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Research Article

Purification and Characterization of Thermostable Laccase from Thermophilic *Geobacillus thermocatenulatus* MS5 and its applications in removal of Textile Dyes

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Abstract: Laccases are multicopper-containing oxidases (EC 1.10.3.2.) and they are widely found in fungi, higher plants and bacteria. Because of their high pH stability and thermostability, laccases from this strain have a potential for the treatment of textile bleaching effluents. In this research, the thermophilic test strain isolated from the Manikaran thermal springs, in Himachal Pradesh, were subjected to analysis of 16S rRNA sequence. The obtained sequence data were compared with data from the database in the Genbank and as a result it has been identified that this organism is a member of *Geobacillus* genus (GenBank number KC515152). The laccase from *Geobacillus thermocatenulatus* MS5 was purified by using precipitate of saturation $(NH_4)_2SO_4$, DEAE-cellulose and Sephacryl S200 and the molecular mass of the enzyme were determined by SDS-PAGE. It has been depicted that purified laccase enzyme preparations can degrade textile dyes successfully, playing a significant role in the bioremediation of synthetic dyes, thus can be used further for bioremediation of industrial effluents.
Keywords: Laccase, *Geobacillus*, Purification, Characterization, Textile dyes.

INTRODUCTION

Laccases (benzenediol: oxygen-oxidoreductase; EC: 1.10.3.2) are multicopper enzymes that catalyse the oxidation of phenols, polyphenols, aromatic amines and different non-phenolic substrates by using molecular oxygen from the air as an electron acceptor [1, 2]. Industrially important laccase enzyme p-diphenol, dioxygen oxidoreductase belongs to a gene family named as the blue-copper proteins of the oxidases [3] and is produced by 4 type of living organisms including bacteria, insects, higher plants and fungi [4]. Although fungal and bacterial laccases have similar structures, their amino acid sequences are quite different [5]. In addition, bacterial laccases often occur as monomers, whereas certain fungal laccases occur as isoenzymes that normally oligomerize to form multimeric complexes [5][6]. So far, very few bacterial origin laccase enzymes have been purified and characterized. The first study on this subject is the prokaryotic laccase which was derived from the *Azospirillum lipoferum*, a rizospheric bacterium. In this bacterium it was determined that the enzyme was composed of a catalytic subunit and is a multimeric enzyme, consisting of 1 or 2 larger chains [7].

In recent years, bacterial laccases have gained increasing attention for their potential in biodegrading environmentally important phenolic pollutants because of their relatively high production rate, high thermostability, and wider pH range, among others [8, 9]. Laccase enzyme was purified from the *Marinomonas mediterranea*, which is a melanogenic marine bacterium. It has also been determined that these bacteria produce two different polyphenol oxidases, capable of oxidizing the substrates of both laccases and tyrosinases [7]. Ruijssenaars and Hartmans [10] have identified a protein of *Bacillus halodurans* as a potential bacterial laccase. There are very few studies performed with thermophilic bacteria. Recent studies have been conducted with thermophilic organisms including Laccase derived from *Streptomyces lavendula* REN-7 [11], laccase-like protein derived from *Pyrobaculum aerophilum* IM2, which is a thermophilic archaea [12], laccase 31-like protein derived from *Aquifex aeolicus* VF5 which is a hyperthermophilic bacterium [13] and laccase derived from *Thermus thermophilus* [7, 14]. Other bacterial laccases have been described in *Azospirillum lipoferum*, *Escherichia coli*, *Bacillus licheniformis*, *Bacillus halodurans*, *Streptomyces coelicolor*, *Thermus thermophilus* and g-

Proteobacterium JB, to mention the most relevant examples [7, 15, 16].

