Optimum Conditions for Phenol Oxidase (Laccase) Production from *Azotobacter chroococcum* by Submerged Fermentation

تحديد الظروف المثلى لإنتاج انزيم فينول اوكسيديز(اللاكييز) من بكتيريا *Azotobacter chroococcum* باستخدام تخمرات الحالة المغمورة

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Abstract

Phenol oxidases (laccase), copper-containing oxidase enzymes, are found in many plants, fungi and microorganisms. Many factors that influence laccase production from *Azotobacter chroococcum* are determined in this study, these factors are: carbon source, nitrogen source, pH, incubation temperature and incubation period. The results showed that the best carbon and nitrogen sources for laccase production are sucrose and yeast extract respectively, and the best factors for laccase production conditions are pH 7.0 with an incubation period of 6 days at temperature 30 C°.

Key words: *Azotobacter chroococcum*, Phenol oxidase, Submerge fermentation.

الملخص

يعد الفينول اوكسيديز (اللاكييز) من انزيمات الاكسدة المحتوية على النحاس والموجودة في نباتات عديدة, الفطريات والاحياء المجهرية. وثمة عدة عوامل تؤثر في انتاج انزيم اللاكييز من بكتريا *Azotobacter chroococcum* حددت في هذه الدراسة والمتضمنة المصدر الكربوني، والمصدر النيتروجيني، والاس الهيدروجيني، ودرجة حرارة الحضن ومدة الحضن. اظهرت النتائج بان افضل المصادر الكربونية والنيتروجينية لإنتاج انزيم اللاكييز هما السكروز ومستخلص الخميرة تباعاً, اما افضل عوامل انتاج انزيم اللاكييزفتمثلت بالاس الهيدروجيني 7 مع فترة حضن 6 ايام وبدرجة حرارة 30 درجة مئوية.

الكلمات الدالة: *Azotobacter chroococcum*, بولي فينول اوكسيديز, تخمرات الحالة المغمورة

Introduction

Phenol oxidases (laccase) are oxidizing phenolic compounds that use oxygen as an electron acceptor. These enzymes include fungal laccases and prokaryotic laccase-like enzymes that typically have multiple copper (Cu) atoms at the reaction center [1]. 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 [2]. This enzyme is classified as protein that catalyzes the oxidation a wide variety of organic and inorganic compounds by using molecular oxygen as the electron acceptor [3,4]. Laccase exhibit broad substrate specificity toward aromatic compounds containing hydroxyl and amine groups including diphenols, polyphenols, diamines, and aromatic amines [5,6]. Laccase represents an example of a "moonlighting" protein that has multiple functions in addition to its primary catalytic function [7]. Laccases are widely distributed among fungi, plant, insect and bacteria [8]. The first bacterial laccase was detected in the plant root associated bacterium, *Azospirillum lipoferum*, which was shown to be involved in cell pigmentation and utilization of plant phenolic compounds [9]. Laccases play an important role in several industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant and removal of endocrine disruptors. Recently laccases have been efficiently applied to nano biotechnology due to their ability to catalyze electron transfer reactions without additional cofactor [10]. In future, laccase is a useful enzyme for biotechnological application in decolorization and biodegradation of contaminating environmental pollutants. Laccases have many biotechnological applications because of their oxidation ability towards a broad range of phenolic and non-phenolic compounds [11].

Azotobacter is a genus of free-living diazotrophic bacteria whose resting stage is a cyst. It is primarily found in neutral to alkaline soils, in aquatic environments, and on some plants. It has several metabolic capabilities, including atmospheric nitrogen fixation by conversion to ammonia. *Azotobacter spp*. has the highest metabolic rate of any organisms [12]. Fermentation technique is best suited for microorganisms such as bacteria that require high moisture. Submerged fermentation (SmF) utilizes free flowing liquid substrates, such as corn steep liquor, molasses and nutrient broths. The enzymes and bioactive compounds are secreted into the fermentation broth. The

substrates are utilized quite rapidly and hence need to be constantly supplemented with nutrients. The aim of this study is to determine the optimum conditions for phenol oxidase (Laccase) production from *Azotobacter chroococcum* by submerges fermentation.

Materials and Methods

Chemicals

Pyrogallol, nutrient agar, Coomassie blue G 250, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$ and all other chemicals that purchased from Sigma-Aldrich and Hi–Media, India, respectively.

