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Research Article

Screening and Characterization of Bacterial Laccase for Aromatic Compound Degradation

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Abstract

The textile dyeing industry produces a large volume of waste water from the different steps in the dyeing process. The discharge from the dyeing industry constituting unfixed dyes, inorganic salts, heavy metal complexes etc., spoils the surrounding areas of industrial sites. There are 100 different Bacterial colonies were isolated and screened for Laccase-production using guaiacol drop assay. The isolated strain 5B and AM produced white on Nutrient agar. Upon gram staining the cells were found to be gram positive. The biochemical chacterization were done to identify the bacteria at genus level. Two bacterial laccase positive isolates were identified by molecular identification with 16S rDNA sequencing. The sequences obtained were analyzed using BLAST search to identify the corresponding Phylogenetic relatives. Based on the Identity score the Phylogenetic affiliations (Firmicutes) were confirmed. The whole cells were developed color change in both ABTS and SGZ within 5 minutes at pH 7.2.The Absorbance spectrum of ABTS and SGZ after oxidation by whole cell shows the characteristic laccase activity in the solution. The Sodium dodecyl sulphate polyacrylamide gel electrophoretic analysis of the partially purified laccase enzyme revealed the presence of different protein bands with molecular weights of 56.4 kDa respectively. The zymogram of the partially purified laccase enzyme revealed that the enzyme was in its active form which is seen as green color .The proposed work focused on the isolation and characterization of bacterial laccase from dye polluted environment.

Keywords: Laccase; waste water; Aromatic compound; Molecular oxygen.

Introduction

One of the major problems that humans are facing is the restoration of the contaminated environment. The textile dyeing industry produces large volumes of wastewater from different steps in the dyeing processes. The discharge from the dyeing industries constituting unfixed dyes, inorganic salts, and heavy metal complexes etc., spoils the surrounding areas of industrial sites. The recalcitrant, residues of dyes in the effluent pose a threat to the environment. Textile dyes contribute as the most important environment-polluting agents. Several classes of such contaminants have been synthesized, and still new products are being synthesized now and then. The textile industry is a large water consumer and produces large volumes of contaminated water. One of such examples is the Ankleshwar Industrial Estate, Ankleshwar, Gujarat and Tiruppur Tamilnadu India, which is a seriously industrialized area and produces millions of liters of improperly treated effluents that are released directly without giving proper treatment [1].

Synthetic dyes released into the environment in the form of effluents by textile, leather, food, paper and printing industries cause severe ecological damages. Wastewater resulting from dyeing and finishing processes has an adverse impact in terms of total organic carbon, biological oxygen demand and chemical oxygen demand. Azo dyes are the main constituents of such pollution because of their wide applicability and usages and therefore these are present majorly in textile industrial effluents. Moreover their toxicity and resistance to degradation offer great challenge for removal technologies. In many cases the products formed after the degradation of the parent azo dye molecule are more toxic. These products are mainly in aromatic amine form. Azo dyes have

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been shown to be mutagenic to the human hepatoma cell line [2].

Material and methods

Isolation of microorganisms

Laccase producing microbes were screened from various environmental samples taken from dye polluted water and soil area at Tiruppur. Bacteria were isolated by standard serial dilution methods using Nutrient agar. The different morphological colonies were further tested for Guaiacol positive by Guaiacol drop assay as mentioned below.

Bacterial identification

The standard biochemical test were performed to identify the Bacterial strain along with 16s rRNA gene sequencing.

Biochemical characterization

The standard biochemical test was performed to identify the bacterial strains morphological, physiological and biochemical characteristics with reference to Bergey's manual of systematic Bacteriology. Gram staining, Indole production test, Methylred test, voges-proskauer test, Simmons citrate test, Oxidase, Catalase test, Triple sugar ion test were performed according to standard protocol. Gram's characterstics and cell morphology of the isolated strain were determined by microscopy. The strip test was also done to identify the bacterial strain. In the strip test, the culture was inoculated with the strip and incubated at 37°C [3].

Bacterial Strain identification by 16s rRNA gene sequencing

Bacterial DNA was extracted using CTAB method. The quality of extracted DNA was determined on 1% agarose gel electrophoresis. The 16s rRNA gene sequencing was amplified using universal 16s rDNA polymerase chain reaction. All PCR amplification was carried out in a thermal cycler. Negative control (PCR Mixture without template DNA) was included. DNA was amplified during 30 cycles sequencing results of 16s rRNA gene sequencing were analysed [4].

