Original Article

International Journal of Biosciences and Nanosciences
Volume 2 (2), 2015, pp. 41-47.

ISSN-2349-5251

Isolation, optimization and production of laccase from *Halobacillus halophilus*

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Received: 29 Dec 2014; Revised 16 Jan 2015 / Accepted: 10 Feb 2015

Abstract

The objective of the project was to isolate the laccase producing bacteria, characterize it, produce the enzyme and identify it. Maximum laccase production was observed after 72 h of incubation. The optimum pH and temperature for growth were found to be 7 and 67 °C respectively. Thus the laccase producing isolated bacteria is thermostable at higher temperature. The laccase producing organism was isolated and identified as *Halobacillus halophilus*. The GenBank accession number allotted for the 16S ribosomal RNA gene of *H. halophilus* was KP796423.

Keywords: bacteria, *Halobacillus halophilus*, laccase, thermostable.

1. Introduction

Laccases (EC 1.10.3.2) are polyphenol oxidases that catalyse the oxidation of various aromatic compounds, particularly those with electron-donating groups such as phenols (−OH) and anilines (−NH2), by using molecular oxygen as an electron acceptor (Gianfreda et al., 1999). Laccase enzymes are widespread among plants, fungi and bacteria, and have various biological functions. Most of the laccases reported thus far are of fungal origin, especially from white rot fungi such as *Phlebia radiata*, *Pleurotus ostreatus* and *Trametes versicolor* (Desai et al., 2011). In contrast to fungal laccases, some bacterial laccases can be highly active and much more stable at high temperatures, at high pH as well as at high chloride concentrations (Bugg et al., 2011; Dwivedi et al., 2011; Reiss et al., 2011; Sharma et al., 2007). Only a few bacterial laccases have been isolated, although genomic analyses have revealed that it is probably a widespread enzyme in bacteria. The first report on bacterial laccase was from the non-motile strain of *Azospirillum lipoferum* isolated from rice rhizosphere (Givaudan et al., 1993). This enzyme was identified as a laccase by using a combination of substrates and inhibitors (Givaudan et al., 1993; Diamantidis et al., 2000). Discovery of novel laccases with different substrate specificities and improved stabilities is important for industrial applications. Recently some bacterial laccases have also been characterized from *A. lipoferum* (Givaudan et al., 1993), *Bacillus subtilis* (Martins et al., 2002), *Streptomyces lavendulae* (Suzuki et al., 2003) and *Streptomyces cyaneus* (Arias et al., 2003). Laccases are used in several industrial applications, such as pulp delignification, textile dye bleaching, effluent detoxification, biopolymer modification and bioremediation (Gianfreda et al., 1999). The objective of the present investigation is to isolate and identify the laccase producing bacteria and to optimize the production of laccase.

2. MATERIALS AND METHODS

2.1. Soil Sample Collection: Soil samples from decaying wood were collected from 3 different places of Coimbatore, Tamil Nadu, India to isolate bacteria producing laccase.

2.2. Enrichment and isolation of micro-rganisms: One gram of the soil sample was diluted by transferring the sample into 100 mL of sterile distilled water. About 1 mL of diluted sample was transferred to 20mL of basic medium containing 0.5% ammonium tartarate, 0.1% malt extract, 0.001% calcium chloride, 0.1% sodium chloride, 0.001% ferric chloride, 0.1% lignin, incubated in shaker incubator at 37 °C for 3 days. The lignin was prepared from peanut hull (Thomas and Triplett, 1983). The enriched samples were then serially diluted by transferring into test tubes so that sample concentrations were obtained from 10^{-1} to10^{-10}. About 0.1 mL aliquots of 10^{-2} to 10^{-10} were pipetted out into sterilized nutrient agar plates containing (0.02%) catechol and spread plated. The plates were then incubated at 37 °C. The coloured zone formed around the colony indicates the production of laccase.

2.3. Screening for laccase producing microorganisms: The morphologically different colonies were isolated and screened for laccase activity. Each isolated colony was cultured on basic medium containing 0.5% glucose (production medium) at 37 °C for 24h in orbital shaker and laccase activity was checked. The highest laccase producing bacteria was further taken for analysis.

2.4.Production of laccase: The pre-inoculum was prepared prior to enzyme production by taking a loop full of colony from the master plate and inoculating in nutrient broth. The inoculated broth was left in the shaker at 37 °C for 24 h. About 1mL of the pre-inoculum was added to 100 mL of enzyme production media at 120 rpm at 37 °C orbital shaker.