Laccase mediated catalysis occurs with reduction of oxygen to water, accompanied by the oxidation of substrate. Laccases thus oxidize polyphenols, methoxysubstituted phenols, aromatic diamines and a range of other compounds [17]. Consequently, it is possible to use the laccase enzyme in many industrial areas such as the removal of textile dyes, phenols removal and waste detoxification, since it does not have the substrate specificity. Dye effluents from the textile industry represent a major environmental pollutant and conventional degradation processes suffer from inefficiency, are not price competitive or result in toxic by-products [17-20]. To date, only fungal laccases are industrially relevant for the detoxification of synthetic dyes and other applications owing to their higher redox potential compared to bacterial laccases [7, 21, 22]. However, more recently bacterial laccases have also been shown to successfully oxidize dyes in the presence and absence of redox mediators [15, 23, 24]. Developing bacterial laccases for biotechnological applications will be advantageous because they are sustainable and can be produced in a short time in inexpensive media.

For this reason, purification and characterization of the laccase enzyme produced by *Geobacillus thermocatenulatus* MS5 (Genbank number KC 414152) which were isolated in the Manikaran thermal spring of Himachal Pradesh and investigating the usability of this enzyme in removing textile dyes in wastewater were aimed in this study.

MATERIALS AND METHODS

Sampling

Samples in the form of water, soil, pebbles and rock mattings from different sites of Manikaran hot water spring were collected in sterilized screw capped vials and jars, which were kept in an icebox immediately during sampling and brought to the laboratory and kept at 4^oC in refrigerator till further processing. The temperature and pH were recorded at the time of sampling.

Isolation of strain

The thermophilic bacterial strain used in this study was isolated from Manikaran Hot water spring of Himachal Pradesh. The water temperature of this hot spring was around 95°C. All the samples were enriched on Nutrient Broth and then incubated in covered water bath incubator shaker at 60°C for 24-48 hours. Enriched samples were spread on Nutrient Agar medium plates and the plates were incubated at 60°C for 24-48 hours. Individual colonies were streaked and restreaked repeatedly and the axenic cultures were stored at 4^oC till further experimentation.

Qualitative and quantitative screening for laccase activity

All the thrermophilic bacterial isolates were further screened for laccase activity by both qualitative and quantitative methods. The culture supernatant was obtained by centrifugation of overnight cultures of selected bacterial isolate at 10,000 x g, 4^0C for 10 mins and used as crude extracellular enzyme. Crude extracellular enzyme preparation was used for both qualitative as well as quantitative assays.

Qualitative determination of laccase activity

Plate assay is a rapid determination of the presence of laccase enzyme. Fifteen ml of sterile agar containing 1.0% dimethoxy phenol was poured into petriplates and after solidification wells of appropriate size were made. Hundered µl of crude enzyme extract was added to first well and commercial laccase enzyme was added to the second well followed by incubation at 60^0C for 12-24 hrs. Orange coloured zone would be formed due to the oxidation of substrate which interprets laccase activity.

Quantitative determination of laccase activity

Laccase activity was measured by monitoring the oxidation of ABTS [25, 26]. Catalase was added to the assay solution and incubated for 1 hour at 60^0C to remove the possible effect of H_2O_2 produced by the bacteria. Laccase activity was determined spectrophotometrically at 420 nm with ABTS as a substrate. The reaction mixture contained 200µl aliquots of crude extracellular enzyme preparation and 0.2 mM ABTS in 0.1M sodium acetate buffer (pH 4.5) making final volume to 1.0 ml. The reaction was held at 60^0C for 10 mins followed by addition of 0.5 ml of 80% trichloroacetic acid to stop the reaction. One unit of enzyme was defined as the amount of enzyme required to oxidize 1.0 µmol of ABTS per min. The molar extinction coefficient of ABTS was found to be 36,000 M^{-1} cm^{-1}.

Morphological and biochemical characterizations of laccase producing thermophilic bacterial isolate

Selected thermophilic bacterial isolate was studied for various morphological characteristics viz., colour, gram reaction, shape, spore formation and motility [27]. Various biochemical tests were carried out for the biochemical characterization of selected bacterial isolates viz., catalase test, urease test, oxidase test, indole test, citrate test, MR-VP test and fermentation of sugars [28, 29]. All the biochemical test reagents were procured from Himedia, Mumbai (India).

