enzyme forms showed decreased enzyme activity at any other pH. This enzyme was totally inactive at pH 4 and 5 (unpublished). The enzyme forms were prepared in different pH buffers a day before the assay. The enzyme kinetics of the 137-kDa form showed a Vmax of 7.0 ± 0.5 IU/mL and a Km value 2.1 ± 0.3 mM. The ν_{max} of 90-kDa and 43-kDa forms were found to be 5.5 ± 0.5 IU/mL and 4.96 ± 0.5 IU/mL when assayed at 90°C and 60°C, respectively. The Km for 90-kDa and 43-kDa forms was 2.0 ± 0.3 mM and 1.9 ± 0.4 mM, respectively. It can be seen that the Km of all the three enzyme forms were closely similar, whereas the ν_{max} varied by as much as 25%, suggestive of probable protein conformational changes affecting the enzyme active site and substrate binding. All the three enzyme forms exhibited a loss in enzyme activity of 8–16% within the first 5 min of denaturation. These results show that the 137-kDa form's, activity is not equivalent to the 43-kDa form at the end of the assay period. As zymogram analysis does not show the 137-kDa band (heated at 80°C, 5 min) and time-dependent denaturation study show detectable 137-kDa activity (90°C, 30 min), structural studies are required to come to a conclusion. Despite this, the sustained retention of enzyme activity over a 30-min period and above the $t_{1/2}$ value allows this enzyme to be considered for use in industrial applications.

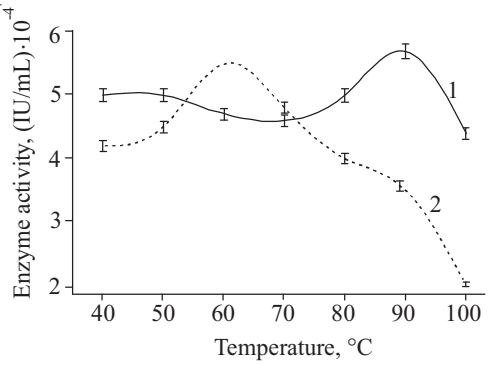

Fig. 3. Effect of temperature on 137-kDa, 90-kDa, and 43-kDa laccase enzyme forms. 137-kDa and 90-kDa enzyme forms showed similar profiles individually and so are shown here as a single profile. The data represents mean of three independent determinations, Mean±SEM. 137- and 90-kDa enzyme forms (1); 43-kDa enzyme form (2).

In comparison to the authors′ earlier work on the laccase enzyme forms in another xerophyte, *Opuntia vulgaris*, the 43-kDa form is detectable in this study in Sephadex G-100 gel permeation chromatography. The major differences between the enzyme forms in both xerophytes are: 1) ν_{max} of the enzyme forms in *Opuntia vulgaris* is lower compared to the enzyme′s ν_{max} in the present study. 2) As said earlier, the 43-kDa enzyme form can be fractionated in Sepahadex G-100 chromatography compared to the earlier work in *Opuntia vulgaris*. 3) *Cereus pterogonus* enzyme forms with molecular weight 137, 90, and 43 kDa exhibit maximum enzyme activity at 90, 90, and 60°C, whereas the earlier work reported shows the maximum enzyme activity at 80 and 70°C [15]. Therefore, these enzyme forms generate more interest and find greater applications in industries.

EXPERIMENTAL

Cladodes of the xerophyte *Cereus pterogonus* plant were obtained from the habitats surrounding Pondicherry University, Puducherry, India, and served as the source of the enzyme. 2,6-Dimethoxyphenol used as substrate in this study was purchased from Sigma-Aldrich, St. Louis, Mo, USA. All other chemicals used were of analytical grade and were purchased from reputed manufacturers in India. Glass-distilled water was used for the preparation of all reagents.

The cladodes were weighed and chopped into small pieces after dethorning and removal of the hard cuticular layer, then homogenized in 0.1 M glycine–NaOH buffer, pH 10.0, containing 1 mM phenylmethylsulfonyl fluoride used as a protease inhibitor using a Waring blendor (Remi motors, Mumbai, India) to prepare a 20% w/v cladode homogenate. The homogenate was filtered through a muslin cloth and centrifuged at 10,000 × *g* for 20 min at 4°C in an Eppendorf refrigerated centrifuge, and the clear supernatant obtained was used as the enzyme source.

Cladode homogenate (20% w/v), 10,000 × *g* supernatant, was taken for ice cold acetone precipitation to a final concentration of 80% v/v, at –4°C. The contents were placed in ice for an hour. The acetone-precipitated proteins were collected by centrifugation at 10,000 × *g* for 30 min at –4°C. The pellet obtained was reconstituted in 1 mL 0.1 M glycine–NaOH, pH 10.0 and taken for further use.