2.4.1.Preparation of crude extract and assay of laccase: Following incubation of the production medium for appropriate period, the contents in the flask were centrifuged at 8000 rpm for 5 min and the supernatant was collected which is an extracellular enzyme. Laccase assay was carried out using guaiacol method. About 50 µL of guaiacol, 500 µL of ethanol, 1.5 mL of phosphate buffer and 1.5 mL of enzyme sample was taken and incubated after thorough mixing at 37 $^{\circ C}$ for 10 min. In the control assay, the enzyme was replaced by 1.5 mL of water. The absorbance value was read at 470 nm (ϵ=12000M $^{-1}$ cm $^{-1}$) (Xiao et al., 2004). One unit of enzyme was defined as the amount of enzyme that liberated 1µM of product / minute/mL in the above mentioned assay condition. Specific activity was defined as the amount of enzyme that liberated 1µM of product/min/mg of protein. The concentration of protein in crude sample was determined by Lowry et al., 1951 method using Bovine Serum Albumin as standard. Each reaction was taken twice. Each experiment was carried out twice. The mean value was mentioned in graph and error bars indicate standard deviation.

2.5. Optimization of laccase production medium:

2.5.1.Effect of incubation period on laccase production: The effect of incubation period on laccase production was investigated by checking the enzyme activity for every 24 h for 4 days. Optimum incubation period for laccase production was found out from the graph drawn against incubation period on the X-axis and laccase activity on the Y-axis.

2.5.2. Effect of pH on laccase production: The effect of pH on enzyme production was investigated by using the laccase production medium prepared with various pH ranging from 5-10. The optimum pH for enzyme production was calculated from the graph drawn against different pH in the X-axis and laccase activity on the Y-axis.

2.5.3. Effect of temperature on laccase production: The effect of temperature on laccase production was investigated by incubating the production medium at various temperatures such as 27 ^{o}C, 37 ^{o}C, 47 ^{o}C, 57 ^{o}C, 67 ^{o}C and 77 ^{o}C respectively. Optimum temperature for enzyme production

was calculated from the graph drawn against temperature on X-axis and laccase activity on Y-axis.

2.6. Identification of Microorganisms: In order to identify the isolated strain, morphological observation, physio-biochemical test and 16S rDNA gene sequence analysis were carried out. The colony morphology i.e. colour, shape, size, nature of colony and pigmentation were observed. The gram characteristics, motility and endospores were examined microscopically. Indole production, Methyl Red test, Voges proskauer test, utilization of citrate, Oxidase test and Catalase test were carried out. The isolated strain were determined according to the methods described in Bergey's Manual of Determinative Bacteriology.

2.6.1. Extraction of genomic DNA and identification of bacteria by 16S rDNA sequencing: The genomic DNA was isolated from the isolated organism. About 50 mL LB broth was inoculated with a single bacterial colony and grown to an absorbance 600 nm of 0.5–1.0 and cells were collected by centrifugation at 5000 rpm, at 4°C, for 10 min. The pellet was suspended with 400µL of STE buffer and vortex well. Add 50 µL of 10% SDS and mix gently by inversion and incubate in water bath for 55 ºC for 15min. About 450 µL of freshly prepared Tris saturated phenol: chloroform: isoamyl alcohol was added, vortexed well and centrifuged at 14,000 rpm for 10 min at 4 ºC. Aqueous phase (400 µL) alone was transferred to another 1.5 mL tube and 2 volumes of ice cold 100% ethanol was added, mixed gently and incubated at 4ºC for 30 minutes. Centrifuged at 12000 rpm for 15 min, the supernatant was decanted and the pellet was air dried. The pellet was dissolved in 50 µL of nuclease free water and 5 µL was loaded into 0.8% agarose gel and electrophoresed. To assign strains to bacterial species for each isolate, the entire 16S rRNA gene was amplified. Amplification of the 16S rRNA gene was performed using the universal primers: forward primer –8F- 5'AGA GTT TGA TCC TGG CTC AG 3' and reverse primer –U1492R-5'GGT TAC CTT GTT ACG ACTT 3' (Chromous Biotech Pvt. Ltd). PCR reaction mix in 0.2 mL microfuge tubes with following components: 5 µL of template DNA, 1µL of each primer (10 pmol/ µL conc), 25µL of 2X PCR master mix (containing Taq DNA polymerase, PCR buffer, dNTPs and $MgCl_2$) and 28µL of nuclease free water for a 50µL reaction. PCR reaction was performed with initial denaturation of 2 min at 94 °C, which was followed by 30 cycles 94 °C for 30 s, 58 °C for 45 s, 72 °C for 90 s and a final extension for 10 min at 72 °C. The presence of the PCR products were examined and visualized by electrophoresis in 1.5% agarose gel and viewed in Gel Documentation System (Biorad Gel Doc. 2000 system). The 1528-bp amplification product was sequenced using forward and reverse direction (ABI Prism, Chromous Biotech Pvt. Ltd) and subjected to nucleotide database analysis. Related sequences were obtained by comparing the non-redundant National Center for Biotechnology Information (NCBI) database by using Basic Local Alignment Search Tool (BLASTn), with the default settings used to find the most similar sequence and were sorted by the E score. The phylogenetic tree was obtained using the distance tree method of BLAST.

