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CLONING OF *BACILLUS MEGATERIUM* LACCASE GENE ISOLATED FROM TEXTILE WASTE WATER REPLENISHED SOIL

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Abstract

In the present study, 180 soil samples were collected from various sites contaminated with dye-containing waste to isolate laccase producing bacteria. On nutrient agar containing 0.001% (w/v) copper sulfate and 2 mM syringe filtered guaiacol, 41 bacterial isolates produced a brown zone indicating laccase production. A bigger brown zone of oxidation producing 5 bacterial isolates were selected to find out the one with the highest laccase production. After submerged fermentation, a strain gave 0.18 U/mL activity with 50 mM guaiacol which was the highest among all laccase producers. The 16S rRNA sequencing showed that this maximum laccase producing bacteria was *Bacillus megaterium*. Its laccase gene was isolated and cloned into pET 21a and transformed into BL21 codon + where after enzyme assay with 50 mM guaiacol it gave 0.31 U/mL activity which depicted a successful increase in the enzyme activity.

Key words: Cloning, *Bacillus Megaterium,* Waste water, Bacterial isolates

Introduction

Laccase (EC 1.10.3.3) also called oxygen oxidoreductase is benzenediol (Dong *et al*., 2023). It is a versatile multi-copper enzyme and is also known as blue oxidase (Cuprys *et al*., 2022). It is found capable of oxidizing a wide variety of phenolic as well as non-phenolic compounds including amines, di-phenols, and polyphenols (Patil *et al*., 2016; Hakulinen & Rouvinen, 2015). So, it is well known as an excellent biocatalyst playing an important role in many biotechnological applications (Bilal & Iqbal, 2019). It is highly efficient in terms of catalytic activity. The molecular oxygen (O_2) is its only co-substrate.

Laccases are generally present in various species of plants, fungi, and bacteria (Shekher *et al.*, 2011; Zhuo *et al*., 2022). The presence of laccases in bacteria was reported in 1993 for the first time. Several bacterial genera reported as laccase producers include *Bacillus* (Muthukumarasamy *et al.*, 2015), *Aquisalibacillus* (Rezaei *et al.*, 2017), *Streptomyces griseus* (Endo *et al.*, 2003), *Alteromonas* sp. (Sanchez-Amat & Solano, 1997), *Thermus thermophilus* (Miyazaki, 2005), *γ Proteobacterium*, *Pseudomonas* (Neifar *et al.*, 2016), and *Enterobacter* (Devasia & Nair, 2016) etc.

Laccase comprises four copper atoms that make an active site for the catalytic activity. Its catalytic activity extends over three steps. In the beginning, T1Cu center accepts electrons from a reducing substrate due to which type 1 copper acquires its reduced form. Then, electrons are transferred internally from type 1 copper to type 2 and then to type 3 copper complex (T2/T3). In the last stage, the molecular oxygen is reduced to water. This process occurs in the trinuclear T2/T3 cluster (Sun *et al*., 2023; Guan *et al*., 2018).

The soil from compost or the one replenished with a textile, tannery, or paper and pulp waste can be used to isolate laccase-producing bacteria. The laccase producing bacteria can be identified as they develop reddish brown zones around their colonies. The color development usually requires incubation of plates at 37ºC for a suitable time period which varies according to the metabolic activity of those laccase producers (Demissie & Kumar, 2014; Neifar *et al.*, 2016; Vignesh *et al*., 2020).

The applications of bacterial laccase are enhancing speedily because, in contrast to fungal laccase, bacterial laccase can catalyze under a diverse range of pH and temperature. It is stable against enormous inhibitory agents (Jeyabalan *et al*., 2023). Furthermore, bacterial laccase is economical in industrial applications because it is produced in a short time and is cloned easily (Prins *et al.*, 2015).

Laccase is known as a "Green catalyst" due to its environmentally friendly and biotechnologically important industrial applications (Agrawal *et al*., 2018). It can replace many chemicals in procedures like denim finishing, rove scouring, wool dyeing, and anti-shrink treatment for wool (Arregui *et al*., 2019). It also has applications in the food industry in fruit processing, and wine and beer stabilization (Moreno *et al*., 2020). It can be used as a bleaching agent in the textile, paper pulp, and food industries (Shekher *et al*., 2011). It is known to be efficient in breaking many chemicals present in wastewater from the textile, leather, and paper industries (Moreno *et al*., 2020). The overproduction of laccase from recombinant organisms has a promising role in its industrial applications. Many factors that support the cloning of the laccase gene include easy cultivation, economical production, and higher productivity (Dubé *et al*., 2008; Arregui *et al*., 2019). The cloning of bacterial laccase is preferred over fungal one as fungal cloning is a hard task due to the unavailability of sequence information, exon intron structure, disulfide bridge formation, and glycosylation of enzyme (Koschorreck *et al*., 2008). To date, several bacterial laccases have been successfully expressed in *E. coli* (Santhanam *et al*., 2011).

