

Cloning of Laccase Gene from a Newly Isolated 2, 4-Dichlorophenol Degrading *Bacillus subtilis* from Dyeing Industry Sites

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Abstract: In an attempt to isolate 2, 4-Dichlorophenol degrading bacteria, 30 soil samples were collected from the near by places of dyeing industry in Erode district of Tamil Nadu, India. Out of 2400 bacteria, around 200 from each sample, 13 isolates showed 2, 4-Dichlorophenol degrading activity. Among these the most promising isolate was identified, morphologically as an aerobic gram positive motile rod shaped bacterium and biochemically respective results made it confirm that isolate is *Bacillus subtilis*. Molecular characterization using 16S rRNA further confirmed its identity as *Bacillus subtilis* strain. Extracellular laccase activity was measured spectrophotometrically with ABTS (2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulphonate) as substrate. Maximum production of enzyme (200 U/ml) and protein 123 mg/ml were observed at pH 8.0. Different carbon and nitrogen sources tested revealed fructose and peptone more supportive for the production of Laccase from *Bacillus subtilis*. The cell free extract of *Bacillus subtilis* was extracted with 50mM sodium acetate buffer and protein precipitated with 70% ammonium sulphate saturation. The laccase enzyme plate assay showed the presence of laccase in cultural filtrate. The SDS PAGE analysis revealed presence of extracellular 62kDa Laccase enzyme. Amplification with Laccase specific primer gave a product of around 1.5 kb which was cloned into *E. coli* DH5 α . Sequencing and blast analysis of cloned product further confirmed presence of laccase gene.

Key words: Laccase Gene • 2, 4-Dichlorophenol Degrading Bacteria • *Bacillus subtilis* • Dyeing Industry • 62kda Laccase Enzyme

INTRODUCTION

Laccase (Benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a multi-copper-bearing lignolytic enzyme, which catalyzes the one-electron oxidation of many phenolic compounds with concomitant reduction of oxygen to water [1]. Biochemically, laccase [EC.1.10.3.2] is a blue copper oxidase that catalyzes the one-electron oxidation of organic substrates coupled to the four-electron reduction of molecular oxygen to water [2]. These are used in an enormous ranges of processes such as pulp production, papermaking, cellulose ethanol production [3], treatment of pollutants such as polycyclic aromatic hydrocarbons (PAHs) [4] bioremediation of xenobiotic compounds [5] treatment of wastewater [6] decolourization and detoxification of textile dyes [7]. Currently laccase is chiefly produced from fungi but the

slow growth rates of these organisms cannot meet the market demand [8]. In recent times more attention has been focused on recombinant laccase by using a highly productive strain as host using stronger promoters to control the desired gene by recombining more gene copies and by selecting the gene sequence [9, 10]. Diverse laccase gene sequences are found within the same species as well as in different species, more than 10 laccase genes have been found in white rot fungus. The enzyme sequences and their activities have been always associated with gene sequences; the same goes for laccase [11]. The current study reports isolation, identification by morphologically, biochemically and molecular characterization of a 2, 4-Dichlorophenol degrading bacterium. It also describes the cloning of laccase gene from this new indigenous *Bacillus subtilis* TRL isolate.

MATERIALS AND METHODS

Soil Samples Collection: A total of 30 soil samples were collected from different dyeing industry localities of Erode in Tamil Nadu, India.

Isolation of 2, 4-Dichlorophenol Degrading Bacteria:

Samples were brought to the laboratory; 1 g of soil was weighed, transferred to a vial containing 10 ml of sterile water and kept on a rotary shaker at 100 rpm for 30 min, to dislodge bacterial cells from the soil particles. Denser soil aggregates were then allowed to settle for 30 min. The supernatant was diluted 10-fold and 0.1 ml (10^{-5} , 10^{-6} , 10^{-7} dilutions) was spread on the isolation medium consisting of bacteriological peptone, yeast extract, beef extract, sodium chloride and agar-agar pH 7.0 supplemented with 200 micro liter of guaiacol as substrate. These plates were incubated at room temperature for 48 hrs. Formation of dark brown color indicates the production of extra cellular laccase activity by the isolated bacteria. The positive isolates were purified by quadrant streaking and stored in glycerol stocks as well as on nutrient agar stubs. The bacterial isolate TMR which exhibited a fast and large oxidation of Guaiacol on agar plates, as demonstrated by the dark reddish brown color appearance on the plates, was taken for further study.