3. RESULTS

3.1. Enrichment and isolation of bacteria from soil: The enriched soil contained 24 different colonies of bacteria isolated on nutrient agar plate containing (0.02%) catechol after enrichment of the soil.

3.2. Screening of laccase producing bacteria: Out of 24 colonies, single colony (Isolate 18) with higher laccase activity was chosen and characterized (Table 1).

3.3. Optimization of production medium

3.3.1. Effect of incubation period on laccase production: Of various incubation periods tested, an incubation period of 72 h was

found to be optimum for laccase production (Fig.1).

3.3.2. Effect of pH: Of various pH tested, production medium with a pH of 7 was found to be optimum for laccase production (Fig.2).

3.3.3. Effect of temperature: Of various temperatures tested, the optimum temperature for laccase production was found to be 67 °C (Fig. 3).

3.4. Characterization of laccase producer: The morphological and biochemical characterization of isolated organism was performed and the following results were obtained (Table 2).

Table 1: Laccase activity of different bacteria isolated

Isolate	Laccase activity (µM/min/mg protein)
1	0.714
2	3.147833
3	0.918
4	0.5015
5	0.918
6	1.292
7	1.575333
8	2.516
9	2.8305
10	0.663
11	0.3825
12	0.558167
13	3.258333
14	0.351333
15	0.371167
16	0.4675
17	0.388167
18	5.9415
19	0.541167
20	3.162
21	1.768
22	3.3065
23	0.291833
24	3.298

