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Microbial Decolorization of Various Dyes by a *Bacillus subtilis* Strain Isolated from an Industrial Effluent Treatment Plant

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Abstract

A *Bacillus subtilis* strain exhibiting laccase activity was isolated from an industrial effluent treatment plant. M9 medium containing Cu^{2+} was used for enrichment and isolation of bacterial strains capable of oxidizing syringaldazine, a known laccase substrate. An isolated strain was identified as *Bacillus subtilis* based on the results of physiological and biochemical tests and sequence analysis of the 16S rRNA gene. The strain could grow at temperatures ranging from 20 to 55°C and showed optimal growth temperature and pH at 25°C and 7.0, respectively. The rate of strain sporulation clearly correlated well with laccase activity. The half-life of the spore laccase was 2.5 h at 80°C and the pH half-life is 15 days at pH 9.0. The spore laccase could discolor 50-90% Remazol brilliant blue R, Alizarin, Congo red, methyl orange and methyl violet, suggesting the possible application of the spore laccase in the treatment of dyestuff.

Keywords: Bacterial laccase; *Bacillus subtilis*; Spore; Decolorization

Introduction

Laccases are multi-copper proteins that can oxidize a wide range of inorganic and aromatic compounds, especially phenols, while reducing molecular oxygen to water [1]. Laccases catalyze the removal of one hydrogen atom from phenolic substrates and aromatic amines by an abstraction of electrons. Free radicals formed during the reaction are also capable of undergoing depolymerization, further repolymerization, demethylation, or quinone formation [2-4]. The low substrate specificity of laccases and their ability to oxidize various pollutants suggest their industrial-technological and biotechnological applications [5,6]. Laccases are widely distributed in fungi and plants [7]. However, it has been found that laccases are also widespread in bacteria [1]. To date, laccases have mostly been isolated and characterized from plants and fungi, but only fungal laccases are currently used in biotechnology applications. In contrast, only a few bacterial laccases have been characterized. Bacterial laccases have the ability to oxidize syringaldazine and 2, 6-dimethoxyphenol, which are typical substrates for laccases, and also possess the canonical four areas for the binding of copper. Nevertheless, overall sequences of bacterial laccases show little resemblance to fungal laccases. Therefore, they are often called "multicopper oxidases" or "(poly) phenol oxidases" and their activity is generally defined as "laccase-like" [8]. The first report of bacterial laccase was from the strain *Azospirillum lipoferum*, which was isolated from the rhizosphere of rice [9]. This enzyme has been identified as a laccase using a combination of substrates and inhibitors [9] [10]. Laccase activities have also been found in *Bacillus sphaericus* [11], *Escherichia coli* [12], *Bacillus halodurans* [13], and *Streptomyces psammoticus* [14] to name a few. CotA, the *Bacillus subtilis* endospore layer component, is the most studied bacterial laccase [15]. Since spores allow microorganisms to survive in harsh conditions, spore coat enzymes can also withstand high temperatures or extreme pH values. As most fungal laccases are unstable at pH values greater than 7.0, their detoxification efficiency for pollutants often decrease under alkaline conditions. This limits the potential industrial application of fungal laccases as many processes are performed under alkaline conditions. Alternatively, spore laccases that are active in the alkaline pH range could be used for bioremediation or application in membrane reactors [4]. Compared to fungal laccases, bacterial laccases have the advantage of being less sensitive to halides and alkaline conditions and the producing strain typically exhibits a rapid growth rate [16]. Despite the importance of bacterial laccase in the degradation of pollutants, only a few new bacterial strains with "laccase-like" activity have been discovered. The lack of a robust and inexpensive commercially available laccase is a major obstacle to the widespread application of laccase in various industrial sectors [17]. Since bacterial genetic tools and biotechnological processes are well established, the development of bacterial laccases would be of great significance [18]. The present study was therefore conducted to isolate and characterize the strain, *Bacillus* sp. ETL 1979, which was isolated from an industrial textile effluent treatment plant. The spore laccase of this strain was characterized and used to decolorize various synthetic dyes.

Materials and Methods

Sample collection

Soil samples used in this study were collected from textile

effluents from the textile industry, Ankleshwar, Gujarat, India. Collected soil samples were stored at 4°C aerobically.

