

CLONING AND EXPRESSION OF LACCASE GENE PRODUCED FROM *Bacillus subtilis* ZHR IN *E. coli*

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ABSTRACT

Ten isolates of Laccase producing bacteria were obtained from 50 isolates belongs to *Bacillus* sp. These isolates were isolate from different local areas of soil in baghdad. The isolates were subjected to primary screening , and revealed high production of enzyme based on consumed time during the reaction with syringaldazine (SGZ) reagent at 37 ° C. These isolates were subjected to secondary screening. The results revealed that isolate 136 was the best producers with highest enzyme activity of 47.0 U/ml. Identification of this isolate was achieved by studying morphological, microscopic characteristic , Vitek2 compact system and studying gene sequencing analysis of 16S rRNA gene .The results revealed that the isolate was belongs to *Bacillus subtilis* and identified as *Bacillus subtilis* ZHR. The laccase gene from *Bacillus subtilis* ZHR was extracted and amplified by PCR technique, then cloned in expressed *Escherichia coli* to produce biologically active enzyme with enzyme activity of 108.6 U/ml and specific activity 332.14 U/mg .

Key Words: Isolation, Gene identification, vitek2 compact system, 16S rRNA, PCR technique,
*Part of Ph.D dissertation of the first author

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مجلة العلوم الزراعية العراقية - 2018 : 49 (5): 879-886

كلونة وتعبير جين اللاكيز Laccase المنتج من بكتريا *Bacillus subtilis* ZHR في بكتريا *E. coli*

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المستخلص

امكن الحصول على 10 عزلات بكتيرية منتجة لأنزيم Laccase من أصل 50 عزلة والتي تعود إلى جنس *Bacillus* من نماذج ترب مختلفة من مناطق محلية في بغداد وأخضعت هذه العزلات لعملية الفريلة الأولية إذ تميزت 10 عزلات بإنتاجيتها العالية للأنزيم بدلالة الوقت المستهلك للتفاعل مع كاشف السرينكلدايزين (SGZ) على درجة حرارة 37 م . اخضعت تلك العزلات لفريلة ثانوية فتبين ان العزلة 136 هي الاكفأ إذ بلغت الفعالية الانزيمية 47.0 وحدة/مل. تم تشخيص العزلة من خلال دراسة الصفات المظهرية والمزرعية والمجهرية والتشخيص بجهاز Vitek2 compact system وكذلك دراسة تحليل التتابعات لجين 16S rRNA . اظهرت النتائج ان العزلة تعود إلى بكتريا *Bacillus subtilis* . تم عزل وتضخيم جين اللاكيز بتقنية PCR من العزلة المنتخبة *Bacillus subtilis* ZHR وتمت كلونته في بكتريا *Escherichia coli* التعبيرية بشكل فعال بايولوجيا ليسجل فعالية انزيمية بلغت 108.6 وحدة/مل وفعالية نوعية 332.14 وحدة/ملغم .

الكلمات المفتاحية: عزل، التشخيص الجيني، جهاز الفايترك2، 16S rRNA، تقنية PCR

*البحث مستل من اطروحة دكتوراه للباحث الأول

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper-containing enzymes that can catalyze the oxidation of an extensive range of phenolic and non-phenolic aromatic compounds, along with the reduction of molecular oxygen to water (7, 22). Laccases belong to the multicopper oxidase family, which typically contains four copper atoms: a type I copper (blue copper), a type II copper, and a pair of type III copper centers (23). Their extensive substrate range makes laccases excellent candidates for various industrial and biotechnological applications, such as biological bleaching in the pulp and paper industry, textile dye decolorization, construction of biosensors for detecting phenolic pollutants, detoxification of recalcitrant environmental pollutants, and bioremediation (19). Laccases are widely distributed among fungi, higher plants, and bacteria (3). To date, most laccases studied are of fungal origin, and only fungal laccases are used in industrial processes (22). However, fungal laccases are usually unstable at high temperatures and alkaline conditions. This characteristic limits their practical applications. Although bacterial laccases have a lower redox potential than fungal laccases and are less frequently investigated, they are more stable to high temperatures and a wider pH range, less dependent on metal ions, and less susceptible to inhibitory agents. Thus, bacterial laccases have significant potential in various industrial applications (21). Furthermore, bacterial laccases are suitable for over production in *Escherichia coli* and their expression level, stability, and catalytic properties are considerably easier to improve by directed evolution compared with their fungal counterparts (2). The bacterial laccase was first discovered in 1993 in the *Azospirillum lipoferum* bacteria and was then discovered in several species of the bacteria (5). The best studied bacterial laccase is CotA, i.e., the endospore coat component of *Bacillus subtilis*. CotA participates in the biosynthesis of brown spore pigment, which is also considered to be a melanin-like product (6), and appears to be responsible for the protection afforded by the spore coat against

