Optimal Conditions for Spore-bound Laccase Production from Local Isolate of *Bacillus subtilis**

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Abstract

Fifty seven of bacterial isolates were isolated from different soils in different sites of Al Diwaniyah and Baghdad in Iraq using nutrient agar medium. The primary screening of bacterial isolates was done on nutrient agar by dropping a few drops of syringaldazine on bacterial colonies proved that, only 10 isolates were developed a pink color within 15 seconds. The secondary screening was done on nutrient broth using syringaldazine as enzyme substrate proved that the isolate 54 developed the highest spore-bound laccase activity (12.8) U/ml. The biochemical tests of bacterial isolate with highest laccase activity showed that the bacteria are *Bacillus subtilis*. The optimization studies revealed that the maximum laccase production was achieved when the production medium were at the following conditions: 3 days of incubation, temperature 37 ˚C, pH 7.0, copper sulphate 0.2 mM, glucose as carbon source 3%, Tryptone as nitrogen source 0.2% , Kcl 1mM and pyrogallol 1mM as enzyme inducer , after these conditions the spore-bound laccase activity was (439.23) U/ml.

***The research is a part of M.sc. thesis in the case of the first researcher**

Introduction

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2.) is a multi copper blue oxidase capable of oxidizing ortho- and para-diphenols an aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical [3]. In most cases laccases are monomeric glycoproteins contain around 500 amino acids with molecular weights in the range 60–85 kDa, depending on the carbohydrate content [7]. Laccases were widely distributed among plants, fungi [8] and bacteria [1]. CotA, which is the endospore coat component of *Bacillus subtilis*, was the most-studied bacterial laccase [5]. Since spores allow microorganisms to survive under drastic conditions, spore coat enzymes might also withstand high temperatures or extreme pH values. Since most fungal laccases are unstable at pH values higher than 7.0, their detoxification efficiencies for pollutants often decrease under alkaline conditions. This limits the industrial potential of fungal laccase as many processes are performed in alkaline conditions. Alternatively, spore laccases which were active in the alkaline pH range could be used for bioremediation or application in membrane reactors [4].

This study aimed to find suitable supplies that can be incorporated in the culture medium and determined the favorable conditions for production of laccase enzyme with high activity.

Material and methods

Nutrient agar, (Syringaldazine, SGZ), and all other reagent grand chemicals were purchased from Hi – Media and Sigma-Aldrich, India.

Soil samples collection and *bacillus spp.* isolation

Samples were collected in containers from different soils in different sites of Al Diwaniyah and Baghdad provided in Iraq, about half a teaspoonful of soil were placed in the screw-capped tube of distilled water and mix well. Finally, 1g of soil from the sample provided was added to 9ml of distilled water, which was then put in a water bath at 80 ℃ for 10 minutes, then cooled dilutions were carried out 6 times; this was done by using 1 ml of the previous solution and 9 ml of distilled water, 1ml of the last 5 samples was spread onto nutrient agar plates These plates were then incubated at 30 ℃ for 24 hours, then a single colonies pick up and

inoculate on nutrient agar plate. The colonies underwent gram staining and spores stain to verify their Bacillus nature

Primary and secondary screening of bacterial isolates for laccase production

Fifty Seven of bacterial isolates Were grown on petri plates containing nutrient agar supplemented with 0.4 mmol/L Cu^{+2}. The plates were incubated at 30℃ for 24 hours. Then 0.5 mmol/L of syringaldazine was dropped on bacterial colonies on the plates for checking its capability to generate laccase activity [15]. The time consumed by each isolate to develop pink color was measured. the secondary screening was done on the bacterial isolates which required less time to display pink color from primary screening were inoculums in tubes containing production medium: 0.8% nutrient broth supplemented with 0.4 mmol/L Cu^{+2}. The tubes were incubated at 30℃ for 24 hours [12]. The spores were collected from the tubes by centrifuging for 20 min at 4000g and then washed with 0.5 mol/L NaCl, and suspended in 0.1 M potassium phosphate buffer (pH 6.8). Finally, 1 ml of spores suspension contained 100 mg wet cell. The bacterial produced high laccase activity was used for the production of laccase enzyme. [16].