Phylogenetic analysis of the isolates

The sequences obtained were analyzed with NCBI (National centre for biotechnology information) online nucleotide BLAST tool to identify the taxonomic hierarchy of the sequences. Taxonomically related 16S rRNA gene sequences were obtained from the NCBI nucleotide database The collected sequences were aligned using Muscle multiple sequence alignment algorithm. The Phylogenetic tree was constructed and inferred using the Neighbor-Joining method. The constructed tree was validated with bootstrap method (1000 replication) to validate. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. All analysis was performed with MEGA 6 [5].

Qualitative assay for laccase

Guaiacol Drop assay Indicator compound Guaiacol 0.001% (v/v) used in the screening was added on the colony only the agar media plates in order to detect microbes that produce laccase enzymes. The positive strains produces Orange/brown colour upon guaiacol oxidation. The syringaldazine (SGZ) assay were carried out by mixing the single colony in the solution containig 50 µM SGZ, 10 µM $CuSO_4$. The positive strain develops pink color. The (ABTS) assay was carried out by mixing the single colony in the solution containing 0.4 mM ABTS, 10µM $CuSO_4$. The positive strain develops green color [6].

Preparation of crude extract

The positive bacteria were grown on Nutrient agar plates. The two day culture was collected from plate and dispensed in the Tris lysis buffer (pH 8) contains 1% Triton x-100, 100 mM NaCl. The suspented cells lyzed by Ultra sonication at 50 W for 8 min with cooling in an ice bath. Total cell extracts were incubated for 1 h at 48°C under gentle agitation in the presence of 1% (v/v, final concentration) Triton X-100. Homogenates from 3 to 4 g bacterial cells treated as above were pooled and used for further analysis of the enzyme [7].

SDS PAGE and Zymogram assay

Prior to SDS PAGE and Native PAGE assay. The crude bacterial isolate was precipitated with 90% acetone. The proteins were precipitated by centrifugation. After air drying, the precipitates were suspended in 50 ml 1 mM Na-phosphate (pH 7.2) after thorough mixing; the solution was centrifuged for 10 min at 12,000 g. The pelleted material was re-suspended in 50 ml phosphate buffer and washed four times in the same buffer in order to eliminate the detergent and any non-bound proteins.

The final pellet was then solubilised in 50 ml solublization buffer containing 1 mM Na-phosphate (pH 7.2), 5 M urea, 2 M NaCl and centrifuged at 20,000 g for 20 min to undissolved debris remaining. Laccase molecular weight was identified by Zymogram followed by SDS PAGE (Laemmli 1970). SDS PAGE of the cell extract was performed on a 10% separating gel and 5% stacking gel. Prior to gel application, the samples were precipitated with TCA and then denatured by boiling them for 5 min. Samples were run with pre-stained standard molecular weight marker in Tris-glycine buffer (pH 9.5) at room temperature and a constant current of for 60 min. Protein bands were visualized by coomassie brilliant blue R230 staining. To detect laccase activity Native PAGE was used. The procedure used was the cell extract without TCA treatment and without boiling the sample (to preserve laccase activity), no SDS was added and then trying an ABTS Staining to detect specifically the band with laccase activity. The gel was rinsed with water and subsequently submerged in 10 mM succinic buffer with 5 mM ABTS where the laccase active bands were highlighted in dark green [8].

Results and discussion

Isolation and screening of bacteria

There are 100 different Bacterial colonies were isolated and screened for Laccase-production using guaiacol drop assay. The bacterial colonies from nutrient agar plates develops the brown colour on the margin of the colonies by addition of guaiacol further assayed for activity against Syringaldazine (SGZ) and ABTS. There were 5 strains shows positive reaction against guaiacol but out of 5 only 2 shown positive reaction against SGZ and ABTS. The activity of laccase was assayed both in extracellualar supernatant and whole cells prepared from nutrient broth cuture. Laccase activity on biofilm was also assayed. The colonies growing on the Agar plates were only given positive against guaiacol [9]. The results were given in the Table 1.

Table 1. Laccase activity assay

Strains	Plate cells	Broth culture		Biofilm
		Supernatant	CELLS	
5B	+	-	-	-
AM	+	-	-	-

Identification of bacteria

The isolated strain 5B and AM produced white colonies on nutrient agar (Fig. 1 and 2). Upon gram staining the cells were found to be gram positive. The biochemical chacterization were done to identify the bacteria at genus level. The results of biochemical test were given in the Table 2. The strain 5B shown to be grown on Bacillus differentiation agar and produced yellow colony by fermenting bacteria. The strain AM produced white colony on Bacillus differentiation agar. Based on the biochemical test and strip test observation it predicted to be belongs to Bacillus genus [10].

