Determination of Optimal Conditions for the Production of Laccase Enzyme by Local Isolate of *Bacillus sp.*

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Abstract:
Fifty isolates of *Bacillus sp*. were subjected to the first and second screening to detect the ability to produce laccase enzyme and select the highest ones production of laccase on Petri plates containing nutrient agar supplemented with Cu^{2+}.

Syringaldazine was used as an indicator and substrate for the determination of laccase activity. Three isolates, which consumed less time to developed pink color were tested for the production of laccase quantitatively. The effective isolate B16 with significant amounts of laccase 1.84 unit /ml was selected for laccase study.

The optimization studies revealed that the maximum laccase production was achieved when the production medium was at the following conditions: 5 days of incubation, temperature 35 C°, pH 7.0, copper sulphate 0.2 mM, galactose 3% as carbon source, 0.2% Tryptone as nitrogen source and K^+ 1mM.

Keywords: Bacillus, Laccase, Syringaldazine, Spore suspension, Optimization, Enzyme production

تحديد الظروف المثلى لانتاج انزيم Laccase من العزلة المحلية *Bacillus sp.*

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الخلاصة
اخضعت 50 عزلة من بكتريا *Bacillus* لمرحلتي الغربلة الاولية والثانوية لكشف قدرتها على انتاج انزيم *Laccase* في وسط مغذي مدعم بأيونات النحاس ولغرض انتقاء العزلة الاكثر انتاجاً للانزيم. استعمل ال Syringaldazine ككاشف ومادة اساس في تحديد فعالية الانزيم. ثلاث عزلات فقط استغرقت وقتاً قليلاً في اظهار اللون الوردي في الفحص النوعي وقد تم اختيارها في دراسة الفحص الكمي لانتاج الانزيم . تم اختيار العزلة B16 ذات الفعالية العالية (1.84)U/ml لدراسة ال Laccase.دراسة الظروف المثلى اظهرت بأن اعلى انتاجية للانزيم كانت عند الحضن لمدة 5 ايام وبدرجة حرارة 35م°وبرقم هيدروجيني 7 في وسط حاوي على :0,2 ملي مول من كبريتات النحاس و3% كالاكتوز مصدراً للكاربون و0,2% تريبتون مصدراً للنايتروجين و 1 ملي مول بوتاسيوم .

Introduction:

Laccase (*p*- benzenediol :oxygen oxidoreductase;EC 1.10.3.2),a multicopper oxidase ,was first detected in 1883 from *Rhus vernicifera* , the Japanese lacquer tree [1]. This enzyme is classified as blue copper protein that catalyze the oxidation a wide variety of organic and inorganic compounds by using molecular oxygen as the electron acceptor [2,3].

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Laccase exhibit broad substrate specificity toward aromatic compounds containing hydroxyl and amine groups including diphenols, polyphenols, diamines, and aromatic amines[4,5].

Laccase represents an example of a 'moonlighting' protein that has multiple functions in addition to its primary catalytic function [6].Laccases are widely distributed among fungi, plant, insect and bacteria [7]. The first bacterial laccase was detected in the plant root associated bacterium, *Azospirillum lipoferum* ,it was shown to be involved in cell pigmentation and utilization of plant phenolic compounds [8]. *Bacillus subtilis* produces endospore coat protein CotA laccase , which participates in pigment production in the endospore coat[2].

Laccases are studied intensively for many environmental and industrial applications. They are play an important role in bioremediation of contaminated soils, production of medical agents [9]. Industrially laccases are attractive significant enzymes used for pulp delignification, organic synthesis and dye decolorization [10,11].

The most investigated studies focused on laccase originate from fungi and plants. In contrast laccases from bacteria are poorly investigated and only a few reports have been published on bacterial laccases [12, 13]. The use of fungal laccases are restricted to acidic reaction conditions and moreover they cannot be produced with bacterial expression systems because they are highly glycosylated enzymes [14,] . Therefore bacterial laccases can overcome the difficulties related with fungal laccase since they have the advantages of being tolerable towards alkaline conditions and its genetic tools are well studied as well as a fast growth rate [13] .

Generally there are a number of approaches to improve production of enzyme like; strain selection, optimization of production medium and growth conditions as well as genetic modification [15]. However various media have been investigated for production of laccase by culture of *Bacillus sp*.[16] but there is limit information in regard the optimization of their production media. Therefore in this study we aimed to find suitable supplies that can be incorporated in the culture medium and determine the favorable conditions for production of laccase enzyme with high activity.