Biochemical tests of *Bacillus spp.*

The fallowing biochemical tests were done to identify the bacteria which was: Catalase, Oxidase, Starch hydrolysis, Gelatin liquefaction, Indole, Anaerobic growth, Urea Hydrolysis, Casein hydrolysis, citrate utilization, Growth at 50 ˚C, carbohydrates fermentation test [15].

Spore-bound laccase activity

Laccase activity of the spores suspension was determined using syringaldazine as the substrate. The oxidation of syringaldazine was detected by measuring the absorbance increase at 525 nm (ε525 = 65,000 L/(mol·cm)) using a spectrophotometer (UV-9200 , UK). The reaction mixture (3 ml) contained 100 µl of spores suspension sample, 2.4 ml of potassium phosphate buffer (0.1 M, pH 6.5) and 500 µl of 0.5 mmol/L syringaldazine. Spore sedimentation was not observed during incubation. Finally, One unit of enzyme activities was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute.

Optimization of culture conditions for enzyme production from *Bacillus subtilis*

Incubation period

The production medium was prepared and inoculated with *Bacillus subtilis* and incubated at 30℃ for 7 days. The spore-bound laccase activity was assayed at every 24 hours interval [12].

Temperature

Laccase production was achieved at different temperatures (20,25, 30, 37,40, 45 and 50℃). and incubated for 3 days [12].

pH Value

The effect of pH on laccase production was determined within a pH range of (5.0 and 10.0) , then the culture medium was inoculated with the isolates and incubated at optimum temperature for 3 days [12].

Copper sulphate concentration

The suitable concentration of copper sulphate for the maximum laccase production was determined by using laccase production medium containing different concentrations of $Cuso_4$ (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7) mM [12].

The media were inoculated with the isolate and incubated at optimum temperature and pH for 3 days. The medium without $CuSo_4$ was used as a control and the laccase and the laccase activity was measured.

Carbon source

Different sources of carbon (sucrose, galactose, lactos , glucose and fructose) were used at the concentration of 3% (w/v) in the production medium containing optimum concentration of $CuSo_4$. After inoculating with *Bacillus subtilis*, the media were incubated at optimum temperature and pH for 3 days [12].

Nitrogen source

The laccase production medium containing optimum concentration of $CuSo_4$ and optimum carbon source was supplied with 0.2% (w/v) of different nitrogen sources (tryptone, peptone, yeast extract, corn, calcium nitrate). The media were incubated at optimum temperature and pH for 3 days [12] .

Metal Ions

Various metals were used to find their effect on the production of laccase by *Bacillus subtilis*, the production medium containing optimum concentration of $CuSo_4$, carbon source and nitrogen source were supplied with different metal sources ($Cacl_2$, $Mncl_2$, $Feso_4$, Kcl and $Znso_4$) at concentration of 1 mM. Finally the media were incubated at optimum temperature and pH for 3 days [12].

Enzyme inducers

The production medium containing optimum concentration of $CuSo_4$, carbon source , nitrogen source and metal ion were supplied with different inducers (Guaiacol, Catechol, Pyrogallol, Phenanthrene and Anthracene) at concentration of 1 mM and inoculated with the isolate. Finally the media were incubated at optimum temperature and pH for 3 days [9].

Results and discussion

Primary and secondary screening of bacterial isolates for laccase production

Primary screening was find that 10 isolates has developed a pink color within 15 second. However, 16 isolates did not develop any color fig. (1). The isolates which consumed less time to develop pink color from primary screening were examined on production medium for the secondary screening by measuring their spore-bound laccase activity. It was found that the isolate 54 produced the highest levels of laccase with activity (12.8 U/ml) fig. (2), so it was selected for further studies.