ultraviolet light and hydrogen peroxide (4, 13). The main purpose of this study is to reduce the production time and cost by achieving over production of laccase enzyme through cloning the laccase gene from *Bacillus subtilis* ZHR to *E. coli* BL21(DE3).

MATERIALS AND METHODS

Isolating the Laccase Producing Strain

The laccase producing bacterial strain was isolated from soil collected from Different areas in City of Baghdad, Iraq. A sample of 10 g soil was added to 90 mL sterile Distilled Water. Serial dilutions were done for each sample using sterilized D.W. then 0.1 ml aliquot from appropriate dilution was taken, spread on nutrient agar plates, and incubation at 37 °C for 48h (1). Then, isolation was carried out via standard serial dilution plate technique. The colonies that exhibit *Bacillus* sp. morphological characteristics were selected and transferred to new plates containing Luria-Bertani (LB) medium supplemented with 0.2 mM CuSO₄ and incubated at 37 °C for 5 days for Primary Screening of laccase producing strains. It was performed by adding 2-3 drops of syringaldazine SGZ (0.5 mM) as a detecting reagent on bacterial colonies of each isolate grew on the plates for checking its capability to produce laccase (27). The time consumed by each isolate to develop pink color was measured (table 1). The bacterial isolates which consumed less time to display pink color were respread on new Petri plates containing (LB) medium supplemented with 0.2 mM CuSO₄ and incubated at 37 °C for 5 days for the secondary screening. The cells were harvested from the agar plate with 5ml of 1M KCl, collected by centrifuge, then washed with 0.5 M NaCl, and suspended in 1 ml of 0.5 mM potassium phosphate buffer (pH 7) (17). Finally, 1 ml cell suspension from each selected isolate was used to measure its laccase activity. The bacterial isolate that exhibited highest laccase activity was used for further studies.

Laccase activity assay

The reactive mixture (3 ml) : 2.4 ml of phosphate buffer (0.5 mM, pH 7), 500 µl of syringaldazine (0.5 mM) and 100 µl of cell suspension (26). Laccase activity was determined for each selected isolate at 25°C

using syringaldazine as the substrate by this formula:

$$\text{Laccase Activity (U/ml)} = A \times V / t \times \epsilon \times l \times v$$

A: Absorbance at 525 nm

V: Total mixture volume (ml)

v: Enzyme volume (ml)

t: Incubation time (3 min)

ϵ : The extinction coefficient of substrates ($\epsilon_{525} = 65,000$ (SGZ) in units of L/(mole.cm), and l: is the cuvette diameter (1cm).

One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute. (10,26). =

Identifying the Strain

The selected isolate with high laccase activity was identified based on its morphological and biochemical characteristics. The morphological characterization involved culturing the isolate on NA plates for studying the appearance of the colonies, following that, cells shape, Gram and Malachite green reaction were studied. Vitek 2 compact system test was used to determine biochemical characteristics of the isolate. Genomic DNA of the isolated strain was extracted using the bacterial DNA extraction kit (Solarbio Co.). The 16S rRNA gene was amplified through primers p1 forward (5' AGAGTTTGATCCTGGCTCAG 3') and p2 reverse (5'ACGGTTACCTTGTTACGACTT3')(8). Amplification was performed by Polymerase chain reaction (PCR) with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1.5 min, and 72 °C for 45 s, and a final extension at 72 °C for 5 min and the Laccase gene was amplified through forward primer p3 (5'ATGACACTTGAAAAATTTGTGGATGCTCTCCC3') and reverse primer p4 (5'CTATTTATGGGGATCAGTTATATCCATCGG3')(29). Amplification was performed by initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 1.5 min, and 72 °C for 45 s, and a final extension at 72 °C for 5 min