Isolation of microorganisms

For the isolation and enrichment of bacterial strains with the ability to produce laccase, 250 ml flasks containing 100 ml M9 culture medium supplemented with 0.2 mmol/l Cu^{2+} were inoculated with 10 g of soil and incubated at 37°C on a rotary shaker (130 rpm) for 2 days. Then 5 ml of the cultures were transferred to 100 ml of Luria-Bertani medium (LB) culture medium containing 0.2 mmol/l Cu^{2+} and incubated at 37°C at 130 rpm for 7 days. Stable enrichment cultures were obtained after sub culturing. To isolate pure cultures, cultivated fortified products were appropriately diluted with a sterile saline solution (0.9% NaCl) before spreading onto LB/Cu^{2+} plates. The plates were incubated at 37°C for 3 days. Bacterial colonies from individual plates were flooded with a 0.1% (w/v) syringaldazine solution to determine whether any of the isolates exhibited laccase activity. Colonies with pink halos were streaked onto new LB/Cu^{2+} plates for purification. Re-inoculation was performed after identification of syringaldazine-positive colonies as described above. The isolation process was repeated several times until the isolates were shown to be pure.

Characterization of isolates

Gram staining was performed according to standard protocol. The characteristics of Gram and cell morphology of the isolated strain were determined by microscopy. For the use of carbon sources, the pure cultures were seeded respectively in peptone culture medium - water containing 1% substrate, and incubated at 37°C for 24 h. The results were determined by varying both turbidity and color of the culture medium. Selected biochemical metabolic capacity properties were determined by inoculating isolated bacteria on media.

Molecular Characterization

The bacterial cells were collected by centrifugation at 10 000 rpm for 2 minutes and incubated with 100 µg/ml lysozyme at 37°C for 1 h, followed by treatment with the lysis solution (1% SDS, 1 mmol/L EDTA, 20 mmol/L CH_3COONa, and 40 mmol/L Tris-HCl (pH 8.0). After addition of 5 mmol/L NaCl to the lysis solution, the mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was harvested and subsequently precipitated with absolute ethanol. The genomic DNA obtained was dissolved in sterile deionized water and stored at -20°C for later use. For the polymerase chain reaction (PCR), specific primers for eubacterial 16S rRNA gene sequence amplification 27F: 5'-GAGTTTGATCMTGGCTCAG-3 '(H = A or C) 1492R: 5'-TACGGYTACCTTGTTACGACTT-3' (Y = C or T) were used [19]. PCR was performed in a Gene Amp PCR System 9700 (Applied Biosystems, Singapore). The amplification reaction consisted of an initial denaturation at 93°C for 5 min, followed by 30 cycles of 94°C for 18 s, 56°C for 15 sec and 72°C for 78 s, and an extension step final at 72°C for 7 min. PCR products were analyzed by electrophoresis in 1.0% (w/v) agarose gel and photographed using a Bio Imaging System (Gene Genius, USA). The amplicons were cloned using a commercially available cloning vector pMD18-T kit and transformed into competent *E. coli* JM109 cells. Positive clones were identified by PCR amplification with the 16S rRNA gene primers specified above.

Nucleotide sequencing, alignment, and phylogeny

16S rRNA gene sequencing of the isolated strain was performed by Bangalore Genei Company, India. Related sequences were obtained from the GenBank database after using the BLAST online tool [20]. Multiple sequence alignment was performed using Clustal X 1.81 [20]. PHYLIP package [20] was used to calculate the similarity values and to build a phylogenetic tree.

Optimization of growth conditions

The optimal growth conditions with regards to pH and temperature were determined. The strain was inoculated in LB media which have been adjusted to various pH values and incubated at 15-55°C. The optical density of growing cultures was observed at 600 nm using a UV spectrophotometer - 1800 (Shimzadzu, Japan) to determine the optimum growth conditions. All assays were performed in triplicate.

Effect of metals and saline solution on bacterial growth

To study the effect of metals on the growth of the laccase-producing strain, 200 µg/ml Zn^{2+}, Fe^{3+}, Ca^{2+}, Mn^{2+}, Mg^{2+} or Cu^{2+} was added to the LB culture medium, respectively. Cultures were grown in 25 ml medium in 100 ml conical flasks at 37°C for 24 h. Cultures grown in the absence of metals was used as a control. Growth was determined by measuring the absorbance at 600 nm against the blank. In addition, the strain was inoculated in LB medium supplemented with 1, 2, 4, 6, 8, 10, or 12 % (w/v) NaCl. The turbidity of the cultures in the growth medium was observed at 600 nm using a UV spectrophotometer - 1800 (Shimzadzu, Japan) to determine the growth state. All assays were performed in triplicate.