Microscopic examination and biochemical tests of *Bacillus subtilis*

The results of the biochemical tests and microscopic examination (Table 1) were compared with the cha.racteristics of *Bacillus spp.* documented by [13,10], so the isolates 54 was identified as *Bacillus subtilis*

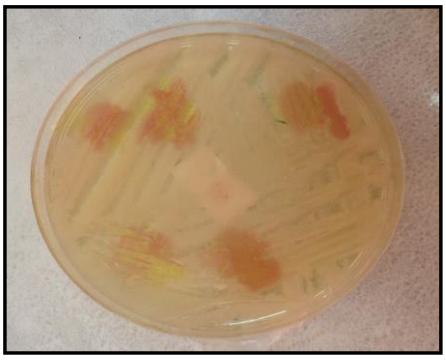

Figure (1): Primary screening of laccase production on solid medium using 0.5 mmol/L syringaldazine after 24 hours of incubation at 30 ˚C

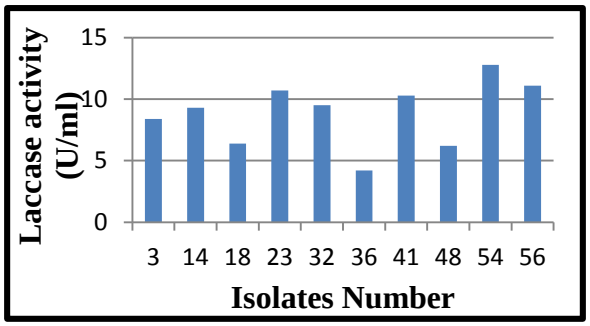

Figure (2): secondary screening of Bacterial isolates for laccase production on production medium after 24 hours of incubation at 30 ˚C

Table (1): Microscopic examination and biochemical tests of *Bacillus subtilis.*

Test	Result
Gram stain	+
Shape	Rod
Spores forming	+
Catalase	+
Oxidase	+
Starch hydrolysis	+
Gelatin liquefaction	+
Indole	-
Anaerobic growth	-
Urea hydrolysis	-
Motility	+
Casein hydrolysis	+
Citrate utilization	+
Growth at 50 ˚C	
Carbohydrates fermentation	
Glucose	+
Lactose	-
Galactose	+
Mannitol	+

Optimization of culture conditions for enzyme production

Incubation period

The maximum laccase production was observed after 3 days of incubation at 30 ˚C with spore-bound activity (59.7)U/ml fig. (3). In contrast to other study [15] observed that the maximum laccase activity of *Bacillus subtilis* WD23 was after 10 days of cultivation.

Temperature

Bacillus subtilis **54 were able to grow and produce laccase at wide range of temperatures from 20 - 50C°, Laccase production was found to be maximum at 37 °C with activity (82.46) U/ml fig. (4). [15] observed that the maximum laccase activity of *B. subtilis* WD23 was at 25 C°. Temperature influencing the rates of biochemical reactions either by inducing or repressing enzyme production [14].**

pH Value

As can be seen in figure (5), higher laccase activity (82.46) U/ml was obtained at pH 7, there was no increasing in spore-bound laccase activity thus for using this pH value before. This finding was agreed with [15] who reported that, the maximum laccase activity of *B. subtilis* WD23 was at pH 7. The pH of the culture influences many enzymatic process and transport of the compounds across the cell membrane [11].

Copper sulphate concentration

The result in figure (6) showed that 0.2 mM of copper sulphate induced the maximum laccase production with (226.61) U/ml spores-bound laccase activity. This suggested a possible role of Cu^{+2} in induction of laccase synthesis and a possible correlation between spore formation and cu^{+2} [16].

Carbon source

Laccase production was detected in the presence of different carbon sources incorporated in the production medium with concentration of 3% (w/v). The laccase activity was significantly increased in the culture supplemented with glucose (265.53) U/ml fig.(7). In contrast to other studies [12] reported that, Among different carbon sources galactose supported good growth and laccase production

Nitrogen source

The effect of different nitrogen sources were evaluated at optimum temperature, pH and carbon source. Based on the results, the spore-bound laccase activity was significantly increased to (345) U/ml in the culture contained tryptone compared with other nitrogen sources fig. (8). [11] observed that addition of inorganic nitrogen source in the production medium resulted in low enzyme production.