Materials and methods

Microorganisms and materials

Bacillus sp. isolates were obtained from Department of Biology, Baghdad University, Iraq. Nutrient agar, (Syringaldazine, SGZ), and all other reagent grand chemicals were purchased from Hi – Media and Sigma-Aldrich, India.

Culture conditions

The stock culture of *Bacillus sp*. was maintained on Nutrient agar (NA) slants and sub cultured periodically to preserve its viability. A loop full of culture from NA slants were spread on Petri plates containing nutrient agar supplemented with 0.4 mmol/L Cu^{2+}. The plats were incubated at 37 C° for 7 days.

Primary screening of bacterial isolates for laccase production

Fifty isolates of *Bacillus sp*. were grown on Petri plates containing nutrient agar supplemented with 0.4 mmol/L Cu^{2+}. The plats were incubated at 37 C° for 7 days. 0.1% (w/v) syringaldazine was dropped on bacterial colonies of each isolate grew on the plats for checking its capability to generate laccase activity [13]. The time consumed by each isolate to develop pink color was measured.

Secondary screening of bacterial isolates for laccase production

The bacterial isolates which required less time to display pink color from first screening were re spread on new Petri plates containing nutrient agar supplemented with 0.4 mmol/L Cu^{2+}.The plats were incubated at 37C° for 7 days. The spores were harvested from the agar plate with 1 mol/L KCl, collected by centrifuge, then washed with 0.5 mol/L NaCl, and suspended in 0.1 mol/L sodium phosphate buffer (pH 6.8) [17]. Finally, 1 ml spore suspension from each selected isolate contained 100 mg wet cell was used to measure its spore laccase activity. The bacterial isolate produced highest laccase activity was used for the production of laccase enzyme.

Spore laccase activity

The reactive mixture contained per 3 ml: 2.4 ml of phosphate buffer (100 mM, pH 6.8), 500 µl of syringaldazine (0.5 mM) and 100 µl of spore suspension (10 mg wet spores) [17].

Laccase activity was determined for spore suspension of each selected isolate at 25°C using syringaldazine as the substrate. The oxidation rate was measured by monitoring the increase in the absorbance at 525 nm (ε525 = 65,000 L/(mol ·cm) after 3 min using a spectrophotometer (U2800,Hitachi, Japan).One unit of enzyme activities was defined as the amount of enzyme required

to catalyze the production of 1 µmol of colored product per min. All assays were carried out in triplicate for each sample [17,18].

Production of laccase

The *Bacillus sp*. with high laccase activity was spread on Petri plates containing basal production medium: 0.8% of nutrein broth, 0.4 mM $CuSO_4$, and 2% agar. The pH of the medium was adjusted to 7.0, autoclaved in flasks and cooled to 55 C° then the $CuSO_4$ was added aseptically. The medium was stirred and poured into Petri dishes. Laccase spore activity were determined after 7 days of incubation at 37 C˚ [9, 19].

Optimization of culture conditions for enzyme production

The following cultural conditions were optimized for maximum enzyme production using above basal production medium. For each experiment loop full from bacterial slant was used as inoculums spread on production medium.

Incubation period study

In order to determine the optimum incubation time for the maximum laccase production, the time course for enzyme production was followed up to 10 days. The production solid medium was prepared and inoculated with *Bacillus sp.* B16 and incubated at 37 C˚ for 10 days. The culture was harvested at every 2 day interval. Finally, 1 ml spore suspension from each time interval contained 100 mg wet cell was used to measure its spore laccase activity.

Effect of temperature on enzyme production

Laccase production was achieved at different temperatures (25, 30, 35, 40, 45 and 50) C˚[13]. The strain was inoculated in production medium and incubated at different temperatures for 5 days. 1 ml spore suspension contained 100 mg wet cells were prepared from the harvested culture for each incubation temperatures and their spore laccase activities were measured.

Effect of pH on enzyme production

The effect of pH on laccase production was determined within a pH range of 5.0 and 10.0 by preparing the production medium at different pH then the culture medium was inoculated with the strain and incubated at optimum temperature for 5 days [5, 13]. Spore laccase activity was measured for each pH value by using 1 ml spore suspension containing 100 mg wet cell prepared from harvested culture.

Effect of various concentration of copper sulphate on laccase production

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 and 0.5) mM [5]. The medium was inoculated with the isolate and incubated at 35 C˚ and pH 7.0 for 5 days. The medium without $CuSO_4$ was used as a control and the laccase spore activity was measured.