Sporulation rate and laccase activity relationship

B. subtilis ETL 1979 was inoculated onto LB plates containing 0.2 mmol/L Cu^{2+} and incubated at 30°C. The amount of spores was calculated daily and the activity of the laccase was determined at the same time. The sporulation rate was determined by the percentage of the quantity of spores opposed to all cells. The spores were removed from the agar with 1 mol/L KCl, washed with 0.5 mol/L NaCl and resuspended in 0.1 mol/L citrate phosphate buffers (pH 6.8). The spore suspension was prepared for the determination of the activity of laccase. All assays were performed in triplicate for each sample.

Spore laccase activity assay

Laccase activity of the spores was determined at 40°C using syringaldazine (dissolved in absolute ethanol, Sigma) as substrate. The oxidation of syringaldazine was detected by measuring the increase in absorbance at 525 nm ($\varepsilon 525 = 65\ mmol^{-1}\ cm^{-1}\ L$) after 3 min using a spectrophotometer (UV spectrophotometer - 1800 Shimzadzu, Japan). The reaction mixture (3 mL) contained

100 µl of spore suspension (10 mg wet spores), 2.4 ml of citrate phosphate buffer (0.1 mol/L, pH 6.8) and 0.5 ml of 0.5 mmol/L syringaldazine. One unit of enzyme activity is defined as the amount of enzyme required to oxidize one µmol of substrate per minute. All assays were performed in triplicate for each sample. The standard deviation does not exceed 5% of the average values.

Effect of pH and temperature

Determination of the effect of pH on laccase activity was performed in 0.1 mol/L citrate buffer - phosphate in the range of pH 4.0 to 8.0 using syringaldazine as substrate. The effect of temperature on the spore laccase activity was determined in the range of 0 to 100°C at the optimum pH value. Syringaldazine was used as the substrate as described before. All assays were performed in triplicate. The thermal stability of the spore laccase was determined by pre-incubation of the spores in 0.1 mol/L citrate buffer phosphate (pH optimum) at 60 and 80°C and the remaining activity was measured by the test described above. pH stability was examined similarly by incubating the spores in different buffers ranging from pH 4.0 to 9.0, at 30°C. All assays were performed in triplicate.

Determination of dye decolorization efficiency

Remazol Brilliant Blue R (RBBR), Alizarin red, Congo red, methyl orange, and methyl violet, were individually prepared at a concentration of 25 mg/L in sterilized distilled water. The prepared dye solution was mixed with 100 g/L spores and incubated at 37°C under mild conditions, shaking for 5 days. Dye samples without spores, which received the same treatment, were designated as the controls. The spectrum of each dye between 200 and 800 nm absorption was measured with a UV spectrophotometer - 1800 (Shimadzu, Japan). Dye decolorization was evaluated by the decrease in absorbance at the maximum wavelength of the dye. All assays were performed in triplicate.

Results

Isolation of the bacterial strain with the highest laccase activity

One hundred and forty colonies were selected from M9 agar plates supplemented with 0.2 mmol/L Cu^{2+}. After a secondary screening, 46 bacterial strains were selected based on the color development reaction to syringaldazine. One potential strain with high levels of laccase activity was named ETL 1979 and selected for further studies. Strain ETL 1979, which formed pink colonies on LB agar, is a gram-positive bacterium, spore-forming, rod-shaped, 1 to 2 µm long, motile, and formed white colonies on LB agar supplemented Cu^{2+} 0.2 mmol/L. The optimum pH for growth was determined to be 7.0 and the optimal temperature 25°C. The 16S rRNA gene amplicon was approximately 1.5 kb (Figure 1). The biochemical, physiological, morphological characteristics (Table 1), and the comparative analysis of the 16S rRNA gene sequence with the available database (GenBank) showed that the isolated strain is a *B. subtilis*. The similarity of the sequence (100%) and phylogeny based on Clustal X indicate that the ETL 1979 strain is a *B. subtilis* (Figure 2).

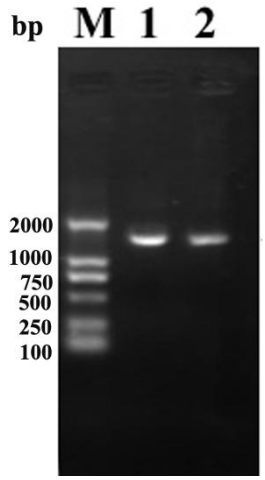

Figure 1: PCR product of the 16S rRNA gene of *B. subtilis* ETL 1979. Lane M: Molecular weight marker (DL2000), lanes 1 & 2: *B. subtilis* ETL 1979.