Characterization of Isolated Bacteria: Morphological, biochemical and physiological characteristics of the potential 2, 4-Dichlorophenol degrading isolate was studied according to Bergey's Manual of Systematic Bacteriology [12]. Biochemical tests such as, fermentation of glucose, arabinose, mannitol and xylose, utilization of citrate, malonate, hydrolysis of starch, decarboxylation of lysine, ornithine, deamination of phenylalanine, arginine dihydrolase, degradation of tyrosine, decomposition of urea, nitrate reduction, oxidase production, production of indole and acetyl methyl carbinol were performed. The ability of the bacterial isolates to grow in 2%, 5%, 7% and 10% of NaCl was also studied.

Molecular Characterization of the 2, 4-Dichlorophenol Degrading Bacterial Strain:

Genomic DNA was extracted from TMR using HiPurA™ Bacterial and Yeast Genomic DNA Purification Spin Kit (Himedia). To determine the sequence of the 16S rRNA gene, a DNA fragment of ~ 1.5-kb was amplified by PCR from the genomic DNA of the sample using universal eubacteria-specific primers: 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-GGYTACCTTG TTACGACTT-3), synthesized at Xcelris Labs Ltd. For polymerase chain reactions (PCR), 0.1 µg of

total DNA from each isolate was mixed with a solution containing each primer at a concentration of 1 µM, 0.25 mM dNTPs and 2.5 units of Taq polymerase (Promega) in PCR buffer with 2.0 mM MgCl₂. PCR amplification was performed in an Eppendorf thermal cycler using the program: a 5 min denaturation step at 94°C, 30 amplification cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, with a final extension step of 10 min at 72°C. The amplified PCR products were purified using GeneJET™ PCR purification kit (Fermentas life science) and sequenced by automated sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd. The forward and reverse sequences were edited using Bioedit program [13]. The sequences obtained from strain TMR was compared to 16S rRNA gene sequences available in the databases of the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) by BLASTN homology search [14]. Phylogenetic analysis included the 16S rRNA gene sequences of the local isolate TMR and the reference strains *Bacillus subtilis* obtained from GenBank. *Alycyclobacillus cycloheptanicus* was used as an outgroup [15]. The evolutionary history was inferred using the Neighbor-Joining method Saitou and Nei [16]. Cluster support was assessed through 1,000 bootstrap replicates [17]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method Kimura [18] and are in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1366 nucleotides positions in the final dataset. Phylogenetic and Evolutionary analyses were conducted in MEGA5 [19]. Sequences were submitted in Gen Bank and accession numbers were obtained.

Purification and Characterization of Laccase

Ammonium Sulfate Saturation: The laccase was purified from culture broth by centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was collected and filtered through membrane filter having porosity of 0.22 µm at 4°C. To the supernatant ammonium sulphate was added slowly with continuous string to the final concentration of 80% saturation. The enzyme solution was allowed to stand for 24h at 4°C and centrifuged at 10,000 rpm for 20 min at 4°C. The precipitate was re-suspended in 50 mM Tris HCl having pH 8.0 and further precipitated with acetone by adding slowly to the final concentration 80% saturation and left for 1 h at 4°C.

Dialysis: The pellet was obtained by centrifugation at 10,000 rpm for 20 min at 4°C and re-suspended in 20 mM Tris HCl pH 8.0 then dialyzed against 500 ml of 5 mM Tris-HCl pH 8.0 containing 1 mM MgCl₂ over night at 4°C with stirring conditions. The dialysate was centrifuged at 10,000 rpm for 20 min at 4°C and subjected to column chromatography.

Sephadex G-100 Gel Filtration Column Chromatography: Sephadex G-100 (Biorad Fine Chemicals, USA) was allowed to swell overnight in sterilized distilled water. The floating gel beads were removed and the gel slurry was packed into a glass column [100 x 2.5 dia cm], which contained sintered filter at bottom. While packing, care was taken to avoid air bubbles. The packed gel column was equilibrated with 0.1M sodium phosphate buffer pH 7.0. Concentrated enzyme was loaded on to the sephadex G-100 column and eluted with 0.1M sodium phosphate buffer (pH 7.0) at the flow rate of 3ml per 15 min. Absorbance of the fractions were read at 280 nm for protein.