Material and Methods

Chemicals: In the present study analytical grade chemicals purchased from Merck (Darmstadt, Germany), Fluka (Switzerland) and Sigma Chemicals Co. (USA) were expended.

Sampling, isolation, and primary screening of laccase producing bacteria: Different locations having tanneries, textile mills, paper mills, drains, compost sites, and denim factories were selected from Sheikhupura, Faisalabad, Lahore, Kahna, and Kasur for the sample collection. From these sites, soil samples were collected from a depth of 5 cm. These soil samples were stored in sterile zipper bags at 4ºC until further processing (Wang *et al*., 2010). A serial dilution of soil samples up to 10^{-7} using a stock of soil suspension (0.1 mg/mL) was made. About 50 µL of the suitable dilutions (10^{-3}, 10^{-5}, and 10^{-7}) was used to inoculate nutrient agar plates having 0.001% (w/v) copper sulfate as an inducer and 2 mM syringe filtered guaiacol as a substrate. The inoculated plates were incubated at 37ºC for 7 days or until the formation of brown zones (indicating laccase production) around the colonies (Sheikhi *et al*., 2012). After purification by quadrant streaking and replica plating the bacterial isolates were stored by making glycerol stocks. These bacterial isolates were subjected to secondary screening to find out the best laccase producer.

Secondary screening by submerged fermentation: The method of Vantamuri & Kaliwal (2016) was adopted for the secondary screening. Inoculum for submerged fermentation was prepared by taking sterile nutrient broth having 0.0001% (w/v) $CuSO_4$ and aseptically inoculating with laccase producing bacteria. This inoculum was incubated at 37ºC and 150 rpm for 16 hours. For the submerged fermentation, sterile yeast extract dextrose copper sulfate (YPD-Cu) medium having 20.0 g/L glucose, 5.0 g/L peptone, 2.0 g/L yeast extract, and 100.0 mg/L copper sulfate was prepared. This medium was inoculated with 2% previously prepared young inoculum. The inoculated YPD-Cu medium was incubated at 37ºC and 150 rpm for 48 hours. The broth culture was centrifuged at 6000 rpm and 4ºC for 20 minutes to get a clear supernatant for the enzyme activity estimation.

Laccase activity assay: The enzyme activity was estimated according to the method suggested by Peter *et al*., (2014). For this 50 mM guaiacol (substrate), 100 mM sodium acetate buffer (pH 7.5) was employed while the enzyme activity was measured at 530 nm. The test tube marked as blank had 3 mL acetate buffer and 1 mL guaiacol. The experimental test tube had 3 mL acetate buffer, 1 mL guaiacol, and 1 mL crude enzyme/supernatant while the control had 3 mL acetate buffer and 1 mL crude enzyme/supernatant. One unit of laccase activity (U) is defined as the concentration of laccase required for the oxidation of 1 µM of substrate per minute under assay conditions (Góralczyk-Bińkowska *et al*., 2020).

The formula given below was used to evaluate enzyme activity in terms of units.

$$Enzyme\ activity\ \left(\frac{U}{mL}\right) = \frac{\Delta \mathrm{A530nm}/min \times \mathrm{Vt} \times \text{dilution factor}}{\varepsilon \times \mathrm{Vs}}$$

where

Vt= Final volume of reaction mixture (mL)

Vs= Sample volume (mL)

ε = Extinction co-efficient of guaiacol (62.5)

Microscopic examination, biochemical and molecular characterization of isolate: The bacterial strain showing maximum units was studied with the help of Gram staining (Siroosi *et al*., 2016). The biochemical characterization was done with the help of capsular staining, endospore staining, catalase test, oxidase test, and urease test (Sondhi *et al*., 2014)

After preliminary identification, the bacterial isolate was subjected to 16S rRNA sequencing. This isolate was sent to Macrogen Korea for 16S rRNA sequencing. The results obtained were subjected to analysis with nBLAST from NCBI (National Centre for Biotechnology Information). Those sequencing results were used to construct a phylogenetic tree with the help of Clustal Omega (Sheikhi *et al*., 2012).