Characteristics	B. subtillis ETL-1979
Colony diameter	1 – 3 mm
Colony color	White
Cell morphology	Rod
Motility	+
Gelatin hydrolysis	+
Urase	+
Lipase	–
Oxidase	+
Catalase	+
Casein protease	+
Amylase	+
NO_3^- reduction to NO_2^-	+
M-R reaction	–
V-P reaction	+
Utilization of:	
Mannite	+
Phaseomannite	+
Sorbierite	+
L-rhamnose	–
Melibiose	+
Lactose	–
Glucose	+
Maltose	+
Xylose	–
Sucrose	+
Gum sugar	–
Fructose	+

Table 1: The morphological and biochemical characteristics of *B. subtilis* ETL 1979.

Effect of metals and saline solution on bacterial growth

Metal cations Zn^{2+}, Fe^{3+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, and Mg^{2+} (200 µg/ml) all showed some degree of inhibition of the growth of the strain. Among them all, Zn^{2+} showed the highest degree of inhibition.

The relationship between sporulation rate and laccase activity

The positive correlation of the activity of laccase and the percentage sporulation was observed in the 10 day old culture as seen in (Figure 3). The result shows that the activity of laccase

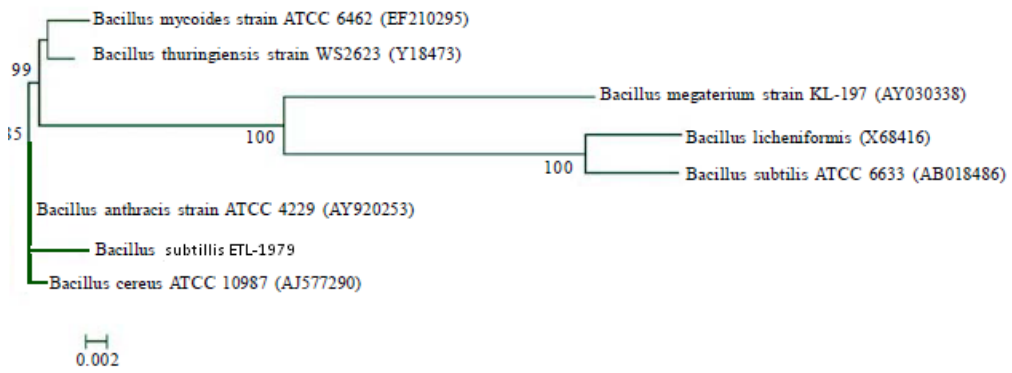

Figure 2: Phylogenetic analysis of the 16S rRNA gene sequences of *B. subtilis* ETL 1979 and related taxa.

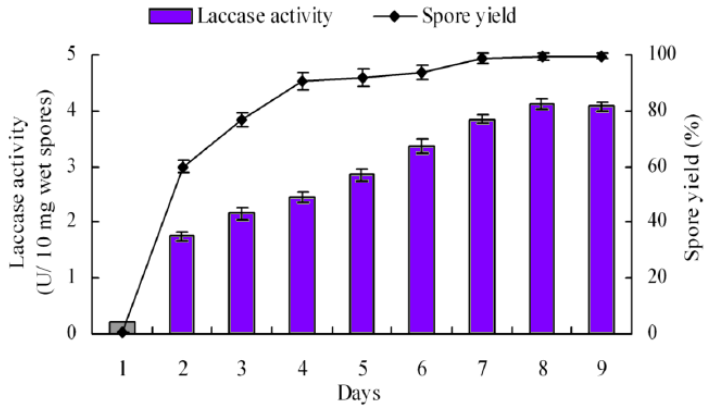

Figure 3: The relationship between sporulation rate and laccase activity of *B. subtilis* ETL 1979.

was derived from spores.

Effect of pH and temperature on the activity and stability of spore laccase

Highest laccase activity was detected at a pH of 6.8 and the optimum temperature was observed at 60°C. Laccase spores showed greater stability under conditions of high temperature and under alkaline conditions that most fungal laccases. The half-life of laccase was 2.5 h at 80°C, while the half-life of the laccase was 15 days at a pH of 9.0.

Efficiency of dye decolorization

To demonstrate the potential application of this bacterium for the treatment of wastewater containing a dye, the spores were used for bleaching RBBR, alizarin, Congo red, methyl orange, and methyl violet. The bleaching rate was 90% in the treatment of RBBR and alizarin red, and 50 to 70% in the treatment of the other dyes (Figure 4). These results indicate that the spore laccase has the ability to decolorize the selected dyes without the need for redox mediators.