Electrophoresis for amplification products

Preparation of Agarose Gel:

The gel was prepared at 1% concentration by dissolving 0.5 g of agarose powder in 50 mL of TBE 1x solution and heat in microwave oven for 1 min followed by the addition of 5 μl of Goldview dye. The previous mixture was

poured into the casting tray after placing the sample comb in its designated place and leaving it to solidify completely. The casting tray was transferred after gel solidification in to the gel tank followed by the removal of sample comb and the addition of TBE 1x solution to cover the surface of the gel (20).

Preparation of the sample

Five microliter of each amplification product was added in addition to the Ladder 1Kb plus (Solarbio Co.) to determine the apparent band sizes

Operation of the device:

The poles of the relay device are set and the power supply is operated after programmed at 60 mA and 100 V, for one hour. After the period has passed, The gel was transferred to the UV-Light transilluminator device to detect the bands locations. The outputs of the amplified gene were sent to the Korean company Macrogen to determine the sequences of the nitrogen bases. The BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used for the sequence similarity search with the standard program default. Multiple sequence alignment and data analysis were performed using the software package MEGA version 6.0 (25).

Molecular Cloning of *B. subtilis* ZHR Laccase Gene

Polymerase chain reaction (PCR) product for Laccase gene was purified from agarose gel using the gel extraction kit (Solarbio Co.). The purified products were inserted into the pMD20 -T vector using the DNA Cloning Vector kit (Solarbio Co.), and then transformed into *E. coli* DH5 α competent cells. Positive white colonies from Blue White Screening were selected on the LB/Amp/X-gal/IPTG plates and validated through PCR assay to ensure the presence of recombinant plasmid pMD20-Laccase gene. The recombinant plasmid was extracted by plasmid extraction kit (Solarbio Co.). Then the product was purified using the gel extraction kit, and sequentially digested by Hind III and Bam HI. The resulting product that contain the laccase gene separated from the pMD20 plasmid was purified from agarose gel by gel extraction kit, and subcloned into digested pET28a+ expression vector (with Hind III and Bam HI restriction enzymes), by the DNA

Cloning Vector kit. This recombinant plasmid, called pET28a+ -laccase, was transformed into *E. coli* BL21 (DE3) to express laccase enzyme the cloned cells were cultured on the LB/Kanamycin plates and validated through PCR assay to ensure the presence of recombinant plasmid pET28a+ -laccase gene. The bacteria were cultured in LB medium supplemented with kanamycin 50 ug/ml with vigorous shaking (220 rpm) at 37 °C until the optical density at 600nm reach 0.6-0.8. The induction was performed by adding IPTG with final concentration 1 mM and supplemented with 0.2 mM of CuSO₄ the time of induction was 4h at 37 °C The harvested cells were suspended in lysis buffer, then the cells were sonicated by using 6-8 cycle of 10 s strokes with 30 s rest, and centrifuged at 12,000×g for 30 min. The supernatant used as crude enzyme (8).

RESULTS AND DISCUSSION

Isolating and Identifying the Strain: Ten different isolates exhibiting high laccase activity were obtained from the soil samples. One isolate, was selected for further study, and it was Gram positive, sporeforming, and had motile long rods (Fig.1). This colony could oxidize SGZ within 22 s with enzyme activity 47 unit/ml (table.1) Several studies have indicated the successful use of the reagent method in primary and secondary screening to isolate the microorganism species producing laccase. Mishra and Srivastava (16) indicated the ability of *Bacillus subtilis* MTCC 1039 to produce laccase by culturing the bacteria on a solid medium supplemented with guaiacol as a substrate, in which the researcher observed the oxidation of guaiacol by forming a reddish brown region on the selected isolate. Another study done by Rajeswari and Bhuvanewari (17) detect the ability of 22 isolates to produce laccase enzyme when cultured on a solid medium supplemented with guaiacol as a substrate. Six isolates showed reddish brown color around the developing colonies. These results are consistent with Allos and Hussein (1) study, that 17 isolates of *Bacillus* species were produce laccase enzyme from 39 isolates grown on the solid LB medium and showed a pink color after dropping the Syringaldazine solution on each isolate. Wang (27) reported that the use of Syringaldazine for the initial