Fig. 1. Growth of strain 5B on *Bacillus* differentiation agar

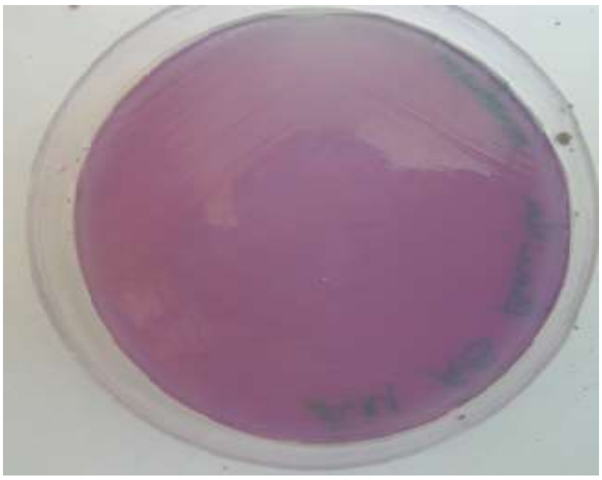

Fig. 2. Growth of strain AM on *Bacillus* differentiation agar

Table 2. Biochemical characteristics for strain 5B and AM

Strain No.	Biochemical Test	Identification	Results
5B	Gram staining	Purple color	Gram positive
	Oxidase	Changes to Dark purple color	Positive
	Catalase	Bubble formation	Positive
	Methyl Red	No change	Negative
	Voges Proskauer	Changes to red color	Positive
	Motility	No change	Negative
	TSIA	Changes to orange color in butt changes to yellow color in slant	Positive
	Citrate utilization	Changes to dark blue color	Positive
	Indole	No change	Negative
Strain No	Biochemical Test	Identification	Results
AM	Gram staining	Purple colour	Gram positive
	Oxidase	Changes to Dark purple colour	Positive
	Catalase	Bubble formation	Positive
	Methyl Red	No change	Negative
	Voges Proskauer	Changes to red colour	Positive
	Motility	No change	Negative
	TSIA	Changes to orange colour in butt changes to yellow colour in slant	Positive
	Citrate utilization	Changes to dark blue colour	Positive
	Indole	No change	Negative

Table 3. Strip test I for strain 5B and AM

Strip No.	Test	Reagents to be added after incubation	Principle	Original colour	Positive reaction	Negative reaction
1	ONPG	-	Detects beta-galactosidase activity	Colourless	-	Colourless
2	Lysine utilization	-	Detects lysine decarboxylation	Olive green to light purple	purple	-
3	Ornithine utilization	-	Detects ornithine decarboxylation	Olive green to light purple	purple	-
4	Urease activity	-	Detects urease activity	Orangish yellow	Pink	-
5	Phenylalanine daamination	2-3 drops of TDA reagent	Detects phenylalanine deamination activity	Colourless	-	colourless
6	H_2S production	-	Detects H_2S production	Orangish yellow	black	-
7	Malonate utilization	-	Detects capability of organism to utilize sodium malonate as a sole carbon sources	Light green	Blue	-

Table 4. Strip test II for 5B and AM

Strip No.	Test	Principle	Reagents to be added after incubation	Original colour	Positive reaction	Negative reaction
1	Esculin hydrolysis	Esculin hydrolysis utilization	-	Colourless	Black	-
2	Arabinose	Arabinose utilization	-	Colourless	-	Red /pink
3	Xylose	Xylose utilization	-	Colourless	-	Red /pink
4	Adonitol	Adonitol utilization	-	Colourless	-	Red /pink
5	Rhamnose	Rhamnose utilization	-	Colourless	-	Red /pink
6	Cellobiose	Cellobiose utilization	-	Colourless	-	Red /pink
7	Mellibiose	Mellibiose utilization	-	Colourless	-	Red /pink
8	Saccahrose	Saccahrose utilization	-	Colourless	-	Red /pink
9	Raffinose	Raffinose utilization	-	Colourless	-	Red /pink
10	Trehalose	Trehalose utilization	-	Colourless	-	Red /pink
11	Glucose	Glucose	-	Colourless	-	Red /pink
12	Lactose	Lactose utilization	-	Colourless	-	Red /pink

To identify the bacterial species based on 16s rRNA sequencing. Genomic DNA was isolated using CTAB DNA extraction method. The isolated DNA was shown in fig. 3(a). The 16s rRNA amplified using bacterial universal primers 27S and 1492 bP. The amplified sequences were shown in fig. 3(b) [11].

