Optimum Conditions for Laccase Production by Local Isolate of *Bacillus Cereus* B5

Mina M. Allos[1] and Asmaa A. Hussein
Department of Biotechnology, College of Science, University of Al-Nahrain.
[1]E-mail: meme_allos@yahoo.com.

Abstract

In this study, fifty five soil samples were collected from different places in Baghdad. Many Gram positive and negative isolates were obtained; of which 39 isolates were identified as *Bacillus* spp. when subjected to morphological and microscopical tests. Laccase enzyme activity was determined by quantitative methods using syringaldazine as substrate for these isolates, results indicated that 17 isolates of them are laccase producer with different specific activity ranged between (98-600) U/mg and the isolate *Bacillus cereus* B5 was the most efficient in the production of laccase with specific activity of 600 U/mg protein therefore, it was chosen to determine the optimum conditions for laccase production. Maximum laccase production was achieved after supplementation of the minimal salt medium (pH7) with 0.5% dextrose, 0.5% yeast extract and incubated at 35ºC in shaker incubator (200 rpm) for 24h. Under these conditions, the specific activity of laccase produced in culture supernatant was sharply increased to 7000 U/mg protein.

Keywords: Laccase, *Bacillus cereus*, laccase activity, optimization.

Introduction

Laccases are belong to the group of polyphenoloxidases and are multi-copper-containing enzymes which reduce molecular oxygen to water (1). This enzyme is classified as blue copper protein that catalyze the oxidation of a wide variety of organic and inorganic compounds by using molecular oxgen as the electron acceptor (2).

Laccase exhibit broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups including diphenols, polyphenols, diamines and aromatic amines (3;4). The syringaldazine [4-hydroxy-3,5-dimethoxy benzaldehyde azine] is considered as the substrate oxidized only by laccase enzyme (5).

Laccases produced by four types of living organisms including bacteria, insects, higher plants and fungi. A very few bacterial origin laccase enzymes have been purified and characterized. The first study on this subject is the prokaryotic laccase which was derived from the a rizospheric bacterium *Azospirillum lipoferum* (6). In recent years, bacterial laccases have gained increasing attention due to overcoming the disadvantages of instability when compared to fungal laccases. They are highly active and much more stable at high temperatures and high pH values. Bacterial laccases become an industrially important enzyme that are applied in various processes like detoxification of industrial effluents, mostly from paper and pulp, textile and petrochemical industries, important tool for medical diagnosed, cleaning agent for certain water purification system and catalyst for manufacturing anti-cancer drugs. The important obstacles to commercialize the bacterial laccases was lack of sufficient enzyme stocks and cost order to achieve cheep over production of this biocatalyst and also alteration of enzyme by chemical means to obtain more robust and active enzyme (7).

According to those mentioned above this study was aimed to isolate a higher laccase *Bacillus* sp. and studying the optimum condition for its production

Materials and Methods

Samples collection

For the isolation of *Bacillus sp*. producing laccase, fifty five soils samples were collected from different location in Baghdad. A quantity of 1 g of each sample were suspended in 9 ml sterile D.W. Serial dilutions was done for each sample using sterilized D.W. then 0.1 ml aliquot from appropriate dilution was taken, spread on LB ager plates, and incubation at 37^0C for 24h.

Determination of specific laccase activity for *Bacillus* sp. isolate (Rid,8)

To screen the ability of local *Bacillus sp*. isolates for extracellular lacces production. Enzyme activity was determined by quantitative methods using Syringaldazine as substrate. A volume of 100 µl of fresh culture of each bacterial isolates was used to inoculate the LB broth in a conical flask and incubated in a shaker incubator (150 rpm) at 37ºC for 24hrs. After incubation, the culture was centrifuged, pellets were discarded, and supernatants were taken and assayed for laccase activity by measuring oxidation of syringaldazine at 530nm for 10min.The assay mixture contained: 0.3ml of 0.216mM syringaldazine, 2.20ml of 100mM monobasic potassium phosphate buffer pH(6.5) and 0.5ml of laccase.

Laccase activity was calculated from the following formula:

Enzyme activity(Units\ml)=

$$\frac{sample\Delta A530nm = (Test - Blank(df))}{(0.001)(0.5)}$$

df = dilution factor
0.001 = the change in A530nm\min. per unit of laccase at pH6.5 at 30^0C in a 3 ml reaction mix.
0.5 = volume (in milliliters) of enzyme used.