Isolation and amplification of laccase gene: The laccase gene was isolated from the fresh overnight culture of *B.megaterium* by DNA purification kit (Thermo scientific K0721) and the isolated DNA was quantified by gel electrophoresis. The isolated gene was amplified by using forward primer 5′-CATATGAATCCTGAGCCATTAAA AAAAAGCCATCACG-3′ and reverse primer 5′-GGAT CCTTACTCCTCCTTATAGCCTATGAAGTTAAACAT GCG-3′. The restriction sites of NdeI and BamHI were added in the forward and reverse primer respectively (underlined) after thoroughly studying the laccase gene sequence in the NEB Cutter V2.0. The primers were analyzed by Oligo Calc for self-complementarity. The DNA was amplified by PCR programmed as 5 minutes at 94ºC, 30 cycles of 3 minutes at 94ºC, 30 seconds at 55ºC, and 1 minute at 72ºC while a final extension at 72ºC for 7 minutes (Lau *et al*., 2018).

Cloning of laccase gene into cloning and expression vector: For cloning of the laccase gene into sub-cloning and cloning vectors, the protocols explained by Hanahan *et al*., (1991) were followed. The amplified DNA was subcloned into PCR vector 2.1 (TA Cloning™ Kit by Invitrogen) and then transformed into DH5α. The cloned colonies were identified by blue white screening. The colony PCR was conducted to confirm the presence of the laccase gene in the transformed colonies. The plasmid was isolated from the selected colony and the presence of the laccase gene was further confirmed by restriction analysis. The double restricted gene was cloned into the pET 21a vector and further transformed into BL21 codon+. The positive colonies were confirmed by colony PCR and restriction analysis. The transformed colony was sent to Macrogen Korea for gene sequencing. The results were analyzed by nBLAST from NCBI.

Expression of recombinant laccase: The positive colonies were subjected to submerged fermentation in an LB medium. For this purpose, a colony was used to prepare inoculum in LB broth having 50 µg/mL ampicillin concentration. Further LB broth with the same ampicillin concentration was inoculated with 2% inoculum and left at 37ºC at 150 rpm until the O.D reached 0.4. The medium was induced with 0.1 mM IPTG and 500 mM $CuSO_4$ and left for 10 hours at 37ºC and 150 rpm. The cells were harvested by centrifugation at 6000 rpm for 20 minutes. The supernatant was separated and the pellet was resuspended in 500 mM Tris-Cl pH 7. The cells were sonicated for 30 minutes with 30 seconds burst and 30 seconds off to obtain total cell lysate. The total cell lysate was separated after centrifugation and the remaining solid fraction was again resuspended in 500 mM Tris-Cl pH 7 and marked as insoluble fraction. These three fractions were subjected to enzyme assay (method described earlier) and SDS PAGE to get insight into the protein expression. For SDS PAGE all the buffers, solutions, and gels were prepared according to Simpson, (2006). The SDS PAGE was performed on 12% resolving and 5% stacking gels. The samples were prepared by boiling and denaturing them for 5-10 minutes along with protein loading dye. The prepared samples were loaded into the wells of pre-set Bio-Rad Mini PROTEAN Tetra Cell along protein molecular weight marker. The protein bands were visualized by staining with Coomassie Brilliant Blue R 250.

Results

Isolation and primary screening of best laccase producer: Among 93 bacterial isolates obtained from the soil samples, 41 produced reddish-brown zones by oxidizing guaiacol. The five bacterial strains (LI-16, LI-23, LI-26, LI-81, and LI-80) were selected for secondary screening as they produced a maximum zone of oxidation as shown in Fig. 1. The isolation of laccase producing bacteria from the industrial waste dumping spots indicates the presence of its substrate in the soil. The presence of dyes is the common thing among soil samples from the leather, paper, and textile waste dumping sites. These dyes include a wide variety of phenolic and non-phenolic compounds (Unuofin *et al*., 2019; Zhai *et al*., 2020). These dyes are degraded by the laccase producers which makes them important for the biodegradation of environmentally harmful compounds commercially (Chauhan *et al*., 2017; Singh *et al*., 2014). The production of reddish brown hallows around the colonies of the bacterial isolates represents the extracellular nature of the laccase (Mehandia *et al*., 2020).