Metal Ions

The effect of metals on the growth and production of laccase from *Bacillus subtilis* 54 was shown in figure (9). Based on the results obtained in this work, Kcl showed the maximum induction on laccase production with highest activity of (397.69) U/ml. Metals can be assimilated as part of enzymatic cofactors which lead to increase in enzyme activity and it may also be adsorbed to surfaces of cells and be precipitated as a result of bacterial metabolism [(2)] .

Enzyme inducers

Laccase production was detected in the presence of different phenolic and hydrocarbons inducers added to the production medium. All inducers was enhanced laccase production, pyrogallol was supported the best laccase production with activity (439.23) U/ml fig. (10). This finding was agreed with [(9)] who reported that pyrogallol substantially enhanced laccase production. Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms [(6)].

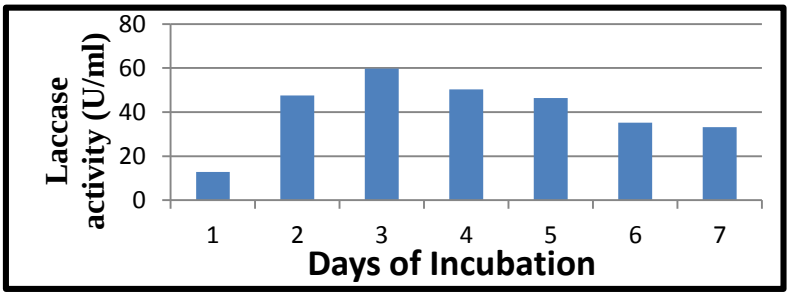

Figure (3): Effect of incubation peroid on laccase production from *Bacillus subtilis* 54 at 30 °C

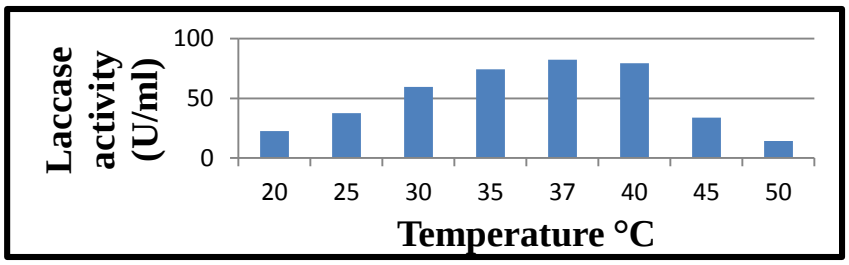

Fig. (4) :Effect of different temperature on laccase production from *bacillus subtilis* after 3 days of incubation

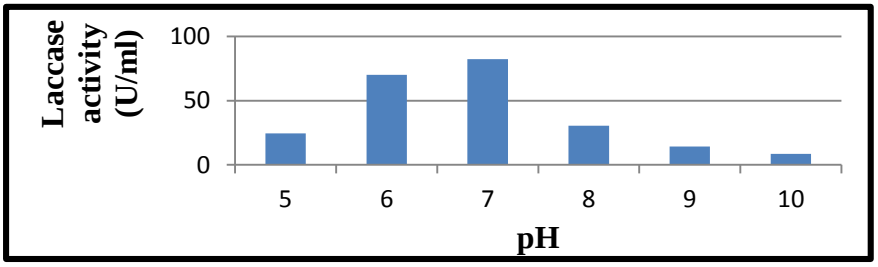

Figure (5) : Effect of pH on laccase production from *Bacillus subtilis*.54 on production medium at 37 °C