Microorganism and culture conditions

The *A. chroococcum* isolate used in this study was provided by the Biotechnology Department/ College of Science/ University of Baghdad. *A. chroococcum* is reactivated with 1.6×10^6 cells/ml in Burks medium (10g/L Glucose, 0.64 g/L K2HPO4, 0.16 g/L KH2PO4, 0.2g/L NaCl, 0.2g/L MgSO4.7H2O, 0.05g/LCaSO4.2H2O, 0.01g/L NaMoO4.2H2O, 0.003g/L FeSO4), pH 7.0, incubated at 30 ^oC for 6 days. After incubation the culture was harvested (4°C, 20 min, 6,000 rpm) and the biomass was washed twice with phosphate buffer saline (pH 7.2). The cell pellet re-suspended in 100 mM sodium acetate buffer pH 5, and disrupted by freezing for 24 hours until cell lysis. The clarified supernatants obtained after centrifugation (4°C, 10000 rpm for 30 min) the cell lysates was used as a source of crude phenol oxidase [13].

Estimation of Laccase activity and protein concentration

Laccase activity was determined at 30°C using pyrogallol as the substrate. The oxidation of pyrogallol is detected by measuring the absorbance increase at 580 nm after 3 min using a spectrophotometer. The reaction mixture (3 ml) contained 100 µl of suspension, 2.4 ml of 0.1 M sodium acetate buffer, pH 5.0 and 0.25 mM/L pyrogallol. The blank (3 ml) is 2.75 ml of 0.1 M sodium acetate buffer, pH 5.0 and 0.25mM/L pyrogallol. One unit of enzyme activity (U) is defined as the amount of enzyme required to oxidize 1 µM of substrate per minute [13]. Protein concentration is estimated according to the method described by Bradford depending on bovine serum albumin for standard curve preparation using Coomassie blue G-250, measured at 595 nm [14]. The specific activity is determined by using following equation:

$$\text{Specific activity}\frac{\text{U}}{\text{mg}}\text{ protein} = \frac{\text{Enzyme activity U/ml}}{\text{Protein concentration mg/ml}}$$

Optimization conditions for laccase production

There are many factors that influence laccase production from *A. chroococcum*; these factors are; carbon source, nitrogen source, pH, incubation temperature and incubation period.

Carbon source

Ten different carbon sources are added to Burks medium separately, and examined to determine the best carbon source for laccase production from *A. chroococcum*; these sources include; glucose, galactose, lactose, maltose, sucrose, starch, fructose, mannitol, sodium benzoate and cellulose at 0.5% (w/v). The cultures (pH=7.0) incubated at 30^oC for 6 days [15].

Nitrogen source

Nitrogen sources are added to Burks medium (containing sucrose) separately, and examined to determine the best source for laccase production from *A. chroococcum*, these sources were; peptone, NH_4Cl, yeast extract, (NH4)2SO4, NaNO3, Ca(NO3), KNO3 and meat extract at 0.5 % (w/v). The cultures (pH 7.0) incubated at 30^oC for 6 days [15].

Optimum pH

Burks medium including yeast extract and sucrose, was distributed into flasks, the pH adjusted to 3, 4, 5, 6, 7, 8 and 9, then inoculated with 1.6×10^6 cells/ml of *A. chroococcum*, and incubated at 30 ^oC for 6 days. The laccase activity and protein concentration is determined after incubation to determine the optimum pH for laccase production [16].

Incubation temperature

The culture which consist of the Burks medium in addition to yeast extract and sucrose, the pH 7 and inoculated with 1.6×10^6 cells/ml of *A. chroococcum*, incubated under different temperature degrees (25, 28, 30, 35, 37 and 40) ^oC, to estimate the optimum incubation temperature for enzyme production. The enzyme activity and protein concentration were determinated according to [16].

Incubation period

After the inoculation of the Burks medium which contained the yeast extract and sucrose (pH 7.0) with 1.6×10^6 cells/ml of *A. chroococcum*, the culture incubated at 30 °C and checked every day for 7 days to estimate enzyme activity and protein concentration [15].

Results and Discussions

Optimum carbon source

Ten carbon sources were tested for their efficiency in laccase production from *A. chroococcum* . Figure (1) showed that sucrose was the most efficient carbon source for enzyme production, with specific activity 59.1 U/mg. followed by fructose, glucose and mannitol with specific activity of 49.4, 48 and 47.3 respectively. Kenkebashvili *et.al*. [15] found that mannitol was the best carbon source for laccase production by *Coriolopsis gallica* with specific activity of 100 U/mg. while Massadeh *et.al.* [16] found that efficient carbon source for laccase production from *Pleurotus* sp. was lignin indulin.