Enzyme activity was expressed in units; 1U being defined as the amount of enzyme causing the formation of 1µmol of product per minute under the assay conditions used (9).

Determination of protein concentration

Protein concentration was determined according to the method described by Bradford, (10) and as follow:

- Standerd curve of Bovine serum albumin (BSA) was plotted by using different concentrations from the BSA stock solution according to the following volumes.

Table (1)
Bovine serum albumin standard curve composition.

BSA (µl)	*Tris-HCL Buffer (µl)*	*Protein Amount (mg)*	*Final Volume (ml)*
20	80	20	0.1
40	60	40	0.1
60	40	60	0.1
80	20	80	0.1
100	0	100	0.1

- Then 2.5 ml of Coomassi brilliant blue G-250 dye was added, mixed and left to stand for 2 min at room temperature.
- The absorbance at 595 nm was measured; the blank was prepared from 0.5 ml of Tris-HCl buffer and 2.5 ml of the dye reagent.
- A standard curve was plotted between the BSA concentrations against the corresponding absorbance of bovine serum albumin.
- Protein concentration was estimated by mixing 0.1ml of the tast sample, 0.4 ml of Tris-HCl and 2.5 ml of Coomasi brilliant blue G-250, left to stand for 2 min at room temperature then measuring the absorbance at 595 nm.

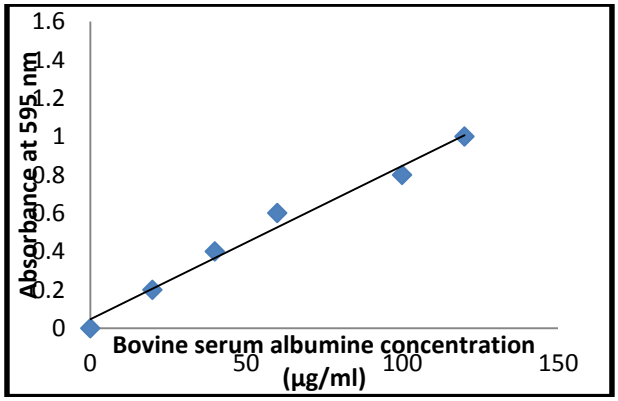

Fig. (1) Bovine serum albumin standard curve.

Identification of the isolate

The selected isolate with high laccase activity was identified based on its morphological and biochemical characteristics. The morphological characterization involved culturing the isolate on LB plates for studying

the appearance of the colonies, following that, cells shape and Gram reaction were studied.

Biochemical characteristics of the isolate was based on indole, methyl red, Voges-Proskauer, citrate utilization, nitrate reduction, starch hydrolysis, urease, casein hydrolysis and gelatin hydrolysis, vitek test was used to ensure the biochemical results according to method described by Logan and Turnbull (11).

Optimum carbon and nitrogen source for laccase production

Various carbon (glucose, dextrose and sucrose,) and nitrogen sources (yeast extract, peptone and tryptone) at initial concentration of 0.5% (w/v). were screened individually in minimal salt medium(12) which compost of g/L (KH_2Po_4 4, Na_2Po_4 4, Nacl 0.5, NH_4Cl.2 and 2ml $Mgcl_2$) the initial pH of the medium was set at 7.0. Laccase activity was determined in the supernatants after inoculation of the medium with the locally isolated *Bacillus cereus* culture, and incubated at 37°C under shaking (200 rpm).

Optimum pH for laccase production

Optimal pH for production of laccase was determined by preparing the medium with different pH values (6, 6.5, 7, 7.5, 8, 8.5, 9). Laccase activity was measured in the supernatant after inoculation of the production medium with *Bacillus cereus* log phase culture, and incubation at 37°C under shaking (200 rpm).

Optimum temperature for laccase production

Bacillus cereus was grown in the production medium and incubated at different temperatures (30, 35, 40, 45,) °C. The laccase activity was determined in supernatants after centrifugation at 6000 rpm for 10min.

Optimum inoculums size for laccase production

Effect of different inoculum size of the selected isolate for the over producer *Bacillus cereus* on laccase production was studied by inoculating the production medium, individually with a serial dilution ranging between (10^2 and 10^9cell/ml).