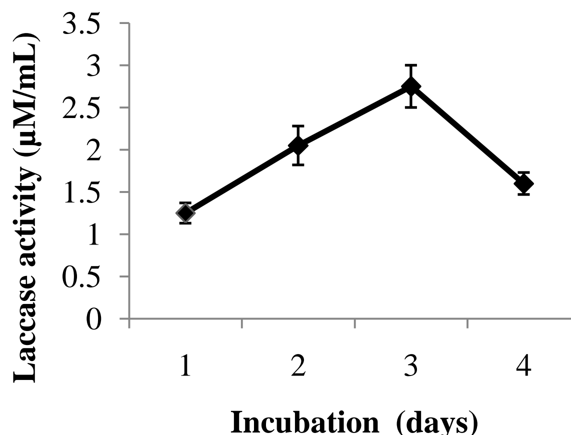

Figure 1: Effect incubation period on laccase production

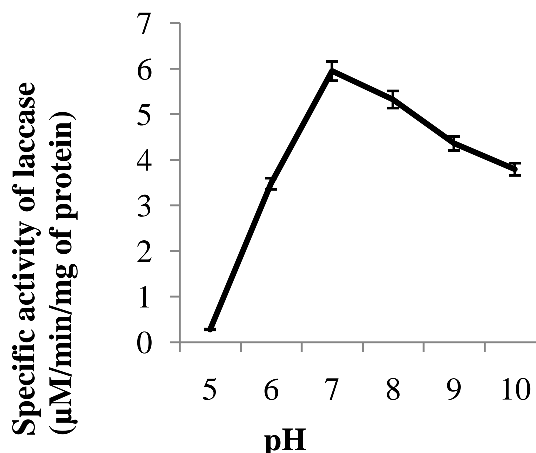

Figure 2: Effect of pH on laccase production

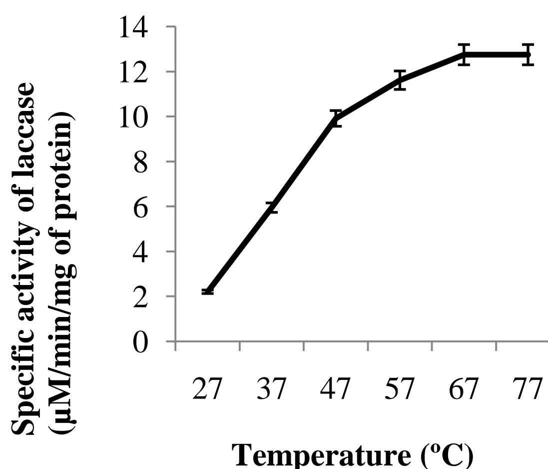

Figure 3. Effect of temperature on laccase production

Table 2: Characterization of Laccase producing bacteria

TEST	RESULT
Gram's staining	Gram positive cocci
Endospore staining	Endospore former
Motility test	Motile
Indole test	Negative
Methyl red test	Positive
Voges proskauer test	Negative
Citrate utilization test	Negative
Oxidase test	Positive
Catalase test	Positive

3.5. DNA isolation and 16S rDNA amplification: The DNA isolated from the given bacteria showed single band in agarose gel observed under Gel Doc system (Fig.4a). The PCR amplification of 16S rDNA showed ~1.5kb product size as shown in Figure 4b by comparing with the 500bp ladder as marker.

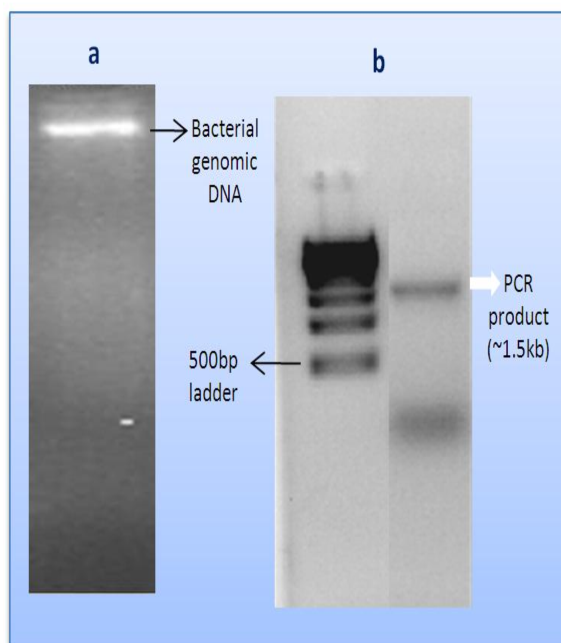

Figure 4: DNA and 16S rDNA amplification a) DNA isolated from bacteria and b) 16S rDNA amplification of DNA from the isolated bacteria

The sequence obtained from the 16S rDNA of the isolated bacteria showed 100% similarity with *Halobacillus halophilus,* DSM 2266 complete genome (formerly *Sporosarcina halophila*).Thus the isolated organism was identified as *H. halophilus* and its 16S rDNA sequence was submitted in Genbank with Accession number KP796423. The Phylogenetic tree has been constructed using the BLAST distance tree method was shown in Fig.5.

4. DISCUSSION

The laccase production was carried out in the basic medium. Similarly the basic medium was used as screening as well as production of laccase by *Serratia* sp. in the work done by Park et al. (2006). The production of laccase was assayed using catechol method similar to guaiacol method done by Xiao et al. (2004). In addition to that, enzyme production was tested with various pH and temperature. Based on the results obtained after this characterization, the production media was designed and production of laccase enzyme was carried out with the isolated bacteria. Similar work was done by Martins et al. (2002).

Maximum enzyme production was observed at 72 h of incubation, while Niladevi et al. (2009) characterized 48 h as the incubation period for the production of laccase. Among the basic and important physical parameters *viz.* pH and temperature, the optimum pH and temperature were found to be 7 and 67 ^{o}C respectively.

Many research works had been carried out with various pH and temperature. In their studies, Dalfard et al. (2006) and Niladevi et al. (2009) concluded the optimum pH to be 7.5, which were very close to our findings. But, the optimum temperature was found to be 37 ^{o}C by Dalfard et al. (2006).

Our bacteria producing the laccase were thermostable at 77 ^{o}C. Similar to our results *Thermus thermophilus* HB27 which was a thermo-tolerant bacteria produces laccase with increased thermostability (Ausec et al., 2011). Similar morphological and biochemical characterization was observed for *H. halophilus* by Hosseini Abari et al. (2012).