Genomic DNA extraction

Thermophilic bacterial culture was inoculated into 20ml Nutrient broth and incubated at 60^0C overnight. Cultures were centrifuged at 14000 rpm for 5 min, cell pellets were washed two times with distilled

water, then used for DNA isolation using Genomic DNA extraction Mini-Kit (Real Genomics) according to manufacturer's instructions.

PCR amplification of the 16S rRNA gene

The PCR amplification of the 16S rRNA gene from purified genomic DNA was carried out in 0.2 ml PCR tubes with 20µl reaction volume by using universal primers *viz*., forward primer (5'-GGTCAGCGGCGGACGGGTGAGTAAC-3') and the reverse primer (5'-GACGGGCGGTGTGTACAGAGGCCCG-3') and all the amplifications were performed using thermal cycler (MultiGene PCR system, Labnet). PCR and molecular biology reagents were procured from Bangalore Genei, (Bangalore) and primers were custom-synthesized and supplied in lyophilized form by Integrated DNA Technologies Inc., USA.

Sequencing analysis

The PCR products obtained through amplification with universal primers targeting rRNA gene were sequenced, using same upstream and downstream primers, by a commercial sequencing facility (Xcleris lab). The sequence of the bacterial isolate after sequencing was blasted using online NCBI BLAST program, http://www.ncbi.nib.gov/blast [30]. Phylogenetic analysis was used for comparative genomics to show evolutionary relationships. The analysis began with aligning of sequences using tools like Clustal W [31] and after alignment, phylogenetic tree was constructed using MEGA software (Molecular Evolutionary Genetics Analysis) [32].

Optimization of culture conditions for maximum laccase production

The bacterial isolate selected by quantitative and qualitative assays depicting maximum laccase activity has further investigated to study effect of different factors such as incubation temperature, pH and incubation time on laccase production as well as growth of the bacterial isolate. The pH range was optimized at 60^{o}C for 24 hrs using NB medium adjusted from 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 separately and temperature range for incubation investigated varied from 40, 50, 60, 70, 80, 90 and 100^{o}C where as effect of different incubation times for the growth of these thermophilic bacteria was studied for 24, 48, 72, 96, 120 and 144 hrs. In all cases optical density was monitored at 600nm on a double beam UV/VIS scanning spectrophotometer.

Purification of Laccase from *Geobacillus thermocatenulatus* MS5

The crude enzyme extract was filtered and centrifuged for 15 min at 5000 rpm and then the ammonium sulphate enzyme homogenate was precipitated at 0-20%, 20-40%, 40-60%, 60-80% and 80-100% intervals. The ammonium sulfate precipitation table [33] was followed to calculate the required amount of ammonium sulfate to be added in cell free extract. Then ammonium sulphate was used to saturate the obtained supernatant at 60-80%. After dissolving the precipitate in 100mM phosphate buffer (pH 6.5), it was dialyzed against the same buffer.

Anion exchange chromatography

After the suspension obtained by ammonium sulphate, precipitation was dialyzed, the 100 mM of phosphate buffer (pH 6.5) and previously equilibrated DEAE-cellulose ion exchange column (2.5 x 30) were studied. The column was washed until the eluate protein detection fails with the same buffer. Following this, NaCl gradient from 0 up to 1 M was applied to elute the proteins attached to the column. The flow rates of the collected fractions were 3 ml with a 3ml/min. The absorbance of protein elution was spectrophotometrically measured at 280 nm using ABTS substrate to measure the activity in the fractions. The active fractions were pooled (combined) and allowed to stand at 4°C.

Gel filtration chromatography

Using an Amicon membrane concentrator with a 10 kDa cut off, the active fractions which have been obtained from anion exchange column were combined, dialyzed, desalted and concentrated. Obtained enzyme solution was applied to Sephacryl S-200 column (120 cm × 1 cm), which has been pre-equilibrated with 100 mM of phosphate buffer (pH:6.5) having 0.5 M NaCl and later the enzyme has been obtained with the very same buffer. And all of fractions have been analyzed as stated above. And for later use, the active fractions have been combined, concentrated and allowed to stand at 4°C.