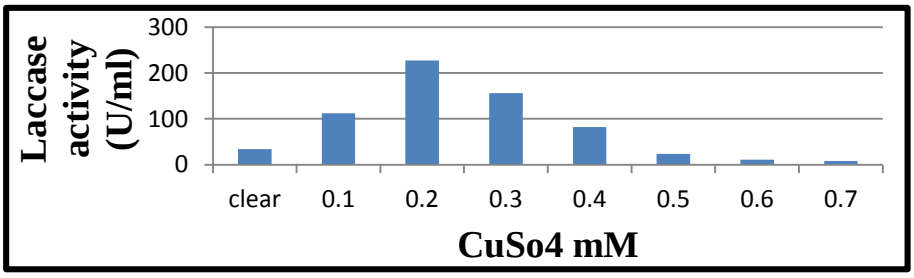

Figure (6) :Effect of different concentration of copper sulphate on laccase production from *Bacillus subtilis* 54 on production medium after 3 days of incubation at 37 °C at pH 7

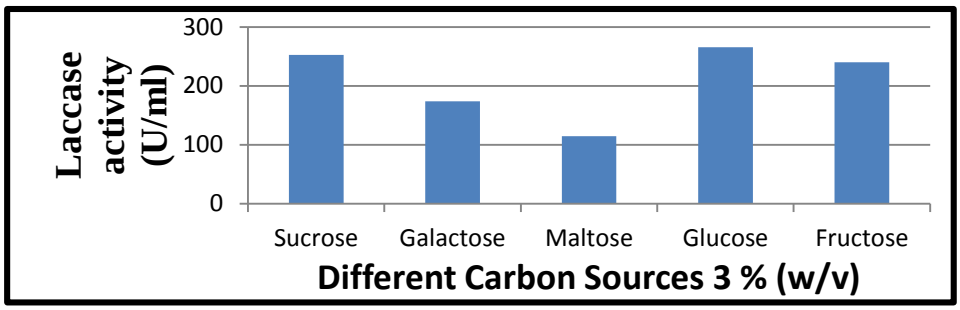

Figure (7) : Effect of different carbon sources on laccase production on production medium after 3 days of incubation at 37 °C at pH 7 containing 0.2 mM of $CuSo_4$

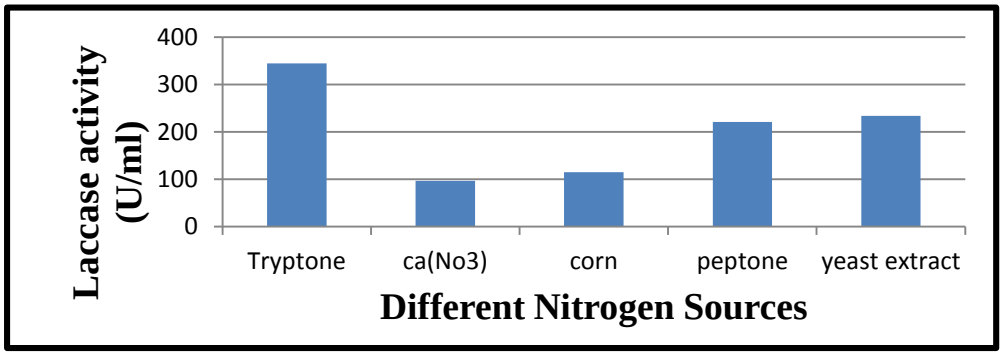

Figure (8): Effect of nitrogen sources on laccase production on production medium after 3 days of incubation at 37 °C at pH 7 containing 0.2 mM of $CuSo_4$ and 3% glucose