Effect of carbon source on enzyme production

Different sources of carbon (glucose, lactose, maltose, fructose, galactose, bran and sucrose) were used at the concentration of 1% (w/v) in the production medium containing 0.2 mM $CuSO_4$. After inoculating with *Bacillus sp*. B16, the medium was incubated at optimum temperature and pH for 5 days. Laccase spore activity was measured to compare the effect of presence of different carbon sources on the production. Finally the optimum concentration of the selected carbon source was determined by using different concentrations of (1%, 2%, 3%, 4% and 5%) (w/v) carbon source,and incorporated in the culture medium.

Effect of nitrogen source on the enzyme production

The laccase production medium containing optimum concentration of galactose (3%) and $CuSO_4$ (0.2 mM) was supplied with 0.2% (w/v) of different nitrogen sources (tryptone , peptone, yeast extract, corn liquor, calcium nitrate and ammonium sulphate)[5]. The medium was inoculated with the strain and incubated at optimum temperature and pH for 5 days. In order to find the suitable nitrogen source for the maximum production of laccase by *Bacillus sp*.B16, the laccase spore activity was measured using spore suspension from each treatment.

Effect of different metals on the enzyme activity

Various metals were used to find their effect on the production of laccase by *Bacillus sp*. B16. The production medium containing optimum concentration of galactose, trypton and 0.2 mM $CuSO_4$ was supplied with different metal sources (KCl, $ZnSO_4$, $MnCl_2$, $FeSO_4$,$CaCl_2$) at concentration of 1 mM and inoculated with the strain [13]. Finally the medium was incubated at 35 C˚ for 5 days and the

laccase spore activity was determined by using bacterial spore suspension obtained from each treatment.

Results and discussion

Primary screening of bacterial isolates for laccase production

Fifty isolates of *Bacillus sp*. were screened on nutrient agar medium containing 0.4 mmol/L Cu^{2+}. It was found that 3 isolates has developed pink color within 15 second after dropping 0.1% (m/v) syringaldazine on them. However, 11 isolates did not develop any color. The results showed that most of isolates were developed the pink color within 20 to 50 second. Only 4 isolates consumed more than 1 minute to develop the color. Developing of pink color by the isolates was considered as an indicator for the laccase production.

Secondary screening of bacterial isolates for laccase production

The strains which consumed less time to develop pink color from first screening as well as the strains which did not develop the color were examined for the production of laccase quantitatively by measuring their spore laccase activity. It was found that the strain B16 produced the highest levels of laccase with activity (1.84) U/ml whereas no spore laccase activity was observed for the isolates that gave negative results in the primary screening. Isolate B16 with high level of laccase activity was selected for further studies.

Optimization of culture conditions for enzyme production

Incubation period study

The activity of laccase for *Bacillus sp*. B16 was measured at different time intervals of growth. The maximum laccase production was observed after 5 days of incubation at 37 C˚ with laccase activity (2.1) U/ml figure 1. In contrast to other study Wang et al [13] observed the maximum laccase activity of *B. subtilis* WD23 was after 10 days of cultivation. The result demonstrated that the laccase activity was derived during the late stage of growth.

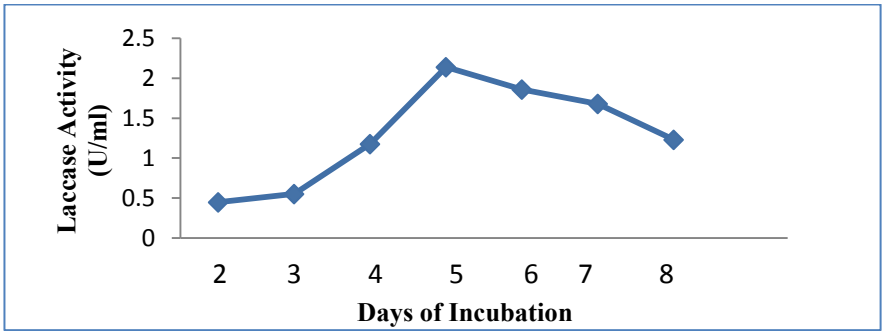

Figure.1-Effect of incubation time on laccase production from *Bacillus sp.*B16 after incubtion at 37 C˚and pH 7.0.

Effect of temperature on enzyme production

Laccase production is found to be maximum at 35 C˚, though they were able to grow at wide range of temperatures from 20 - 55C˚. Wang et al [13] 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 [20]. The spore laccase activity at 35 C˚ was (2.55) U/ml figure 2 .