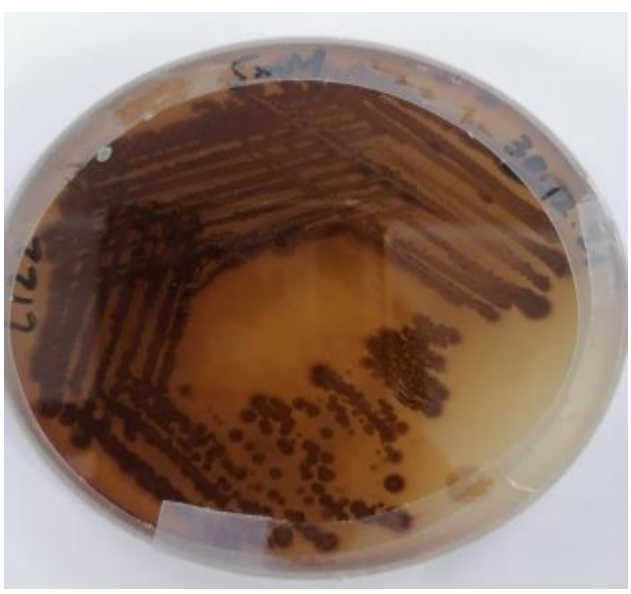

Fig. 1. Reddish-brown zones of oxidation produced by LI-81.

Secondary screening: Submerged fermentation and enzyme activity assay showed that LI-81 had 0.12U/mL activity which was the maximum enzyme activity shown among all other isolates as depicted in the Fig. 2. The submerged fermentation is ideal for the bacterial growth due to simple and easier maintenance. Above all, it is easier to observe and control all the parameters for the optimization (Subramaniyam & Vimla, 2012).

Microscopic examination, biochemical and molecular characterization: The observations of microscopic and biochemical characterization are shown in the below Fig. 3 and Table 1. The 16S rRNA sequencing confirmed the LI-81 to be *Bacillus megaterium* also known as *Priestia megaterium* with 99.57% similarity. The sequence of LI-81 was used along with other correlated bacterial sequences (fetched from NCBI BLAST) to create a dendrogram by using Clustal Omega. This dendrogram also confirmed the isolated strain to be *Bacillus megaterium* shown in Fig. 4.

Gene amplification by PCR: The 822bp long laccase gene fragment was successfully amplified by PCR. Its size was confirmed by gel electrophoresis which is shown in Fig. 5.

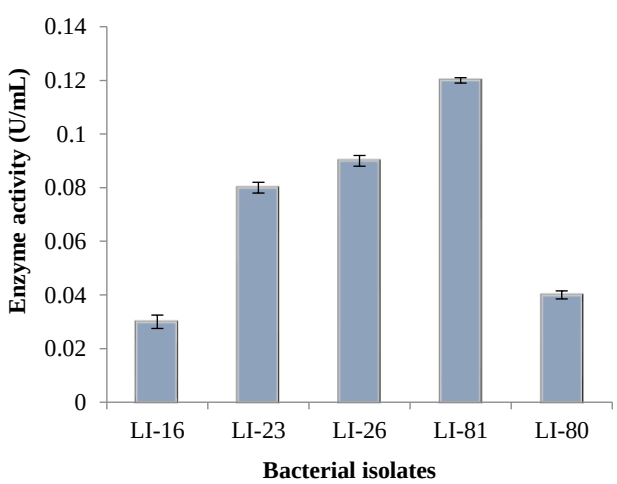

Fig. 2. Graphical representation of results observed after the secondary screening. Standard deviation (±S.D) is shown by error bars for the parallel triplicate experimental reactions differing significantly at $p \leq 0.05$.

Table 1. Cultural, morphological, and biochemical characteristics of LI-81.