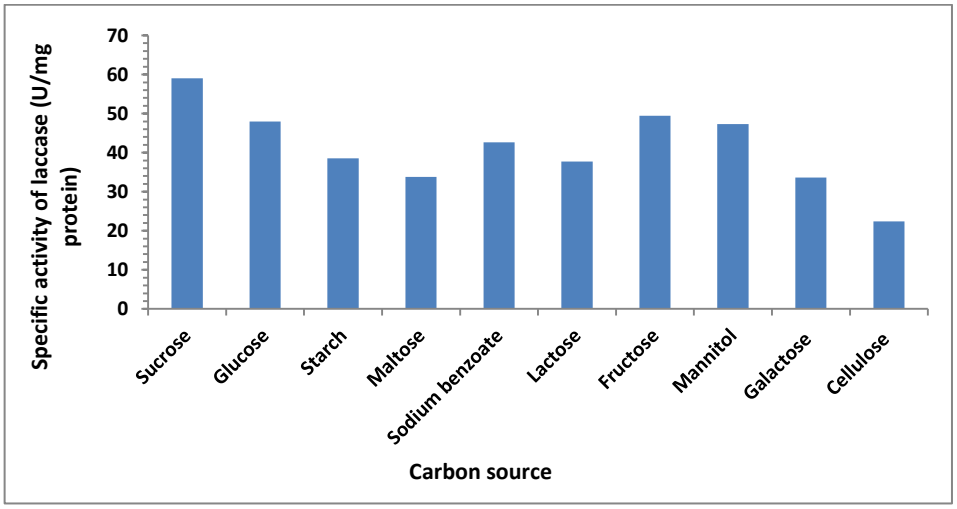

Fig. (1): Effect of carbon source on laccase production by *A. chroococcum*, using Burks medium pH 7.0, incubation at 30 °C for 6 days.

Nitrogen source:

Figure (2) showed that yeast extract was the most efficient nitrogen sources for laccase production from *A. chroococcum*, followed by meat extract and NaNO3 with specific activity of 97.3, 84 and 79.3 U/mg respectively. While other nitrogen sources such as peptone, (NH4)2SO4, NH4Cl, Ca(NO3) and KNO3, had specific activity of 66.6, 66.5, 61.8, 56.6 and 59.8 U/mg, respectively. The presences of nitrogen sources improved laccase production by about 12-46%.

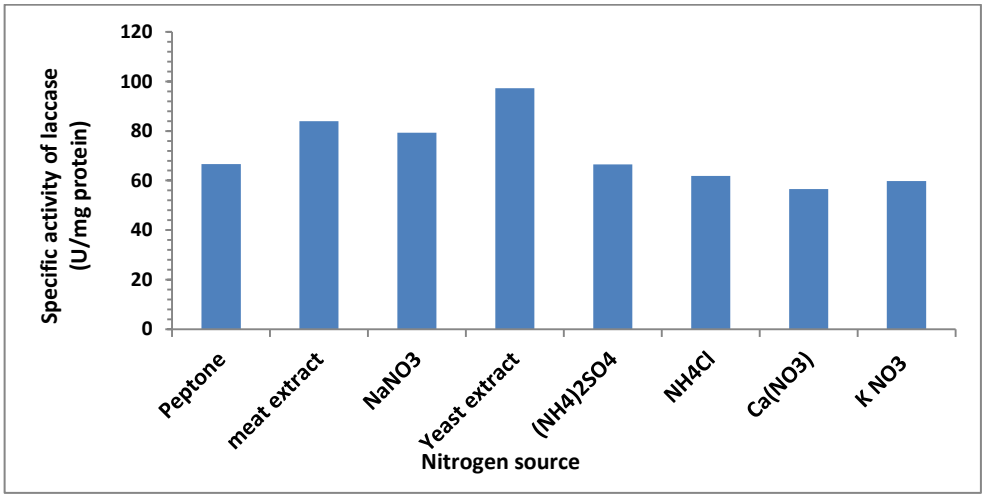

Fig. (2): Effect of nitrogen source on laccase production by *A. chroococcum*, using Burks medium pH 7.0, incubation at 30 °C for 6 days.

Kenkebashvili *et.al*. [15], found that best nitrogen sources for laccase production from *Coriolopsis gallica* was peptone with concentration 1.5%, while Ticlo *et.al.* [17] found that NH4Cl was best nitrogen sources for laccase

production from Marine-Derived Fungus. Furthermore, He, *et.al.* [18] also found that efficient nitrogen sources for laccase production from *Armillariella tabescens* was peptone.

Optimum pH

The specific activity of laccase was estimated to determine the optimum pH for laccase production. The results illustrated in figure (3), showed that the optimum pH for enzyme activity was 7.0 and gave high specific activity (97.7 U/mg), while pH 3, 4, 5, 6, 8 and 9, gave 18.7, 22.3, 35.6, 47.9, 79.5 and 54.3 U/mg respectively. Dhakar and Pandey [19] found that optimum pH for laccase production from *Trametes hirsute* was 7.5, while Diaz *et. al.*, [20] found that best pH for laccase production from *Pleurotus ostreatus* by submerged fermentation was 6.5.