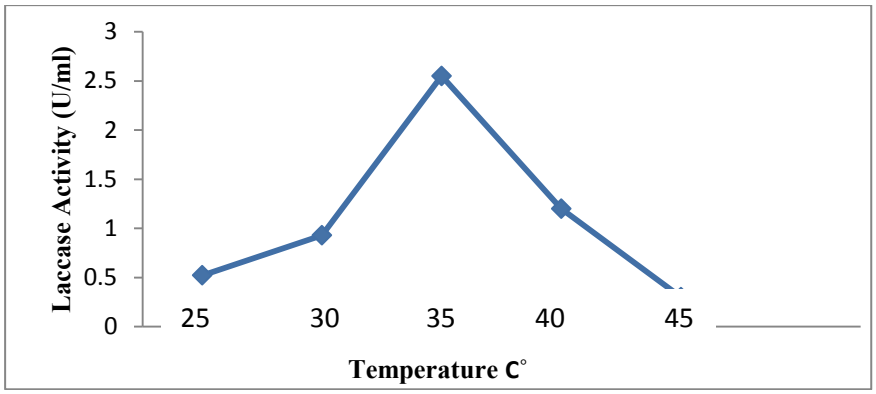

Figure 2- Effect of different temperature on laccase production from *Bacillus sp.*B16 after five days of incubation at pH 7.0.

Effect of pH on enzyme production

As can be seen in figure 3, higher laccase activity (2.86) U/ml was obtained at pH 0.7 which agree with Wang et al [13] who reported that the maximum laccase activity of *B. subtilis* WD23 was at pH 7. The pH of the culture significantly influences many enzymatic process and transport of the compounds across the cell membrane [21].

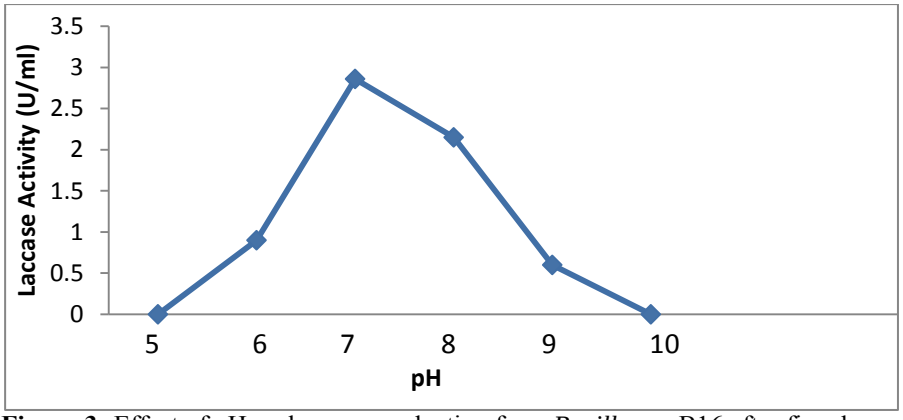

Figure 3- Effect of pH on laccase production from *Bacillus sp.*B16 after five days of incubation at 35 C° and pH 7.0.

Effect of various concentration of copper sulphate on laccase production

The effect of different concentrations of $CuSO_4$ on laccase production were studied. The result in figure 4 showed that 0.2 mM of copper sulphate induced the maximum laccase production with 6.7 U/ml spore activity whereas the laccase activity was (4.6) U/ ml form the spore on the plates without Cu^{2+}.This suggested a possible role of Cu^{2+}in induction of laccase synthesis and a possible correlation between spore formation and Cu^{2+} [22,17]

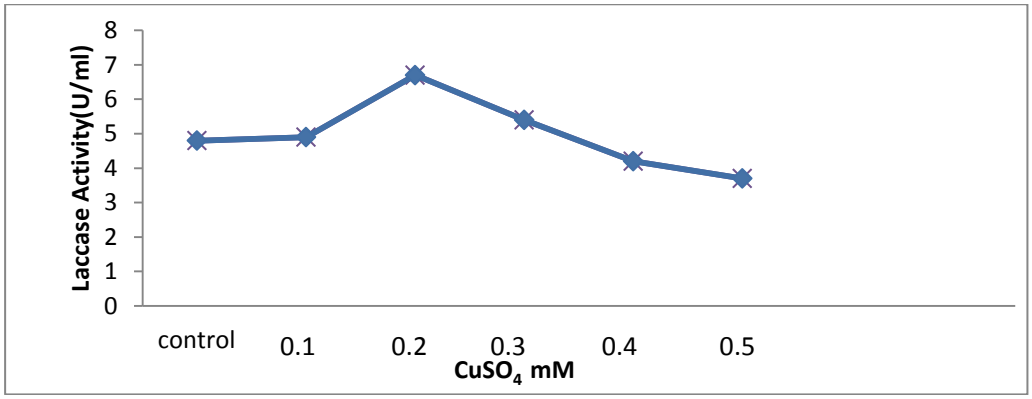

Figure. 4-Effect of different concentration of $CuSO_4$ on laccase production from *Bacillus sp.*B16 after five days of incubation at 35 C° and pH 7.0.