Protein concentration

The protein concentration was determined spectrophotometrically with an absorbance at 280 nm in addition to the Bradford's method [34] using Bovine Serum Albumin (BSA) as a standard.

Determination of laccase activity

The laccase activity has been determined using ABTS substrate [25, 26]. The reaction mixture was prepared by adding 0.5 mL of the enzyme solution on top of the ABTS (3 mM) substrate dissolved in 0.5 mL of 0.1 M acetate buffer (pH = 4.5) and then it was incubated at 60°C. The oxidation of ABTS was determined by monitoring the increase in absorbance at 420 nm. And the one unit of a laccase activity was defined as the required amount of enzyme to oxidize 1 µmol of ABTS/min ($\varepsilon 420 = 36{,}000\ M^{-1}\ cm^{-1}$) [35].

Characterization of purified laccase enzyme

In the characterization the purified laccase enzyme involved determination of molecular weight of laccase enzyme using SDS-PAGE in addition to effect of

different factors such as temperature and pH its activity and stability was studied.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis of purified laccase of *Geobacillus thermocatenulatus* MS5 isolate was performed by using Mini Dual Gel Electrophoresis System (Atto Corporation, Japan) in 10% polyacrylamide gel electrophoresis method which was defined by Laemmli [36]. Gel was stained with Coomassie brilliant blue G-250 and destained with 10% methanol and 10% acetic acid The standard protein was used as protein molecular weight marker for SDS-PAGE for estimation of molecular size of polypeptides of laccase of the selected isolate.

Effect of different factors

To find out the optimum pH for laccase enzyme activity, a pH range of 4.0-10 was investigated. The effect of temperature on enzyme activity was investigated within the range of 30-80°C at a pH of 4.6. The activity was measured using standard ABTS assay.

Remediation of some textile dyes

In this study, it was investigated that whether the purified laccase enzyme can be used in the removal of some dyes used in industry or not. For this purpose, RBBR, Indigo carmine, Congo red, Brilliant green and Bromophenol blue were used as five different dyes. All the dyes were solubilised in distilled water to the final concentration of 50 mg/l. The reaction mixture (6.0 ml) contained 2.0 ml acetate buffer (pH: 4.6), 2.0 ml of individual dye solution and 2.0 ml of laccase enzyme followed by incubation at 60^0C for 3-10 hrs. Control samples were run in parallel without addition of crude extract/laccase enzyme and one sample containing commercial laccase from *Trametes* (SIGMA chemicals) was also run to compare the decolourization efficiency. The decolourization percentage was determined spectrophotometrically as the relative decrease of absorbance at each maximal absorbance wavelength of the dyes. All reactions were performed in triplicates. Dye decolourisation was expressed in terms of percentage calculated according to the equation.

$$\text{Decolourisation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where, A_0 is an absorbance at an appropriate wavelength of the dye immediately measured after adding the enzyme solution and A_t is an absorbance after each time intervals.