Sr. No.	Characteristics	LI-81
	Colony characteristics	
1.	Color of the colony on nutrient agar (without guaiacol)	White
2.	Color of the colony on nutrient agar (with guaiacol)	Reddish-brown
3.	Shape of colony	Circular
4.	Margin	Round
	Morphological characteristics	
5.	Gram's reaction	+ve
6.	Shape	Rod
7.	Capsule staining	+ve
8.	Endospore staining	+ve
	Biochemical characteristics	
9.	Catalase test	+ve
10.	Urease test	-ve
11.	Oxidase test	-ve

Cloning of laccase gene into cloning and expression vector: The amplified laccase gene was cloned into PCR vector 2.1 (cloning vector) which was further transformed into DH5α. The transformed colonies were picked by the means of blue white screening and were subjected to colony PCR. The white transformed colonies were tested among blue ones which worked as a control. The results of colony PCR are given in Fig. 6A. Further the plasmid from the positive colony was isolated and the presence of the laccase gene was reconfirmed by restriction of the plasmid. The PCR vector 2.1 had 3929 bp and the laccase gene had 822 bp length. The PCR vector 2.1 and laccase gene collectively had 4751 bp length. After restriction with BamHI, a band was obtained at 4751 bp which confirmed the presence of the laccase gene in the vector (Fig. 6B). The plasmid was isolated from the positive colony and was subjected to double restriction by BamHI and NdeI (Fig. 7) and was cloned into double restricted pET21a vector. That ligated vector was transformed into BL21 codon+. Again the cloning was confirmed by colony PCR whose results are shown in Fig. 8A and restriction analysis of the isolated plasmid. The vector had a 5443 bp length and with the laccase gene insert of 822 bp it got a 6265 bp length which was confirmed by the gel electrophoresis results (Fig. 8B). The gene sequencing results confirmed the presence of laccase gene in the cloned organism. The results show 99.5% similarity with *Bacillus megaterium* which is also known as *Priestia megaterium.*

Expression of recombinant laccase: The enzyme activity carried out after submerged fermentation showed that the recombinant organism had 0.31 U/mL enzyme activity with guaiacol, which was 0.18 U/mL as exhibited by the wild *B. megaterium*. It shows the successful attempt to increase the production of the enzyme. Almost the same units were reported by Koschorreck *et al*., (2008) who introduced the laccase gene from *Bacillus licheniformis* into pET22 which was further transformed into *E.coli* BL21 (DE3). Their clone gave 0.37 U/mL activity with ABTS as a substrate. Lončar *et al*., (2013) cloned the laccase gene from *Bacillus amyloliquefaciens* into BL21 by ligating it into pET 21a. They reported 0.235 U/mL after cloning which is less than the units reported in the present research.

The SDS PAGE results are given in the Fig. 9 which confirm the expression and production of laccase enzyme by the recombinant organism. The laccase enzyme protein has 30.7 kDa size and the protein band against the same height confirms the expression of the protein. The picture of the gel clearly shows the intracellular nature of the enzyme as it is not present in the lane of the supernatant. However, the presence of the 30.7 kDa protein band in the second lane shows the presence of the laccase enzyme in total cell lysate (TCL). A thicker band of laccase can be seen in the insoluble fraction which can be solubilized by employing various optimization strategies.

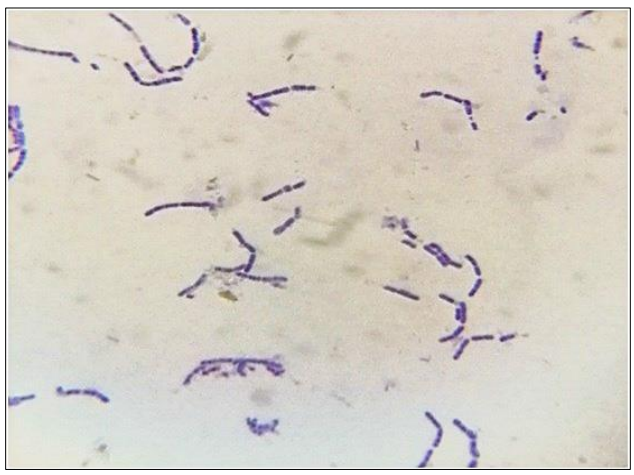

Fig. 3. Gram staining image of LI-81.

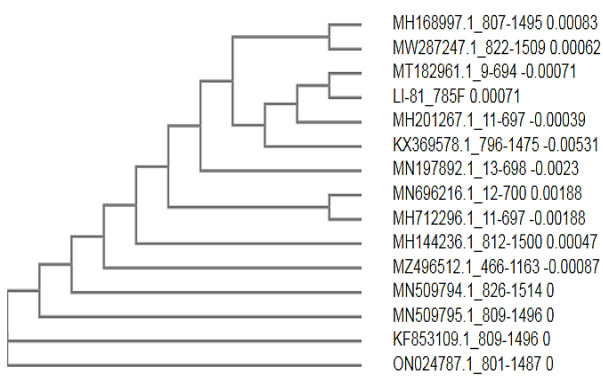

Fig. 4. Dendrogram of *B. megaterium* (LI-81) with other closely related bacterial species.