detection of laccase producing bacteria showed that 46 isolate from 400 strain grown on the M9 medium showed different ability to interact with Syringaldazine and that only one isolate *B.subtilis* WD23 was characterized by high ability to produce Laccase enzyme.

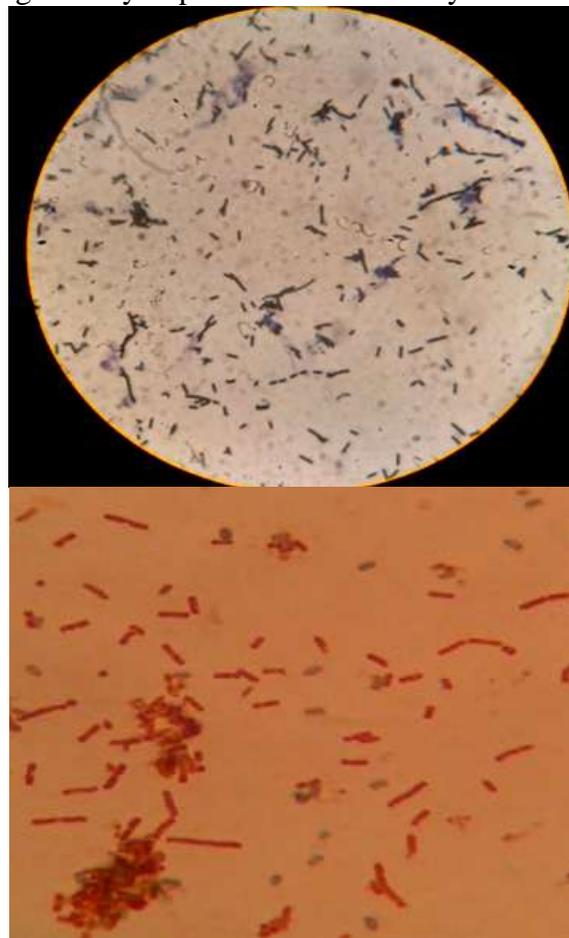


Fig 1. The selected isolate Microscopic characteristics (sporeforming ,Gram positive)
Table 1. The time consumed to oxidized (SGZ) reagent and enzyme activity for each selected isolates

Enzyme activity U/ml	Time consumed to oxidized (SGZ) reagent (s)	isolates No.
41	30	38
33	33	41
25	34	45
39	35	49
21	35	60
29	34	104
37	35	123
42	34	133
47	22	136
35	32	143

The Vitek 2 compact system test and the 16S rRNA sequence of the selected strain showed it belongs to *Bacillus subtilis*. The Vitek 2 Biochemical test showed 97% similarity to *B. subtilis*, The 16S rRNA gene was sequenced and the BLAST search indicated that this

strain belonged to the genus *Bacillus*.(Table.2).The isolated strain was finally identified as *B. subtilis* ZHR (GenBank no. MG735442.1).based on the results of the morphological ,observation, physiological, and biochemical tests, as well as the 16S rRNA sequence analysis .The *B. subtilis* ZHR laccase gene consists of Approximately 1,542 bp (Fig.2) , which encodes a protein of 513 amino acids (13, 21, 22). The sequence of the laccase gene from *B. subtilis* ZHR Showed identity between 85-94% with *B. subtilis* laccase gene at the BLAST search results (Table. 3). Previous studies have differed in the determination of the molecular size of the laccase gene due to the different sources of the gene in terms of the type of organism produced, the variation in the environmental