Effect of carbon source on enzyme production

Laccase production was detected in the presence of different carbon sources incorporated in the basal production medium with concentration of 1%. As can be noticed in figure 5, the laccase activity was significantly increased in the culture supplemented with galactose (7.98) U/ml compared with glucose containing medium which was (4.63) U/ml. Among the seven different carbon sources galactose supported good growth and laccase production. Furthermore, the optimum concentration of galactose for the production of laccase was also determined. It was found that 3% (w/v) galactose has induced a maximum production of laccase under the experimental conditions used in this work figure 6.

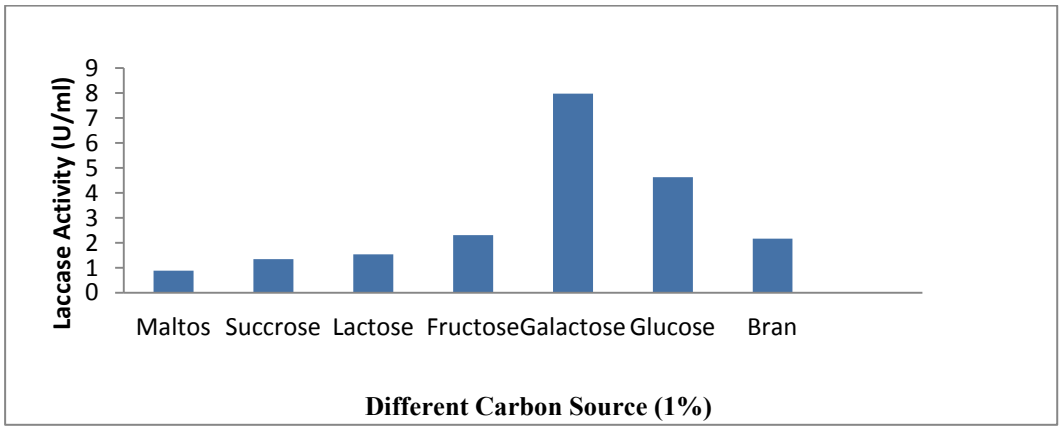

Figure.5-Effect of different carbone source on laccase production from *Bacillus sp.*B16 after five days of incubation at 35 C° and pH 7.0.

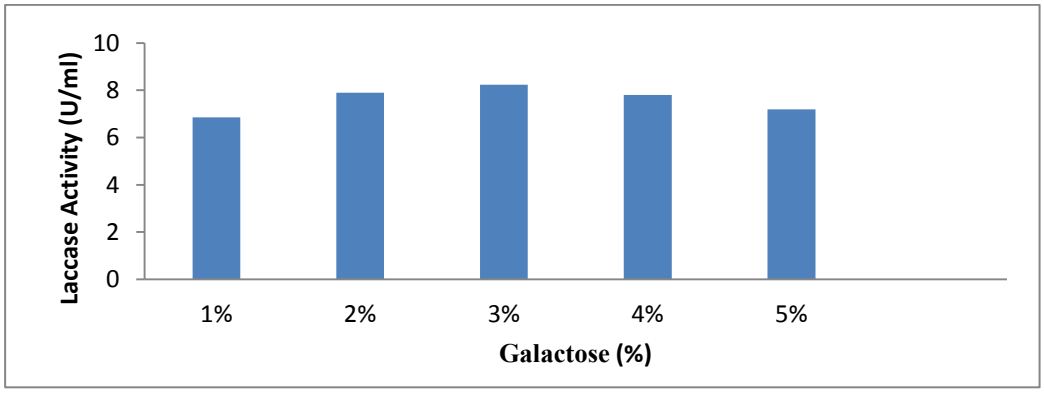

Figure.6-Effect of different galactose concentration laccase production from *Bacillus sp.*B16 after five days of incubation at 35 C° and pH 7.0.