RESULTS AND DISCUSSION

In this present study, thermophilic bacterial strain was isolated from Manikaran hot water spring of Himachal Pradesh and subjected to a range of morphological and biochemical tests. It was revealed in the results that test strain was gram-positive, catalase and oxidase positive where as rest of biochemical descriptors *viz*., amylase test, urease test, MR-VP test and fermentation of sugars were found to be negative and also is of type endospore forming and mobile rod. The optimal conditions observed for the maximum growth of selected thermophilic bacterial isolate, from Manikaran thermal spring, were found to temperature range of 60^oC-65^oC, optimal pH range of 6.0-8.0 and optimal time 96 hours. These findings comply with the criteria of thermophilic bacteria which were living at temperatures higher than 50°C [37]. The 16S rDNA of the isolates was amplified by using the forward primer (5'-GGTCAGCGGCGGACGGGTGAGTAAC-3') and the reverse primer (5'-GACGGGCGGTGTGTACAGAGGCCCG-3'). 1151 nucleotides of 16S rRNA from the test isolate (MS5) were aligned to compare with the sequences of the related bacteria. Stackebrandt and Goebel [38] have concluded that the strains belonging to the same genus, which were exhibiting less than 97% 16S rRNA gene sequence similarity, should be considered as the members of different species. The isolate (MS5) has exhibited 99% resemblance to *Geobacillus*, according to 16S rRNA gene sequence analysis [37]. The strain MS5 GenBank accession number is KC515152. 16S rRNA sequence analysis method was found inadequate to reveal the distinction between the species which are in a very close relationship with each other, however, it was found adequate for identification at the genus level again in the same literature [37]. Therefore, these data should be supported with more advanced genomic analysis methods which allow identification at a strain level.

Purification and characterization of laccase enzyme from *Geobacillus thermocatenulatus* (MS5)

The laccase enzyme was purified from the *Geobacillus thermocatenulatus* (MS5) bacteria extracellularly in 3 steps as the ammonium sulphate precipitation, DEAE-cellulose and Sephacryl S200 gel filtration chromatography. All purification steps of the laccase enzyme are shown in table 1. The ammonium sulphate saturation of the laccase enzyme in the range of 0-90% was determined in the first step. Laccase enzyme was precipitated in the 60-80% range which exhibits the highest rate of precipitation and purified 3.85 times with a 38.2% yield and applied to DEAE cellulose ion exchange column. Enzyme had a peak at DEAE cellulose ion exchange column in the second step and further purified 13.1 times with a 34.8% yield. And in the last step, the enzyme fraction was obtained and concentrated from the ion exchange column applied to the Sephacryl S 200 column. A single peak was obtained and 1535 EU/mg protein specific activity was reached with the 93.03 times purification and 30.6% yield. SDS polyacrylamide gel electrophoresis was carried out to determine the purity as well as molecular weight of laccase enzyme. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that the laccase enzyme consists of 2

subunits of sizes 42.5 kDa and 65 kDa (Fig. 1). An optimum pH was found to be 4.0-5.0 and optimum temperature was found to 55-60^{0}C for maximum laccase enzyme activity.

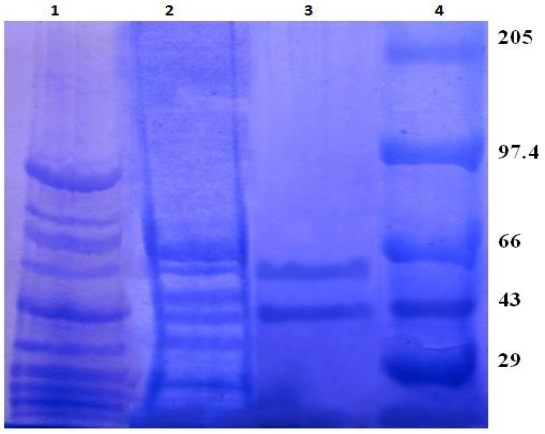

Figure 1: SDS polyacrylamide gel electrophoresis of laccase enzyme *Geobacillus thermocatenulatus* MS5
[Lane 1: Extracellular crude extract, Lane 2: Protein fraction after ammonium sulphate fraction, Lane3: Partially purified protein eluted from ion exchange chromatography, Lane 4: Protein molecular weight marker (kDa)]