Results and Discussion

Isolation of *Bacillus* sp.

Fifty five soil samples were collected from different places in Bagdad. Many Gram positive and negative isolates were obtained; of which 39 isolates were identified as *Bacillus* spp. when subjected to morphological and microscopical tests in which growing colonies on the LB agar had a rough surface with an unacceptable odor. After cells were stained with Gram stain, results showed that they were Gram positive.

Screening ability of *Bacillus* sp. for laccase production

To screen the ability of local *Bacillus sp*. isolates for lacces production. Enzyme activity was determined by quantitative methods using Syringaldazine as substrate. Results indicated that 17 of 39 isolates are laccase producing with different specific activities Table (2). Depending on these results, the isolate named B5 was found to be the most efficient in the production of laccase with specific activity about 600 U/mg protein, therfore it was chosen for further study.

Table (2)
Specific activity of laccase produced by 17 local isolates of Bacillus sp.

Bacillus sp. isolates	*Laccase specific activity(U/mg)*	*Bacillus sp. isolates*	*Laccase specific activity(U/mg)*	*Bacillus sp. isolates*	*Laccase specific activity(U/mg)*
B1	470	B7	120	B13	188
B2	400	B8	140	B14	399
B3	460	B9	123	B15	250
B4	110	B10	89	B16	207
B5	600	B11	300	B17	336
B6	200	B12	114		

Harkin and Obst (13) were the first researchers to demonstrate the use of Syringaldazine as a substrate for laccase assay and later several other workers have also used syringaldazine as an assay substrate for laccase. Laccase catalyses the oxidation of syringaldazine to tetramethoxy-azo-bis (methylene quinone) that is measured spectrophotometrically at 530 nm. However, focusing on the quantitatively production of laccase, differences were showed comparing some bacterial species. *Pseudomonas putida* was able to produce 11 U mg^{-1} (14), *Bacillus sp*. HR03 exhibited a DMPO-specific activity equal to 50 U mg^{-1} (15).

Identification of the isolate

To identify selected *Bacillus* sp. B5 isolate with high laccase activity. It was subjected to the biochemical test and vitek. Results in Tables (3) and (4) shows that this isolate was *Bacillus cereus* according to the criteria of Bergys Manual of systematic bacteriology and vitek test (16,11).

Table (3)
Biochemical characterization of Bacillus cereus B5.

Test	*Catalase*	*Oxidase*	*MR*	*VP*	*Nitrate reduction*	*Indole*	*Gelatine*	*Citrate utilization*	*Urease*	*Starch hydrolysisi*	*casein hydrolysis*
B5	+	-	+	+	+	+	+	+	-	+	-

(+) positive results (-) negative results.

Table (4)
Vitek test result for identification of Bacillus cereus B5.

Test			*Result*	*Test*	*Result*	*Test*	*Result*	*Test*	*Result*
BXYL	-	IRHA	-	PyrA	+	GLYG	+	AMAN	-
BGAL	-	dTAG	-	CDEX	+	MTE	+	NAG	-
APPA	+	NACL 6.5%	+	MdX	-	PLE	-	PVATE	+
ELLM	+	ProA	-	dMLZ	-	AGLU	+	dRIB	+
DMNE	-	BNAG	+	PHC	-	PSCNa	_	TTZ	-
BMAN	-	MdG	-	dGLU	+	POLYB-R	+	Phe	+
INU	-	DMAN	-	ESC	+	AspA	+	TyrA	+
OLD	-	BGLU	+	LeuA	-	AGAL	-	INO	-
LYSA	-	dTRE	+	ALaA	+	dGAL	-	Glya	-
KAN	+								

(+) positive results (-) negative results.

Optimum conditions for production of laccase production

The over producer *Bacillus cereus* characterized with its high ability in laccase production was used to determine the optimum conditions for laccase production.