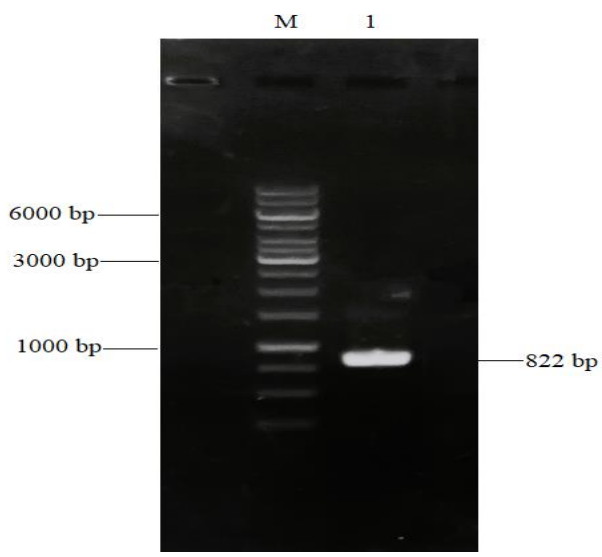

Fig. 5. A clear band of amplified DNA at 822 bp height.

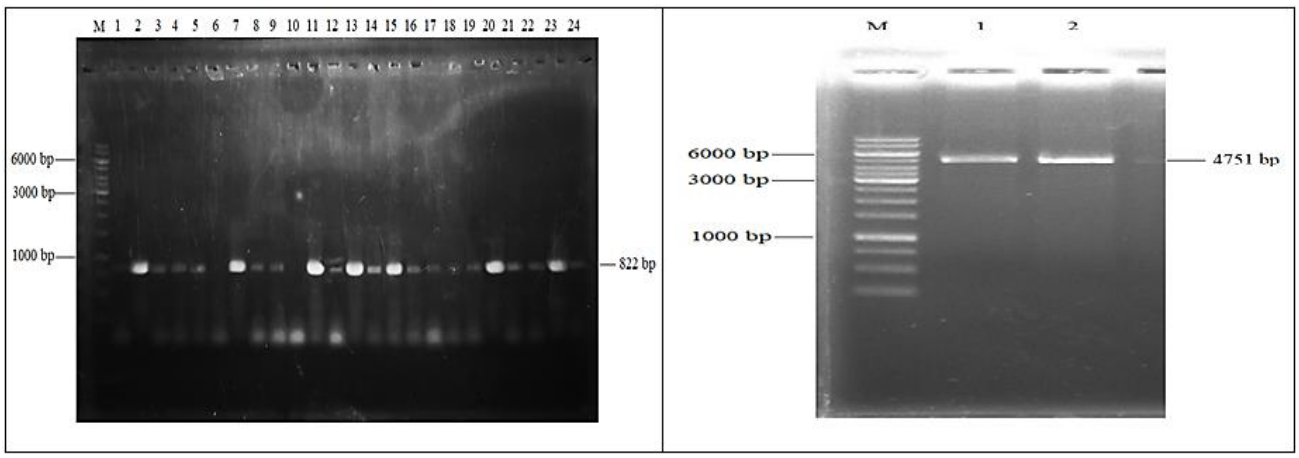

Fig. 6. **A**: Colony PCR of laccase gene cloned into PCR vector 2.1 **B**: The laccase gene containing PCR vector 2.1 restricted with BamHI.