Discussion

In this study, a new strain of *B. subtilis*, strain ETL 1979, was isolated from soil collected at an industrial effluent treatment plant. This strain was unable to use xylose and sugar gum, while the type strain of *B. subtilis* according to Bergey`s manual Unlike other *B. subtilis* strains in our laboratory which showed little laccase activity, strain ETL 1979 exhibited high laccase activity. Laccases as biocatalysts have received much attention because of their great capacity to oxidize phenolic and other aromatic compounds. This advantage makes laccases highly suited for certain biotechnological applications, such as the biodegradation of xenobiotics, including aniline, methoxyphenols and benzenethiols [21, 22]. In contrast to fungal laccases, bacterial laccases are very active and much more stable at high temperatures and high pH levels. As indicated above, most of the effluents from textile industries are characterized by a neutral to alkaline pH (about 7-11) [23, 24]. For many industrial applications it is necessary that catalysts such as laccases are kept active throughout the process or via immobilization onto intermediate membrane reactors [18]. The spore laccase of *B. subtilis* ETL 1979 has a high thermal stability and high stability under alkaline conditions. These characteristics could be of great importance for biotechnological applications.

It is well known that copper ions are toxic to a number of bacteria, even when present at low concentrations. However,

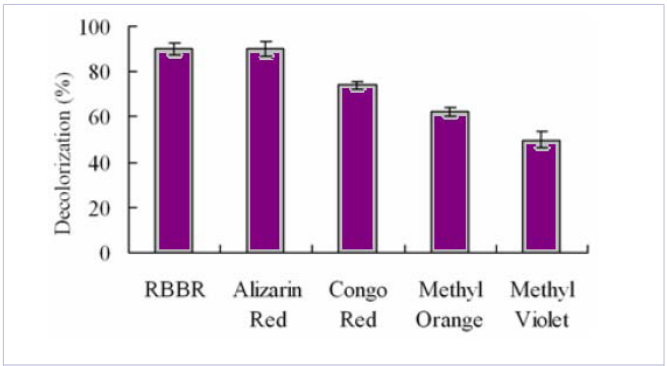

Figure 4: Decolorization of dyes with spore-bound laccase from *B. subtilis* ETL 1979.

certain bacterial laccases, such as CopA and CueO play a role in copper tolerance [20]. The regulation of copper homeostasis in *E. coli* was analyzed, and although the mechanism is still uncertain [25], it has been postulated that CueO is involved in the main mechanism of copper resistance and involves the oxidation of Cu^{+} to Cu^{2+} [26]. This method is effective for copper resistance because Cu^{+} is more harmful than Cu^{2+} [27]. The present study showed that *B. subtilis* ETL 1979 can survive in a medium containing copper. However, the ETL 1979 strain is unable to form melanin in the medium containing copper ions. CotA in *B. subtilis* has been associated with the formation of a brown pigment [28, 29]. Strain ETL 1979, also showed strong resistance to high concentrations of NaCl; it can survive in 10% NaCl. This advantage makes it potentially useful for dealing with saline wastewater and reduces the pretreatment time. To date, bacterial laccases have only been found in *A. lipoferum*, *Alteromonas* sp. MMB-1, *Pseudomonas* sp. KU03, *E. coli*, and some species of *Streptomyces* and *Bacillus*. There is little information on the use of enrichment culture methods and the sampling of soil from industrial effluent treatment plants for the prospecting of bacterial laccases. In this study, soil samples were taken at the factory of an industrial effluent treatment plant. Other reports on the isolation of bacterial species with laccase activity was focused on the rhizosphere of rice [9], seawater [21], river mud or top-soil containing organic waste [13], contaminated soil with dye and textile industry, and lignocellulosic waste effluents [30]. In our study, the spore laccase was used for the bleaching of an anthraquinone dye and azo dyes without the addition of nutrients or redox mediators. Our results indicate that the spore laccase can decolourize the dyes effectively in 5 days. This result is similar to that of the spore laccase from *Bacillus* sp. SF [4]. However, few spore laccases can be reused because it is difficult to separate the laccase from the decolorized solution. Immobilized enzymes are highly effective in bleaching stains, because immobilization can improve the utilization rate of the enzyme despite the reduction in enzyme activity. Future studies would therefore focus on the immobilization of the ETL 1979 spore laccase.

The results presented here demonstrate that the spore laccase has a potential application in the treatment of aqueous solutions that contains dye. In summary, the *B. subtilis* ETL 1979 strain that exhibited laccase activity was isolated from soil collected from an industrial effluent treatment plant and was characterized during the course of this study. The strain showed the ability to catalyze a substrate that is considered to be a typical laccase substrate (syringaldazine), it exhibited good growth at 55°C, and its spore laccase can decolorize selected dyes without the addition of redox mediators.

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