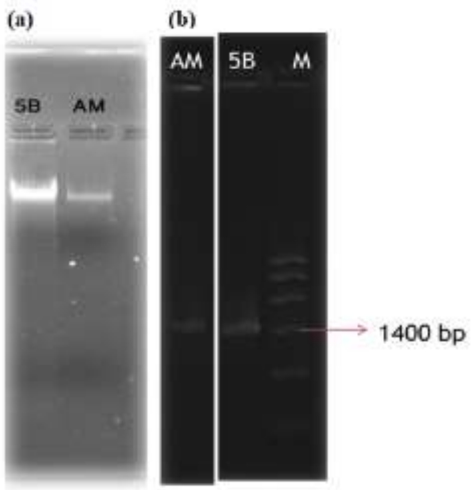

Fig 3. (a) Genomic DNA isolation (b) 16s rRNA gene amplification (Strain: 5B and AM; M – Marker; bp - Base pair)

Phylogenetic analysis of the isolates

Two bacterial laccase positive isolates were identified by molecular identification with 16S rDNA sequencing. The sequences obtained were analyzed using BLAST search to identify the corresponding Phylogenetic relatives. Based on the identity score the Phylogenetic affiliations (Firmicutes) were confirmed. The related sequences were obtained from NCBI nucleotide database and Phylogenetic tree was constructed using Neighbor-Joining method. The relationships among the sequence of related organisms computed using Maximum Composite Likelihood method. The isolates AM strain exhibited a high nucleotide sequence identity with *B. tequilensis* (99%), *B. subtilis* (99%), B. mojavensis (99%) and *B. axarquinensis* (98%) in the Blast analysis and 5B strain exhibited 99% identity with *B. subtilis and B. tequilensis*. The phylogenetic tree analysis revealed the AM strain close relation with *B. tequilensis* (99%) and 5B strain exhibited a close relation with *Bacillus subtilis*. *E. coli* 16s rRNA gene sequence was used as out group. Further the

Biochemical and morphological observation confirms the strain AM is *B. tequilensis* and strain 5B is *B. subtilis* [11]. The optimal tree with the sum of branch length = 0.26186748 is shown fig. 4. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1386 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.

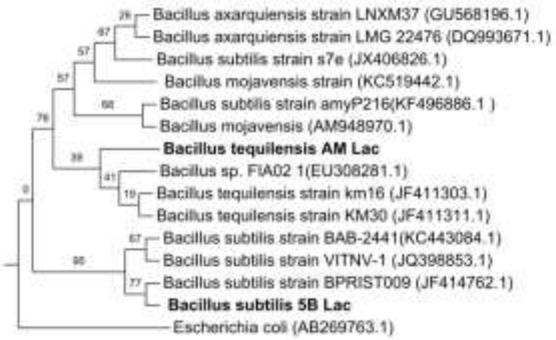

Fig 4. The Phylogenetic relationship inferred using the Neighbor-Joining method

Whole cell laccase activity assay

The laccase activity of isolated strains

The bacterial cultures were grown on Nutrient agar plates grown at 37°C used to confirm the laccase activity and to study the substrate range of the enzyme. The whole cells were used in the assay against ABTS and SGZ at pH 7.2 Phosphate buffer. The whole cells were developed color change in both ABTS and SGZ within 5 min at pH 7.2. The Absorbance spectrum of ABTS and SGZ after oxidation by whole cell shows the characteristic laccase activity in the solution [12]. Guaiacol test brown color appeared after oxidation, SGZ assay pink color develops after oxidation, ABTS assay green color develops after oxidation as shown in fig. 5.

Guaiacol test brown color appeared after oxidation, SGZ assay pink color develops after Oxidation, ABTS assay green color develops after oxidation as shown in fig. 6.

Laccase activity using SGZ

Laccase activity determination using syringaldazine. The specific activity of laccase was assayed spectrophotometrically by monitoring the absorbance increase from oxidation of syringaldazine at 530 nm (Fig. 7). SGZ assay develops pink colour after oxidation [12].