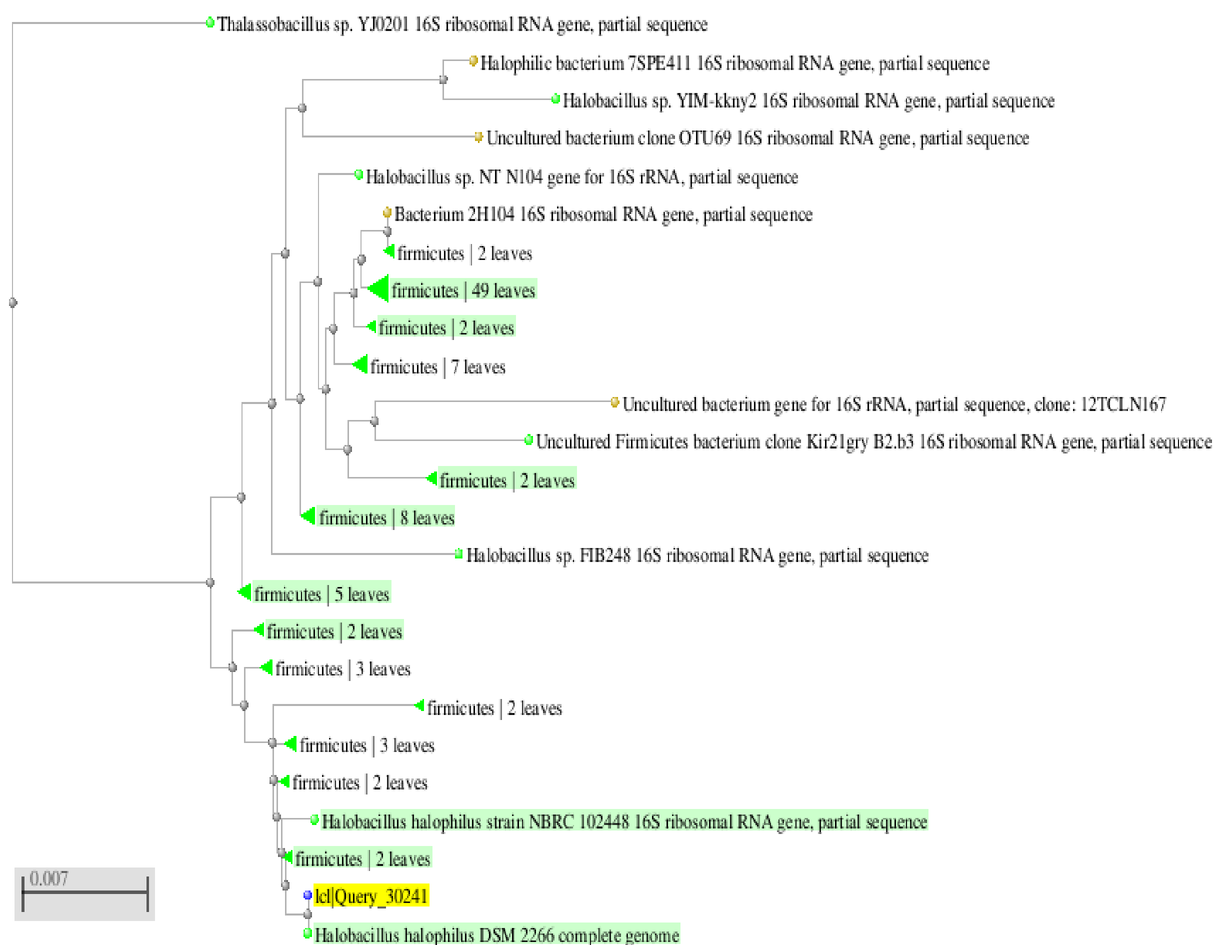

Figure 5: Phylogenetic relationship of the bacterial isolate no 18

5. CONCLUSION

In the present study, *H. halophilus* was isolated for production of laccase. Its optimum incubation period was 72 h, optimum pH was 7 and the optimum temperature was 67°C for production of laccase.

Acknowledgement: We acknowledge DBT-STAR college scheme, Department of Biotechnology (DBT), Government of India, New Delhi for their financial support and encouraging research in undergraduate level. We also thank our Management and Department of Biotechnology of Kongunadu Arts and Science College for providing facilities and encouragement to carry out this research work.

Conflict of Interest: The authors declare that they have no conflict of interests.

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