Optimum carbon and nitrogen sources

Optimum carbon and nitrogen source was investigated by using sucrose, dextrose, glucose and sodium citrate (as carbon source) and yeast extract, tryptone, peptone and sodium nitrate (as nitrogen source). *Bacillus cereus* B5 was cultivated in a minimal media containing 0.5% from each of these various carbon and nitrogen sources. Results in Table (5) shows that, this isolate was capable of utilizing different carbon sources as a sole source for carbon and energy, while production of laccase was varied according to the type of the carbon source. Since, dextrose was the best carbon source for laccase production, while glucose and sodium citrate were the less effectives. The type of nitrogen source also affected enzyme production, among the various nitrogen sources, maximum laccase specific activity was obtained when yeast extract was added to the medium. Also, good level of enzyme activity was obtained with other nitrogen sources.

Nature and type of carbon and nitrogen sources are among the most important factors for any fermentation process (17). Chhaya and Modi (18) have been reported that dextrose was comparatively less repressive for laccase production from *Sterptomyces chartreusis*, which yielded 4.6 U/ml while all the other carbon sources reduced the enzyme yield considerably. This was probably due to the reason that dextrose was a readily utilizable substrate which would promote the biomass production. Replacement of yeast extract with peptone failed to elicit laccase production. This confirmed the suitability of yeast extract as the nitrogen source (5.2 U/ml).

Table (5)

Effect of carbon and nitrogen source on laccase production by Bacillus cereus B5.

Carbon source	*Specific activity (U/mg protein)*	*Nitrogen source*	*Specific activity (U/mg protein)*
Glucose	2261	Yeast extract	2084
Dextrose	3200	Peptone	971
Sucrose	1500	Tryptone	1800
Sodium citrate	1200	Sodium nitrate	900

Optimum pH

In order to investigate the effect of the initial medium pH on laccase production by the B5 *B. cereus*, the production medium was adjusted to different pH values ranged between pH 6 and pH 9. Fig.(3) shows that maximum laccase production was obtained when the pH value of the production medium was adjusted to 7.5, at this value the enzyme specific activity in culture filtrate was 6478 U/mg protein.

A decrease or increase in hydrogen ions (H+) concentration causes pH changes in the culture medium which may lead to drastic changes in the three-dimensional structure of proteins because H+ and/or OH- compete with hydrogen bonds and ionic bonds in an enzyme, resulting in enzymes denaturation (19). Kaushik and Thakur (20) observed that highly acidic and alkaline conditions were not suitable for the bacterium to produce the laccase enzyme, whereas neutral pH was the best condition for laccase production.

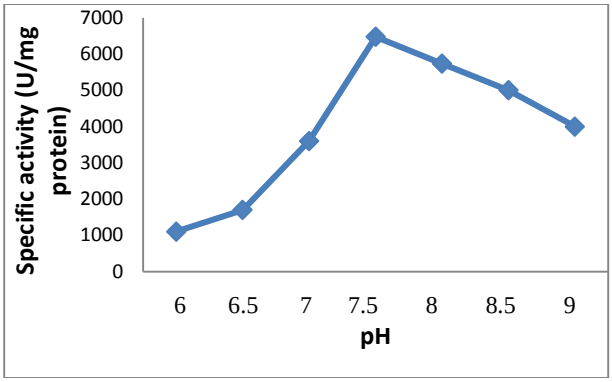

Fig.(3) Effect of pH on laccase production by Bacillus cereus B5.

Optimum incubation temperatures

In this study, different incubation temperatures (30, 35, 40, and 45) ºC were used to determine the optimum temperature for laccase production by *B.cereus* B5. Fig.(4) shows that laccase specific activity is increased with increasing temperature from 30-35℃, since the specific activity was 5800 u/mg protein at 30℃ has increased to 6700U/mg protein at 35℃, but decreased at higher temperatures.

Generally, for any enzymatic reaction, temperature below or above the optimal temperature will drastically reduce the rate of reaction. This may be due to the enzyme denaturation, or to losing its characteristics of three-dimensional structure. Denaturation of a protein involves the breakage of hydrogen bonds and other non-covalent bonds (21). The conditions of laccase activity are necessary for the efficiency of enzymes and their applications. The laccase of the bacteria we examined had optimum temperatures in mild conditions (32 to 45)°C, unlike fungal laccases which have high activities at high temperatures (40 to 70) °C and had good activities in acidic condition similar to fungal laccases (22).