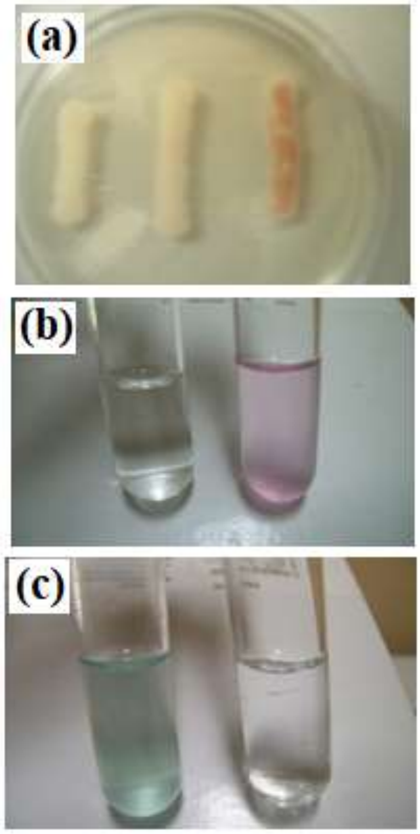

Fig. 5. Whole cell laccase activity assay, Oxidation by 5B strain grown on nutrient agar (a) Guaiacol drop assay (b) SGZ assay (c) ABTS assay

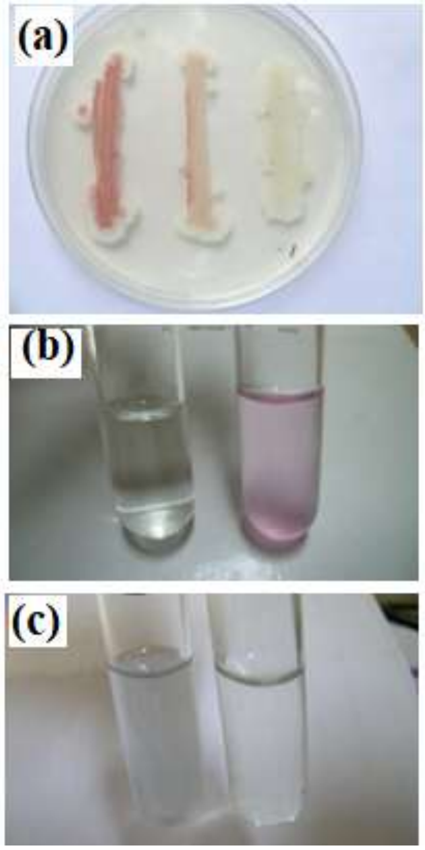

Fig. 6. Oxidation by AM strain grown on nutrient agar (a) Guaiacol drop assay (b) SGZ assay (c) ABTS assay

Laccase activity using ABTS

Laccase activity was determined by the oxidation of ABTS method. The ABTS is oxidized by laccase to the cation radical. The concentration of the cation radical responsible for the green colour and that indicates the enzyme activity [13]. Oxidation of ABTS was monitored by determining the increase in 420 nm. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank (Fig. 8).

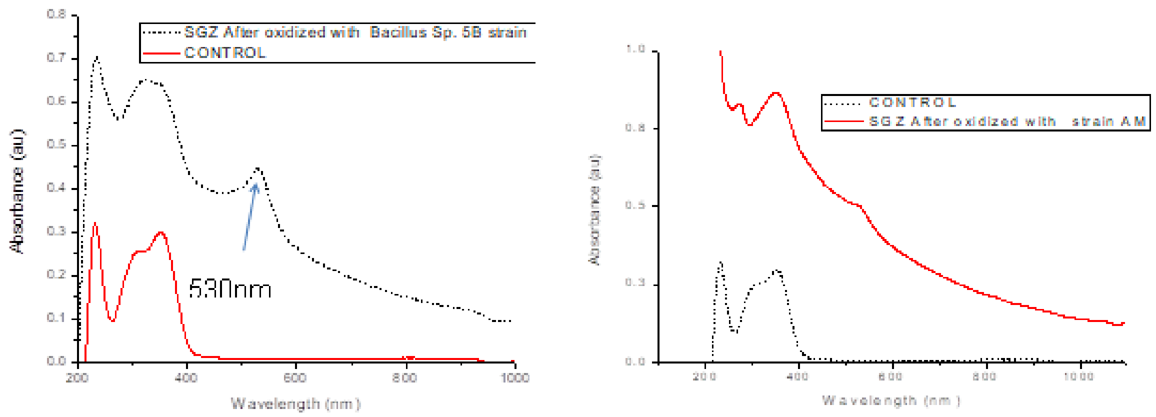

Fig. 7. Confirmation of laccase activity by isolated strains using UV absorbance spectrum of SGZ oxidized with (a) 5B and (b) strain AM