conditions in which the organism grew in , as well as the differences in the structural structure of the enzyme. Yadav et al(28) noted that the molecular size of the laccase gene isolated from *Streptomyces coelicolor* A3 was 1029 base pairs when studying the possibility of cloning laccase gene in *Pichia pastoris* cells while Sun et al(24) found that the molecular size of the isolated laccase gene of the *Bacillus vallismortis* fmb 103 bacteria had reached 1542 bp in an attempt to clone the gene into *E. coli* BL21 bacteria. Returning to the results of the study, the molecular size of the *Bacillus subtilis* ZHR has reached Approximately 1,500 bp , as compared with *Bacillus coagulans* LMCO and *Bacillus pumilus* DSM 27 bacteria with molecular size 1566 and 1530 bp (9,18) respectively.

Table. 2. Identity Percentage of 16S rRNA gene nitrogen bases sequences of NCBI strains with the 16S rRNA gene sequencing of *Bacillus subtilis* ZHR

Strain	Identity (%)	Accession No.
<i>Bacillus subtilis</i> 263XG6	99	KF818638.1
<i>Bacillus subtilis</i> JSD	99	KT894724.1
<i>Bacillus subtilis</i> Gy11	99	KP876486.1
<i>Bacillus subtilis</i> 264AY1	99	KF836541.1
<i>Bacillus subtilis</i> 261AY2	99	KF811042.1
<i>Bacillus subtilis</i> DX2	99	KJ499783.1
<i>Bacillus subtilis</i> amyP216	99	KF496886.1
<i>Bacillus mojavensis</i> A131	99	KC519426.1
<i>Bacillus subtilis</i> HPS 8	99	JQ308559.1
<i>Bacillus subtilis</i> OL-13	99	HQ202817.1

Table. 3. Identity Percentage of Laccase gene nitrogen bases sequences of NCBI strains with the laccase gene sequencing of *Bacillus subtilis* ZHR at NCBI

Strain	Identity (%)	Accession No.
<i>Bacillus subtilis</i> CICC20613	94	JN043511.1
<i>Bacillus subtilis</i> X1 (cotA)	86	KC751428.1
<i>Bacillus subtilis</i> BJ3-2	87	CP025941.1
<i>Bacillus subtilis</i> YG011	86	KF658461.1
<i>Bacillus subtilis</i> ATCC 21228	85	CP020023.1
<i>Bacillus subtilis</i> QB928	85	CP003783.1
<i>Bacillus subtilis</i> CW14	85	CP016767.1
<i>Bacillus subtilis</i> WD23	85	GQ184294.1
<i>Bacillus subtilis</i> CotA	85	U51115.1
<i>Bacillus subtilis</i> TMR	85	KM088071.1