Determination of Protein Content and Assay of Degradation Activity: The protein concentration was determined by taking bovine serum albumin as standard [20]. To study degradation of 2, 4-D compound, 20% acetone that was sufficient to dissolve 15µgml⁻¹ and caused less than 20% enzyme inhibition was used. Different concentrations of enzymes were used to determine the degradation capabilities. 2, 4-D degradation without the treatment of enzyme served as control.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-Poly acrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5% w/v) as described by the method of Laemmli [21]. At the end of electrophoresis, gel was removed and stained by silver staining method Blum *et al.* [22]. Next, the gel was stored in 7% (v/v) acetic acid. Standard protein marker was used to determine the molecular weight.

Screening for the Presence of Laccase Gene: Laccase genes are frequently homologous, principally those that come from the same species [23]. Thus, two a primer pair (5'-ATGACACTTGAAAAATTTGTGGATGCTCTCCC-3' and 5'-CTATTTATGGGGATCAGTTATATCCATCGG-3') designed for the detection of laccase was synthesized [9]. The PCR amplification was carried out in an Eppendorf thermal cycler in a 25µL reaction volume containing 100 ng DNA, 0.5 mM of primers, 100 mM deoxynucleoside

triphosphate, 1XTaq DNA polymerase buffer and 3U Taq DNA polymerase (Genei Bangalore). After confirmation of the size of the amplicon, the amplified PCR products were purified using Gene JET™ PCR purification kit (Fermentas life science).

Cloning and Sequencing of Laccase Gene: InsTAclone™ PCR Cloning Kit (Fermentas Life Science) was used for cloning of purified PCR products. The PCR amplified laccase sequences was ligated in pTZ57R/T vector as per instructions in user manual. The recombinant vectors pTZ57R/T-LAC was transformed in *Escherichia coli* DH5α. Positive clones were identified by blue white screening. The recombinant plasmid (pTZ57R/T-LAC) from positive clone was sequenced using an automated DNA sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd. The forward and reverse sequences were edited using Bioedit program and blast performed using BLASTN [13]. Sequences were submitted in GenBank and accession numbers were obtained.

RESULT

Isolation and Screening of 2, 4 Dichlorophenol Degrading Bacteria: The soil samples collected from the dyeing industry areas of Erode in Tamil Nadu, India, were serially diluted and dilution factor 10⁻⁴, 10⁻⁶ and 10⁻⁷ were used for isolation of bacteria. A total of approximately 2400 bacteria were screened, around 200 from each sample, out of these 13 isolates showed 2, 4-Dichlorophenol degrading activity as demonstrated by the dark reddish brown colour colonies. The isolate which revealed highest degradation ability was selected for further characterizations (Figure 1).



Fig. 1: The *Bacillus subtilis* strain showed the Laccase Screening activity on Guaiacol amended Agar plate assay Dark brown colour indicate laccase

Table 1: Morphological and biochemical characteristics of xenophytic bacterial strain

Test	Result	Test	Result
Colony morphology	Dull white irregular, convex	Malonate	-
Spores	Cylindrical	Ornithine	+
D-Glucose Utilization	+	Tyrosine	-
L-Arabinose Utilization	+	Oxidase	+
D-Xylose Utilization	+	Starch	Hydrolysis
Mannitol Utilization	+	Urease	+
Nitrate reduction	+	Growth in 2% NaCl	+
Catalase	+	Growth in 5% NaCl	+
Citrate	-	Growth in 7% NaCl	+
Phenyl alanine	-	Growth in 10% NaCl	+
Arginine	-	Acetyl methyl carbinol	+
Lysine	-	Indole	+
Bacterium		<i>Bacillus subtilis</i>	

Note where: + Positive; - Negative

Table 2: Summarized Purification of Laccase production from *Bacillus subtilis*

Purification step	Volume in ml	Total Protein in mg	Total activity in U	Specific activity U/mg	Purification fold	Yield%
Crude extract	100	2180	12200	5.5	1	100
70% (NH ₄) ₂ SO ₄	80	1200	10720	8.9	1.60	87.8
Sephadex G-75	30	306	3780	12.3	2.2	30.9

Biochemical Characterization of Potential Isolate:

The xenophytic bacterial isolate was found to be aerobic, gram-positive, motile and rod-shaped bacterium. Based on the morphological and physiological characters, strain TMR was identified as *Bacillus subtilis* summarizes in Table 1. The PCR amplification of the 16S rRNA gene of TMR yielded amplicons of the size of 1.5 kb. BLASTN (NCBI) analyses of TMR sequence indicated a 99% similarity with other *Bacillus subtilis* strains. Sequence was deposited in GenBank under accession number (KM088070). Next, we determined the degree of relatedness of our isolate to different *Bacillus subtilis* species through a phylogenetic analysis. The NJ tree shows a close relationship between the strains isolated in the present study and Group I of Nakamura (Strains B-23269 and B-23287).

Purification of Laccase from *Bacillus Subtilis*: The cell free extract of *Bacillus subtilis* was collected after extraction with 50mM sodium acetate buffer and its proteins were precipitated by salting out with ammonium sulphate (70%). The crude protein preparation was dialyzed, concentrated by lyophilization and used for further analysis. The concentrated protein (19 mg) was loaded on Sephadex G-75 column and each 2ml of 100 protein fractions were collected (Table 2). Four peaks of Laccase were obtained: peak I-fractions from 15 to 45, peak II-fractions from 52 to 75 and peak III -fractions from 85 to 95. The enzyme active fractions from 15-45 were pooled, concentrated by lyophilization, dialysed against sodium acetate buffer (10mM; pH 5.5) and used for further purification.

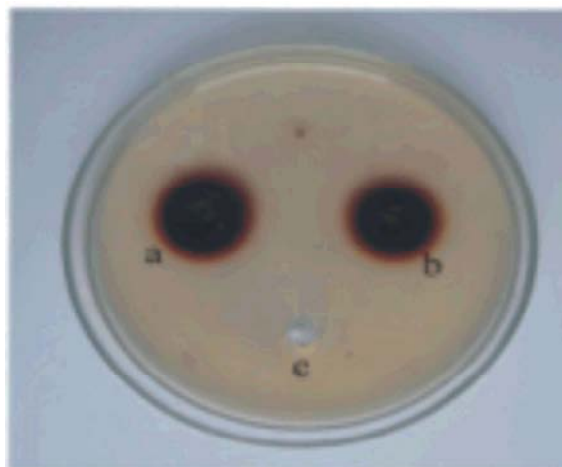


Fig. 2: Laccase enzyme activity on 1 mM Guaiacol amended agar medium containing a. dialysis sample b. purified sample and c. control sample

***Bacillus subtilis* Laccase Assay on Agar Plate:** The laccase enzyme plate assay showed the presence of laccase enzyme in the culture filtrate. The laccase enzyme activity of the dialyzed and purified enzyme exhibited dark reddish brown color, which confirms the presence of laccase (Figure-2).

Enzymatic Degradation of Xenobiotic Compound (2, 4-D Degradation): To study degradation of selected xenobiotic 2, 4-D degradation, its solubility in a mixture of acetone-water with various ratios was measured. A mixture of 20% acetone that was sufficient to dissolve 15µgml⁻¹ of 2, 4-D compound and caused less than 20%

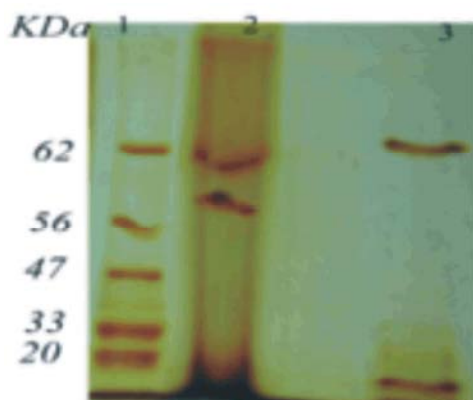


Fig. 3: Molecular mass determination of Laccase from *Bacillus subtilis* by SDS-PAGE showed the presence of 62KDa protein where Lane 1-Standard protein marker, L 2-Dialysed sample and L 3- Purified sample

enzyme inhibition was used. The activity of the enzyme during experiments in the water-solvent mixture was stable. In control (2, 4-Dichlorophenol alone) concentration decreased to $10.1 \mu\text{gml}^{-1}$ after 168 hours. However the enzyme was able to decompose all the studied 2, 4-D at different rates. The highest degradation was observed at pH 8.