Diethylaminoethyl–Sephadex A-50 anion exchange resin was taken in 0.05 M HCl, and the suspension was allowed to stand for 1 h. Following this, the supernatant was discarded and 0.1 M citrate buffer (pH 5.0) was added to the resin and the pH of the resin adjusted to 5.0 and allowed to stand for an additional 30 min. The material was then packed into a column (30 cm × 0.8 cm) without air bubbles. The packed gel matrix was equilibrated with 0.1 M citrate buffer, pH 5, for 3–5 bed volumes. Aliquots (3 mL) of the acetone-precipitated proteins were applied to the equilibrated ion exchange resin column. The protein sample volume was adjusted to 10% of the column bed volume. Ion exchange chromatography of the proteins was carried out at room temperature, and fractions of 2 mL each were collected manually. At the end of sample application, the column was washed with the equilibrating buffer until the absorbance of the wash at A_{280} exhibited < 0.05. The adsorbed proteins were then eluted using a linear gradient of NaCl (0–1.0 M) in 0.1 M glycine–NaOH buffer, pH 10. The protein content

of each fraction was monitored at A_{280} employing a UV-Visible double-beam spectrophotometer. Laccase activity in the fractions was determined using the assay described below. The collected fractions were pooled and concentrated using diatomaceous earth (Celite) employing a dialysis bag (MW cutoff: 30 kDa). This was carried out by placing the active fraction pools in the dialysis bag and making sure that both ends of the dialysis bag are tied tightly. Celite was sprinkled on the dialysis bag, allowing it to absorb the buffer inside the bag, and the process was repeated till the desired concentration of sample was achieved.

Sephadex G-100 was allowed to swell in 0.1 M glycine–NaOH buffer, pH 10, for 72 h at room temperature and then packed onto a glass column (30 cm × 0.8 cm) with a flow rate of 0.9 mL/min. Active fractions in the ion exchange chromatography were loaded onto the column. Fractions of 2 mL were collected, and the absorbance in each fraction was measured at 280 and 469 nm for the enzyme activity. The molecular weight was determined from a standard plot of Ve/Vo vs log molecular weight obtained by the use of standard proteins, Blue dextran, and by determining the Ve of the enzyme fractions.

The molecular weight of the purified sample was determined under reducing conditions (95°C , 3 min) employing 2-mercaptoethanol and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining the gel with 0.25% w/v coomassie blue and destaining in methanol–water–acetic acid (45:45:10) [16]. Enzyme activity was detected by placing the electrophoresed gel (non-reducing) on the substrate (10% w/v of 2,6-dimethoxyphenol) containing agarose gel [14]. A broad-range protein molecular weight marker, commercially available from Bangalore Genei India Pvt Ltd., in the molecular weight range 205 kDa to 29 kDa was used for estimating the molecular weight of the enzyme protein. Densitometric analysis of the stained gel was carried out using Syngene Gene Tools software.

Laccase enzyme activity in the plant homogenate was assayed by the method of Palmieri et al. [17]. The reaction mixture consisted of 2.5 mM 2,6-dimethoxyphenol and the enzyme source in a total volume of 2 mL 0.1 M glycine–NaOH buffer pH 10 (ε_{469} = 27500 M^{-1} cm^{-1}, referred to 2,6-dimethoxyphenol concentration). The reaction was monitored at room temperature for 2 min kinetically at 469 nm. The ΔA/min ratio over the respective molar extinction coefficient was determined to arrive at the enzyme activity. The enzyme activity was expressed as IU. One IU was defined as the number of moles of cation released during the assay per minute per milliliter of the enzyme. The protein content of each enzyme preparation was estimated by the method of Bradford [18]. All the experimental values reported were the mean of three independent experimental determinations.

Effect of Temperature. The laccase enzyme activity was assayed in the temperature range 40–100°C at intervals of 10°C by incubating the enzyme sample at each temperature for 10 min and then taking it for enzyme assay as described earlier.

Effect of pH. The ability of the enzyme to express its activity at various pH was carried out in the pH range 4–12. The enzyme source was prepared in different buffers (0.1 M citrate buffer, pH 4–5; 0.1 M McIlvaine′s buffer, pH 6–7; 0.1 M phosphate buffer, pH 8; 0.1 M *tris*-glycine buffer, pH 9, and 0.1 M glycine–NaOH, pH 10–12). Enzyme assay was carried out as discussed earlier at the respective temperatures employing the respective buffer and 2,6-dimethoxyphenol.

Enzyme Kinetics. Substrate saturation kinetics for laccase enzyme activity was carried out employing increasing concentrations of 2,6-dimethoxyphenol in the assay mixture (0.5–5 mM) and a fixed amount of the laccase enzyme source from *Cereus pterogonus*. From a plot of substrate concentration versus enzyme activity, the ν_{max} and Km values were determined using Graphpad Prism software, Version 5.

Time-Dependent Denaturation Kinetics. The purified enzyme sample was taken for time-dependent denaturation kinetic studies. Aliquots of the enzyme sample were incubated at 60°C and 90°C over a period of 30 min at 5 min interval, and laccase enzyme activity was assayed as described earlier.

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