Effect of nitrogen source on the enzyme production

The effect of different nitrogen sources were evaluated at optimum temperature, pH and carbon source. Based on the results, the laccase activity was significantly increased to 9.35 U/ml in the culture contained tryptone compared with other nitrogen sources which had an inhibitory effect on the laccase production figure 6. In this context, tryptone was the best source of nitrogen that supported the growth and enzyme production from *Bacillus sp.*B16 [9,19]. Shanmugam et al [21] observed that addition of inorganic nitrogen source in the production medium resulted in low enzyme production. The type of nitrogen source play important role in the production of laccase [23].

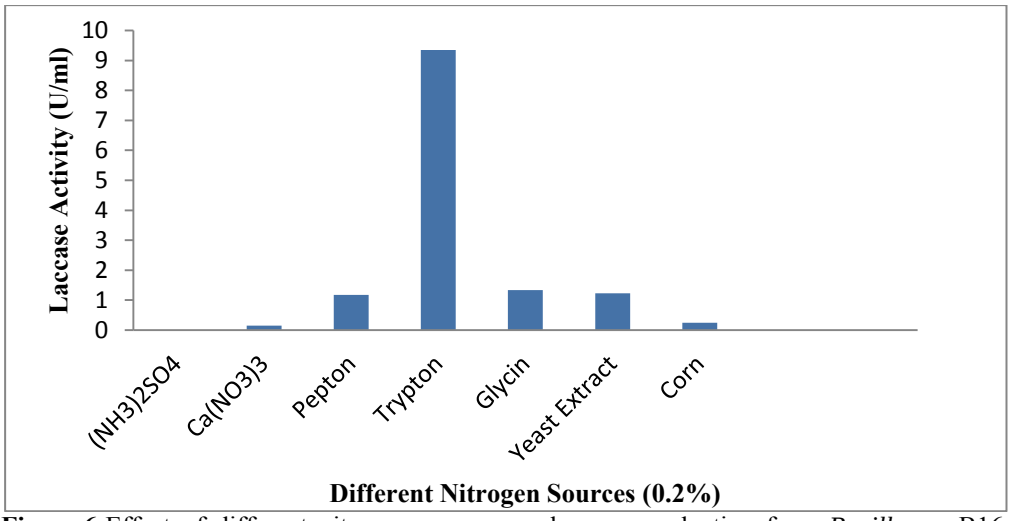

Figure.6-Effect of different nitrogen sources on laccase production from *Bacillus sp.*B16 after five days of incubation at 35 C° and pH 7.0.

Effect of different metals on enzyme activity

The effect of metals on the growth and production of laccase from *Bacillus sp.* B16 was shown in figure7. Based on the results obtained in this work, K^+ showed the maximum induction on laccase production with significant highest activity of (14.8) U/ml. In addition, Mn^{2+}, Zn^2 showed a significant inhibitory effect on the bacterial growth that agrees with Wang et al. [13]**.** 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 [19] .

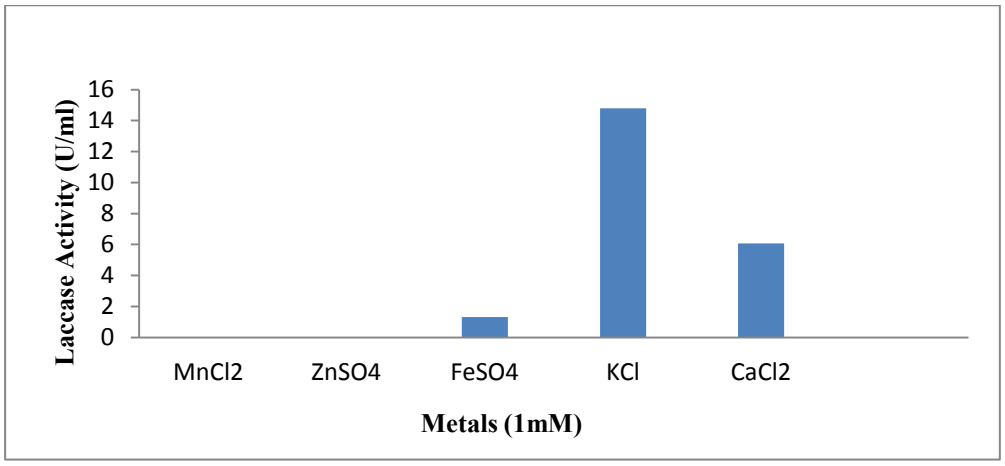

Figure.7-The effect of different metals on laccase production from *Bacillus sp.*B16 after five days of incubation at 35 C° and pH 7.0.

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