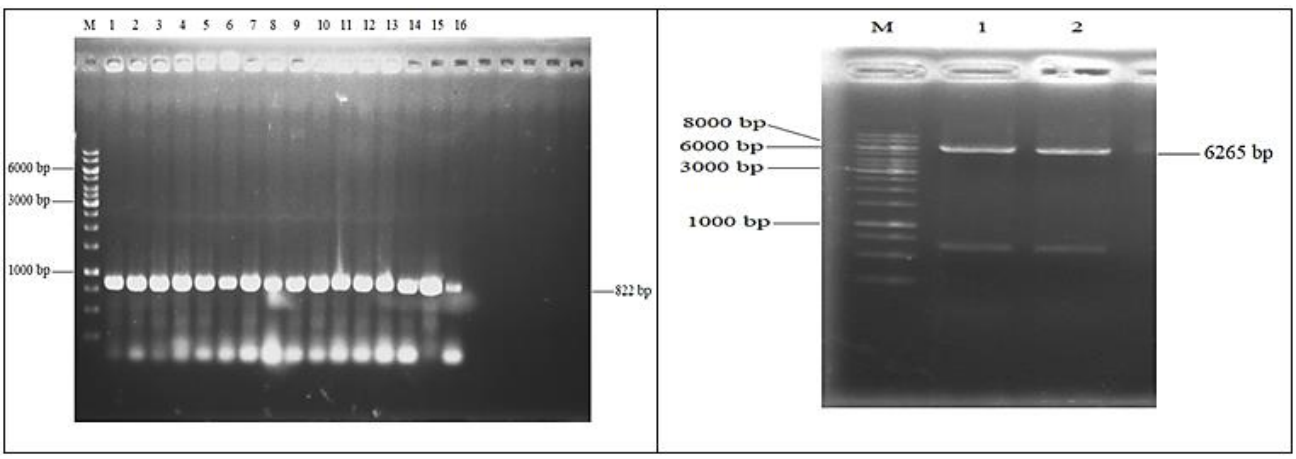

Fig. 8. **A**: Colony PCR of laccase gene cloned into pET21a; **B**: The laccase gene containing pET21a restricted with BamHI.

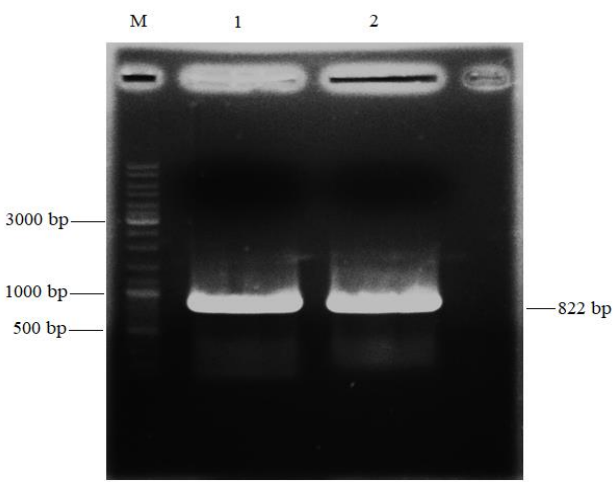

Fig. 7. The DNA fragment obtained after double restriction of PCR vector 2.1 containing laccase gene with NdeI and BamHI.

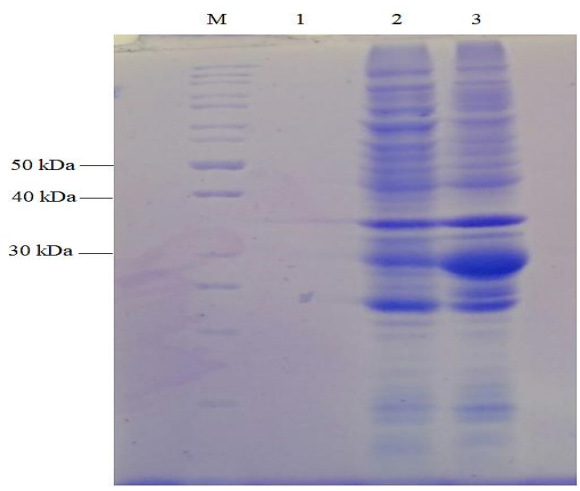

Fig. 9. SDS PAGE gel of laccase production by recombinant BL21 codon+ **M:** protein marker, **1:** supernatant, **2:** total cell lysate, **3:** pellet/insoluble fraction obtained after sonication.

Conclusion

A novel laccase producing bacterium *Bacillus megaterium* was isolated from the soil replenished with textile dye waste water which had good oxidizing abilities. Its laccase gene was successfully cloned into BL21 codon+ and a clear increase in the oxidation ability was observed. However, the SDS PAGE showed more protein trapped in the insoluble fraction which needed to be solubilized. In the future, optimization of various parameters can increase the amount of soluble protein which can have a fair role in textile dye degradation and can have a promising role in reducing the water pollution caused by the textile industries.

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