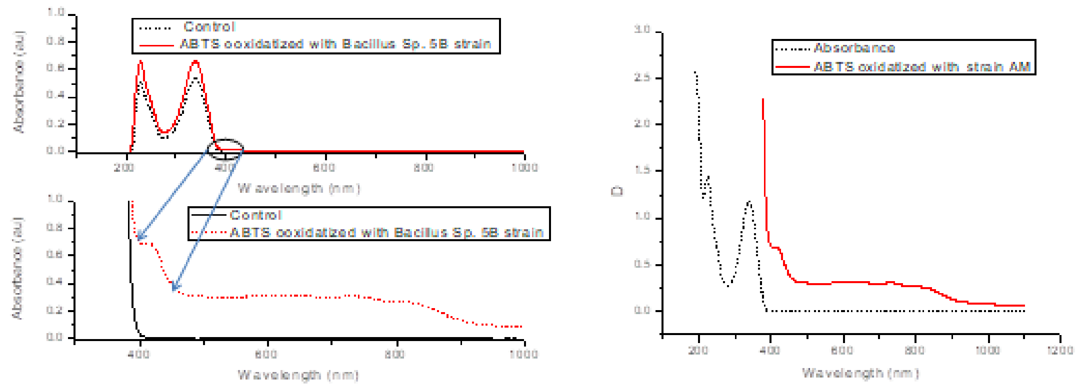

Fig. 8. Confirmation of laccase activity by isolated strains by UV absorbance spectrum of ABTS oxidized with (a) Strain 5B and (b) strain AM

SDS PAGE

The Sodium dodecyl sulphate polyacrylamide gel electrophoretic analysis of the partially purified laccase enzyme revealed the presence of different protein bands with molecular weights of 56.4 kDa respectively. The presence of two separate bands could be attributed to the fact that co-precipitation at 80% $(NH_4)_2SO_4$ saturation could have occurred (Fig. 9).

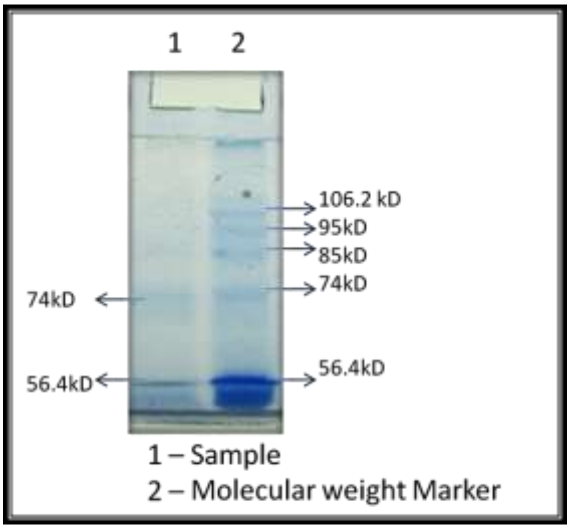

Fig. 9. SDS PAGE

Zymogram

The zymogram of the partially purified laccase enzyme revealed that the enzyme was in its active form which is seen as green color in fig. 10 [14].

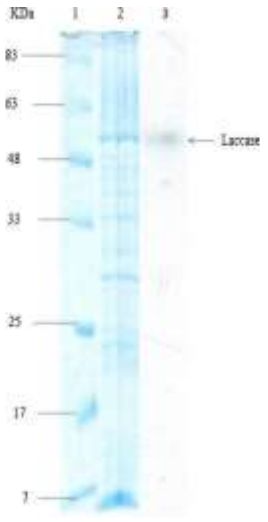

Fig. 10. Native page

Conclusions

From the present study it can be concluded that the ability of laccase to catalyze the oxidation of aromatic compound was investigated. Laccases are ubiquitious in nature, being produced by wide variety of plants, bacteria and also fungi. The function of enzyme differs from organism to organism. Laccase play an important role in carbon cycle and could also help in degrading a wide range of aromatic compound. Laccases have become important industrially relevant enzymes that can be used for a number of diverse applications such as delignification of lignocelluloses', bioremediation application such as waste detoxification, textile dye transformation, food technologic uses, personal and medical care application. The present protocol used for large production of bacterial laccases to degrade the aromatic compound and can be used without any problems.

Conflicts of Interest

Authors declare no conflict of interest.

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