Molecular Mass Determination of Purified *Bacillus subtilis* laccase by SDS-PAGE: The purified Laccase ($3.0 \mu\text{g}$) was analyzed on SDS-PAGE (10% w/v) and stained with silver nitrate. On sodium dodecyl sulfate-poly acrylamide gel electrophoresis the purified protein showed a single band indicating that it was electrophoretically homogeneous. The molecular mass of the purified Laccase was determined as 62 kDa by comparing with relative mobility of the molecular mass of protein ladder (Figure 3).

Screening for the Presence of Laccase Gene: Primers for Laccase gene gave rise to fragments of 1.5kb amplicon respectively, corresponding to the Laccase (62 kDa). The sequences of Laccase gene have showed 99% identity with the same genes found in *Bacillus subtilis*. The sequence has been submitted into the GenBank under the accession number KM088071.

DISCUSSION

Chlorophenols are important industrial raw materials for producing dye, preservatives, pesticides and chemicals. However, the industrial waste water containing

the refractory organics such as chlorophenols is seriously harmful for the environment and human health. A number of physical and chemical processes have been proposed as having potential application in removal of chlorophenols. However, these processes are not environmental friendly. Laccase-mediated removal of chlorophenols is one biochemical process which are low-cost, environmentally friendly and high efficient. Laccase from *Bacillus subtilis* is the major ligninolytic enzyme produced in liquid culture and submerged fermentation. It has been considered as an efficient method for enzyme production in biotechnological process due its potential advantages and high yield [24]. In present study bacterial isolates from soil samples were screened for the degradation of 2, 4 Dichlorophenol and it was observed that *Bacillus subtilis* strain produced highest levels of laccase. This strain also showed a strong endurance to high concentrations of NaCl; it can survive in 10% NaCl. This advantage makes it potentially useful to deal with wastewater containing saline solution and to reduce the time of pretreatment. SDS-PAGE analysis showed the presence of one acidic isoform of *Bacillus subtilis* laccase, whose molecular mass was estimated 62 KDa. Biodegradation of chlorophenols by bacterial species has been studied for long time as it is a rather effective approach. Although bacteria individuals can detoxify these materials, if the pollutants are produced on a large scale, then bacteria may be not the ideal organisms to biodegrade them. It is well-known that laccase-mediated biodegradation systems are succeeding to deal with it. Laccase from *Trametes pubescens* was evaluated for the ability to degrade a chlorophenol mixture and 82% of TCP (15 mg/l) after 4 hours of reaction under the optimum condition [25]. It is well known that copper ions are toxic even at low concentrations to lots of bacteria. However some bacterial laccases, such as CueO and PcoA play a role in copper tolerance [26]. The regulation of copper homeostasis of *E. coli* has been analyzed, although the mechanism is still unclear [15]. The main mechanism of CueO in copper resistance has been postulated to be the oxidation of the Cu^+ to Cu^{2+} [27]. *Bacillus subtilis* can survive in copper-containing medium. This process is effective for copper resistance because the Cu^+ is more harmful than Cu^{2+} [28]. The degradation of xenobiotic by peroxidation enzyme was first described in *Phanerochaete chrysosporium* as a lipid peroxidation-dependent process [29]. The oxidation of 2, 4-D as a temporal intermediate and 2, 4-D degradation experiments showed that MnP isolated from *I. lacteus* was able to efficiently degrade three and four ring 2, 4-D compounds with IP higher than 7.8eV which are not

degraded by the enzyme from *S. coronilla* [30]. The high level of laccase production could be attributed due to the presence of ferulic acid in wheat bran which was shown to be a better platform for *G. lucidum* growth and production of xylanase enzyme [31]. Recently cloning, expression and characterization of the bacterial cotA from *Bacillus clausii*, a supposed alkalophilic ortholog of cotA from *Bacillus subtilis* was reported [32].

CONCLUSION

The ability of *B. subtilis* to degrade xenobiotic compound 2-4 D is evident, but the advantage of laccase treatment is a shorter treatment period. The *Bacillus subtilis* is promising bacterial strain since it produces a high laccase levels in the studied conditions. Currently, the optimization of laccase production from this bacterial strain is being studied xenobiotic compound 2-4 D being treated with the enzyme to check its potential use in detoxification of the effluents.

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