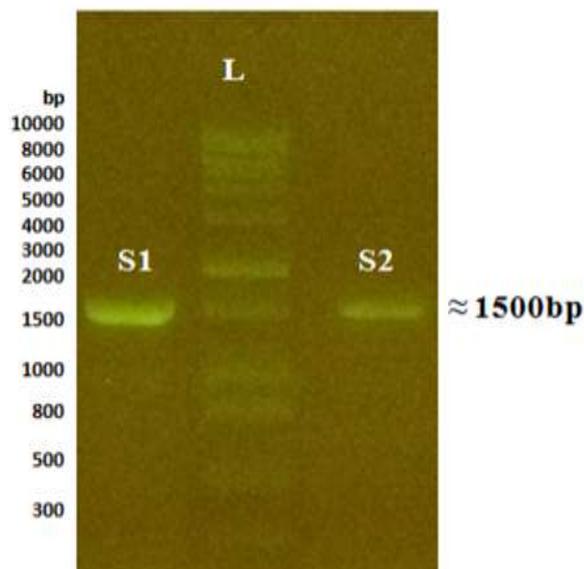


Fig 2. PCR amplification product of laccase gene on Agarose gel (1%) , L is 1Kbp ladder Cloning of the *B. subtilis* ZHR Laccase Gene into *E.coli*: The *B. subtilis* ZHR laccase gene was purified from agarose gel by gel extraction kit then inserted into the pMD20 -T vector and transformed into the competent cell of *E. coli* DH5 α the Positive clones was discovered by Blue – White Screening the Positive white colonies were selected on the LB/Amp/Xgal/IPTG plates for having the recombinant plasmid pMD20 - Laccase gene (Fig.3). The recombinant plasmid was extracted and purified from the agarose gel and digested from specific restriction sites by restriction enzymes Hind III and Bam HI, respectively the two band that appered on agarose gel were pMD20 with \approx 2600 bp and laccase gene \approx 1600 bp compared with 1 Kb ladder(Fig. 4).



Fig. 3. Blue – White Screening for *E. coli* DH5 α .the white colonies contain the recombinant plasmid pMD20 - Laccase gene

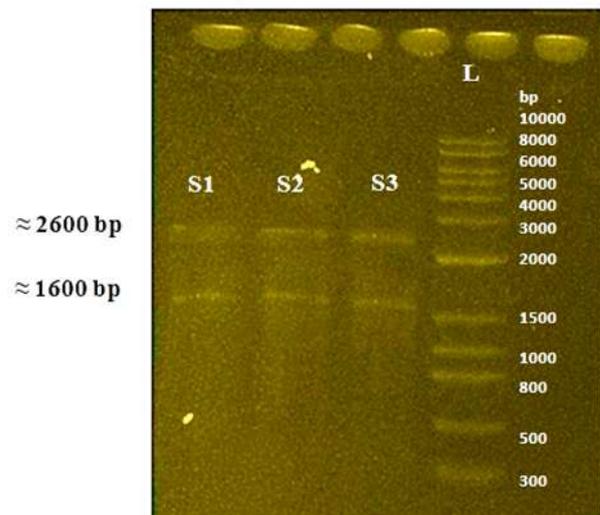


Fig. 4. The digestion products of recombinant plasmid pMD20 - Laccase gene by Hind III and Bam HI

Expressing of Laccase gene

The purified and digested laccase gene was subcloned into the digested pET28 a+ expression vector. The recombinant plasmid, called pET28 a+ -laccase, was transformed into *E. coli* BL21 (DE3) to express laccase enzyme .The colonies that contain the recombinant plasmid were used for the production of intracellular recombinant laccase enzyme. The laccase activity was measured and it was 108.6 U/ml and specific activity 332.14 U/mg. The broad range of practical applications of laccase enzyme and the increasing desire to obtain the species that mimic the needs of these applications have contributed to increased studies to find the appropriate strategy for laccase over production like cloning Technique. The results of this study are consistent with Zeng et al(30) research when studying the possibility of cloning the *Bacillus subtilis* laccase gene by using the digested plasmid pEASY-T1 by *NheI* and *HindIII* restriction enzyme to provide sticky ends to bind the laccase gene with pET28a + plasmid before being transferred to *E. coli* BL21 (DE3) expression cells ,so as Lončar et al(11) who Succeeded to clone laccase gene from *Bacillus licheniformis* ATCC 9945 using the digested expression plasmid pBadNdeIHis with *NdeI* restriction enzyme. The results were also consistent with what was indicated by Mathews et al(14) that the cloning of laccase enzyme produced by *Paenibacillus glucanolyticus* SLM1 bacteria , after ligating the digested laccase gene and

expression plasmid pET22b by in the *Bam*HI and *Xho*I enzymes with T4 DNA ligase Was a successful method to produce laccase in *E. coli* XL1-Blue cells. Menaka et al(15) reported that the positive colonies form blue white screening were selected as evidence for successful cloning process of the *Bacillus subtilis* laccase gene using pTZ57R / T plasmid in the *E. coli* DH5 cells, while Lončar et al(12) used pJET plasmid to subcloning the *Bacillus amyloliquefaciens* laccase gene by digesting the gene and the plasmid by *Nde*I and *Eco*RI enzyme . The gene was attached to the pET-21a expression plasmid before it was inserted into the *E.coli* BL21 (DE3) expression cells.

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