Dye decolourization by crude and partially purified laccase

Purified laccase enzyme was also investigated for dye decolourization ability of selected five different dyes. In case of indigo carmine dye, it was found that 50% of decolourization occurred after 2 hrs of incubation and then after 4 hours of incubation, 99% of decolourization was observed. Similarly in case of congo red dye, 60% of decolourization was measured after 6 hrs of incubation and it decolourized up to 99% after 32 hrs of incubation. In case of RBBR, 40% of decolourization was determined after 12 hrs of incubation and then it increased slowly up to 98% after 32 hrs of incubation. The rest of two dyes, bromophenol blue and brilliant green were found to show lesser decolourization comparatively. Percent decolourization of 70% and 60% was observed in bromophenol blue and brilliant green dyes, respectively even after 48 hrs of incubation (Fig. 2, Table 1). The methyl or methoxy groups bound on the phenolic groups in the azo dye structure give electrons and hence increase the activity of laccase enzyme, whereas the nitro groups in the structure attract electrons and hence inhibit the oxidation reaction catalyzed by the laccase enzyme [39, 40]. Thus it was observed that crude as well as purified enzyme preparation also have ability to effectively degrade the textile dyes. The findings are in line with the findings obtained from the removal of azo dyes from the wastewater [41].

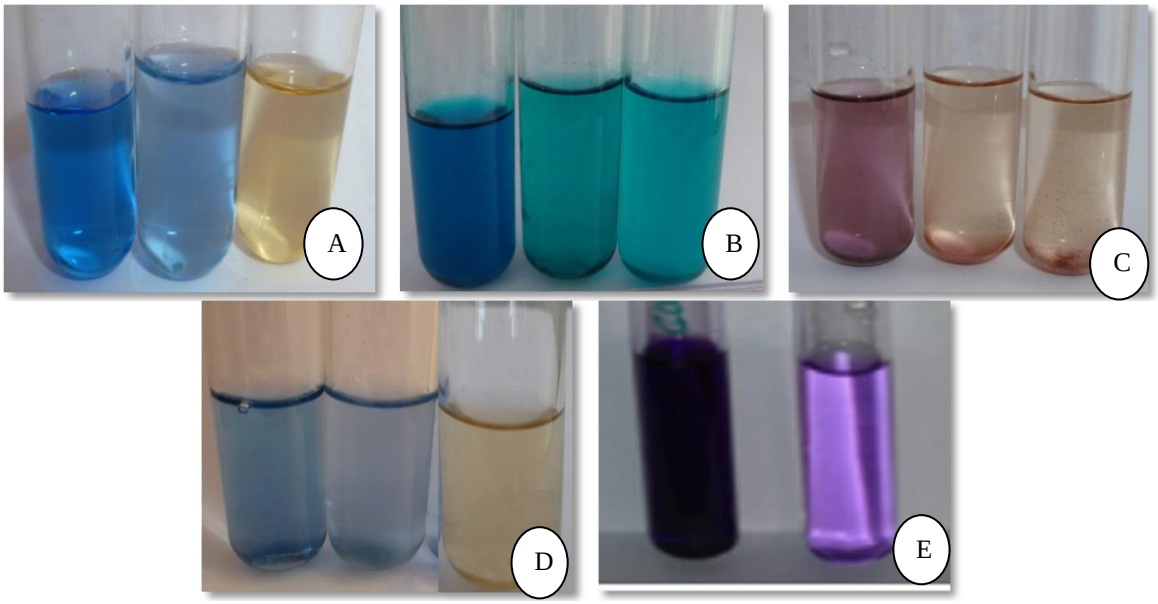

Fig. 2: Decolourization of synthetic dyes using laccase enzyme preparations.
[A) Indigo carmine decolourization, B) Brilliant green decolourization, C) Congo red decolourization, D) RBBR decolourization, e) Bromophenol blue decolourization]

Table 1: Decolourization of dyes by using laccase enzyme preparations

Dye	Decolourization Percentage				
	Purified Laccase Enzyme				
	6hr	12hr	24hr	32hr	48hr
Indigo Carmine	99	99	99	99	99
Brilliant Green	10	24	43	52	60
Congo Red	60	78	98	99	99
RBBR	30	40	79	98	98
Bromophenol Blue	5	24	39	54	70

CONCLUSION

The results suggest that the laccase enzyme purified from *Geobacillus thermocatenulatus* MS5 is of very higher catalytic activity and are economic, highly stable at different temperatures and pH levels and can be used widely and effectively in the removal of the dyes that cause environmental pollution.

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