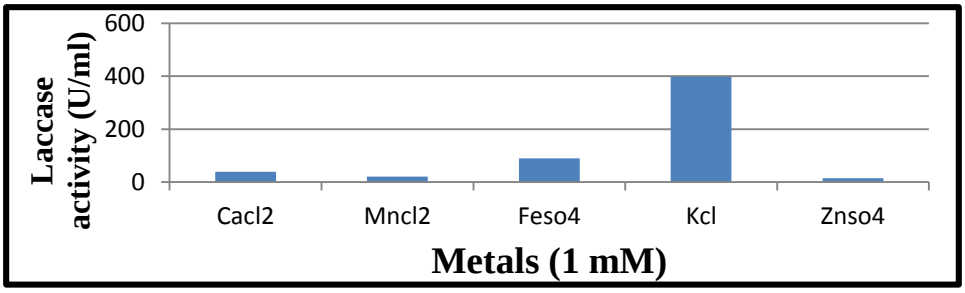

Figure (9): Effect of Metal ions on laccase production on production medium after 3 days of incubation at 37 °C at pH 7 containing 0.2 mM of $CuSo_4$, 3% glucose and 0.2% tryptone

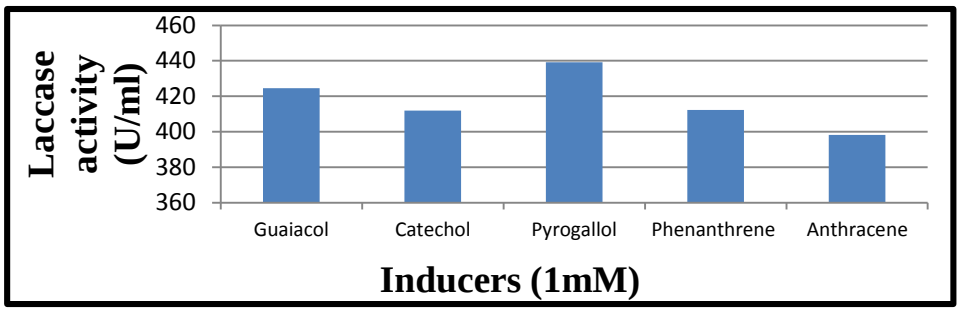

Figure (10) : Effect of Inducers on laccase production on production medium after 3 days of incubation at 37 °C at pH 7 containing 0.2 mM of $CuSo_4$, 3% glucose , 0.2% tryptone and 1 mM Kcl

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الظروف المثالية لإنتاج اللاكيز المرتبط بالسبورات من العزلة المحلية *Bacillus subtilis**

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الكلمات الافتتاحية: ***Bacillus subtilis*** ، الانزيم المرتبط بالسبورات ، الاختبارات الكيموحيوية ، الغربلة ، التحسين.

الخلاصة

سبعة وخمسون من العزلات البكتيرية تم عزلها من ترب مختلفة ومن مواقع مختلفة من الديوانية وبغداد في العراق باستخدام وسط الاغار المغذي. الغربلة الأولية للمستعمرات البكتيرية تمت على وسط الاغار المغذي عن طريق إسقاط بعض القطرات من syringaldazine على المستعمرات البكتيرية أثبتت ان 10 عزلات أظهرت اللون الوردي بأقل من 15 ثانية. اما الغربلة الثانوية تمت على وسط الاغار السائل باستخدام الـ syringaldazine كركيزة للانزيم أثبتت ان العزلة رقم 54 تظهر اعلى فعالية من الانزيم المرتبط بالسبورات U/ml (12.8). تم تشخيص العزلة البكتيرية ذات أعلى فعالية لإنتاج اللاكيز بواسطة الاختبارات الكيموحيوية كـ ***Bacillus subtilis*** . دراسة الظروف المثلى أظهرت بأن أعلى إنتاجية للاكيز كانت عند الحضن لمدة 3 ايام وبدرجة حرارة 37 وبرقم هيدروجيني 7 في وسط حاوي على : من كبريتات النحاس (0.2 ملي مول) و كلوكوز مصدراً للكاربون (3%) وتريبتون كمصدر للنيتروجين (0.2%) و كلوريد البوتاسيوم (1 ملي مول) و البايروجالول (1 ملي مول) كمحفز للاكيز. وكانت فعالية اللاكيز المرتبط بالسبورات بعد هذه الظروف U/ml (439.23).

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*البحث مستل من رسالة ماجستير للباحث الاول