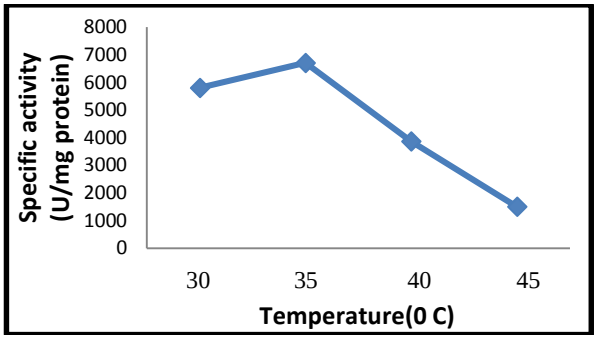

Fig.(4) Effect of temperature on laccase production by Bacillus cereus B5.

Optimum inoculum size

Different inoculums sizes were used to determine the optimum for laccase production by B5. These inoculum sizes were ranged between (10^2-10^9) cell/ml. results illustrated in Fig. (4) shows that production of laccase by B5 was affected by the inoculums size. Laccase production was increased slightly with increasing inoculums size to 10^5 cell/ml, then it was decreased above this size. However, maximum specific activity (7000 U/mg) was obtaind using 10^5 CFU/ml.

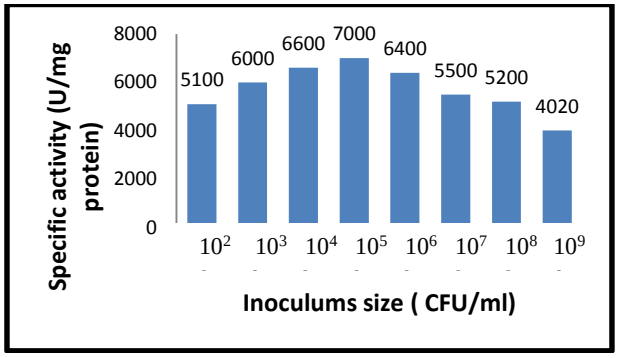

Fig.(5) Effect of inoculums size on laccase production by Bacillus cereus B5.

Production of enzyme in sufficient amount required optimum inoculums size of cells; lowering inoculum size required longer time for cells to multiply for sufficient number and produce enzyme. On the other hand, an increase in the number of the inoculum would ensure a rapid proliferation and biomass synthesis, after a certain time, enzyme production could be decreased because of the depletion in the nutrients which may result in decreased in metabolic activity (23). Niladevi *et al*. (24) recorded that a very low inoculum size was found to be inadequate for enzyme production, while the inoculum level above the optimum reduced the yield probably due to the competition for nutrients. In the same reference, the optimization of inoculum size revealed that 1.5 x 10^7 CFU yielded maximum (33.4 U/mg) laccase production by *Streptomyces pasmmoticus* and the enzyme yield was reduced at lower and higher inoculum levels.

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الخلاصة

في هذه الدراسة، جمعت خمس وخمسون عينه تربه من أماكن مختلفة في بغداد. تم الحصول على عدة عزلات موجبه وسالبه لصبغة كرام. منها تسعة وثلاثون عزله شخصت على انها / *Bacillus* sp عند إجراء الفحوص ألمجهريه والمظهرية لها. تم تحديد فعالية إنزيم اللايكيز لهذه العزلات بطرق كميه باستخدام مادة syringaldazine, أظهرت النتائج أن سبعة عشر عزله منها منتجه لإنزيم اللايكيز مع اختلاف الفعالية النوعية والتي تراوحت بين٩٨ –٦٠٠ وحده/ ملغم وكانت العزلة *Bacillus cereus* B5 هي الأكفأ في إنتاج اللايكيز مع فعاليه نوعيه بلغت ٦٠٠ وحده/ ملغم. لذلك اختيرت هذه العزلة لتحديد الظروف المثلى لإنتاج اللايكيز. تم الحصول على أعلى إنتاجيه للايكيز بعد تدعيم وسط الأملاح الأدنى ذو الرقم الهيدروجيني ٧ بـــ ٠,٥% دكستروز و ٠,٥% خلاصة الخميرة مع حضن بدرجة حرارة 35^0C في حاضنه هزاز بسرعة ٢٠٠ دوره/ دقيقه لمده ٢٤ ساعة. تحت هذه الظروف ازدادت الفعالية النوعية بشده إلى ٧٠٠٠ وحده /ملغم بروتين.