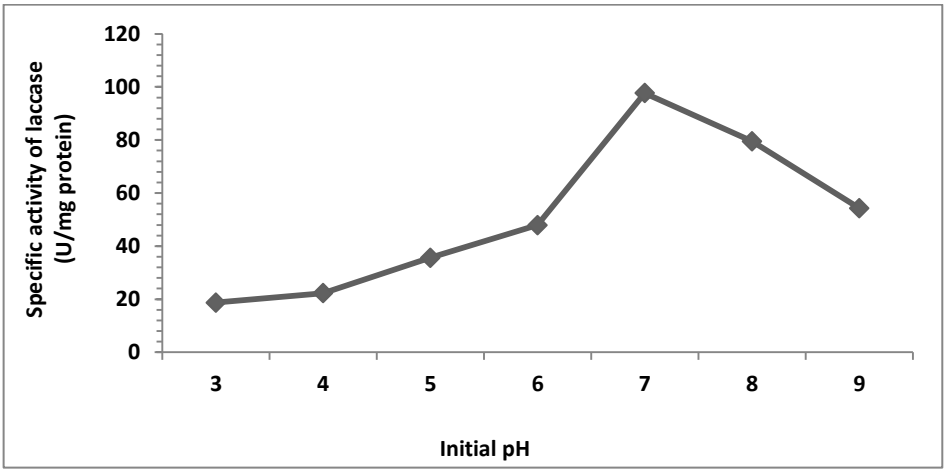

Fig. (3): Effect of pH on laccase production by *A. chroococcum*, using Burks medium incubation at 30 °C for 6 days.

The pH affects in enzyme production because of its role in the solubility of medium substrates and its effect on the ionization of the substrate and it's availability for the microorganisms' growth. Moreover the pH affects the productivity and enzyme stability and due to alterations in three-dimensional protein structure [21].

Incubation temperature

The culture which consists of Burk's medium with pH 7.0, was inoculated with 1.6×10^6 celles/ml of *A. chroococcum* isolate incubated at different temperature degrees (25, 28, 30, 35, 37 and 40±C) to find the optimum incubation temperature for enzyme productivity. The results in figure (4) showed that the optimum incubation temperature was 30±C, which gave the specific activity of 98 U/mg. Lower and higher temperatures decreases the specific activities because of the thermal effects on the microorganism growth and on the enzymatic reaction rate inside the cells which reflected the vital creation of the enzyme. Dhakar and Pandey [19] found that optimum temperature for laccase production from *Trametes hirsuta* was 35±C, while Abdulredha [22] found that the best temperature for laccase production from *Pleurotus* spp. was 25 ±C.

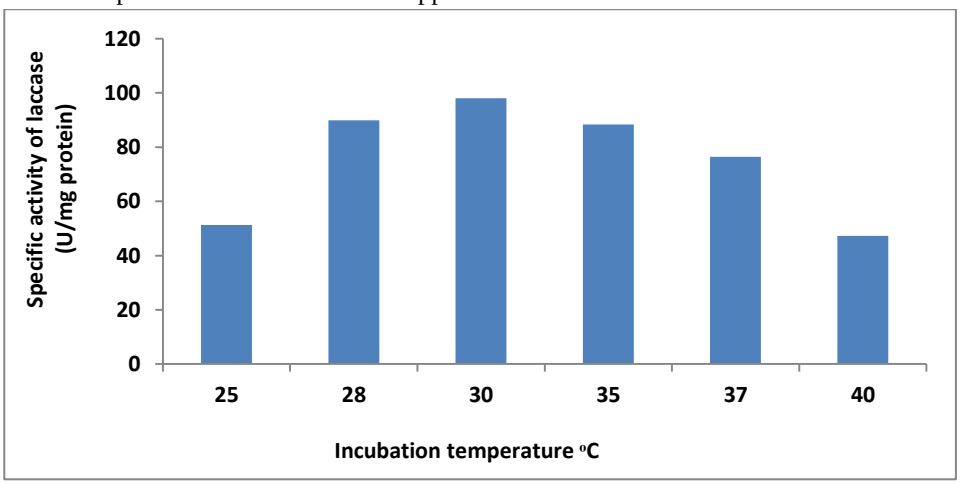

Fig. (4): Effect of Incubation temperature on laccase production by *A. chroococcum*, using Burks medium incubation at pH 7.0 for 6 days.

Incubation period

The results in figure (5) illustrate the effect of incubation period (1–7 days) on laccase production from *A. chroococcum*. The highest specific activitywas 98.2 U/mg, at 6 days of incubation. Packiyam and Ragunathan [23] found that the best incubation period for laccase production from *pleurotus florida* was 7 days, while Elshafei *et.al.* [24] found that the best incubation period for laccase production from *Penicillium martensii* NRC 345 by solid state fermentation was 26 days. Bhuvaneshwari *et.al.* [25] found that the best incubation period for laccase production from *Halobacillus halophilus* was 3 days.

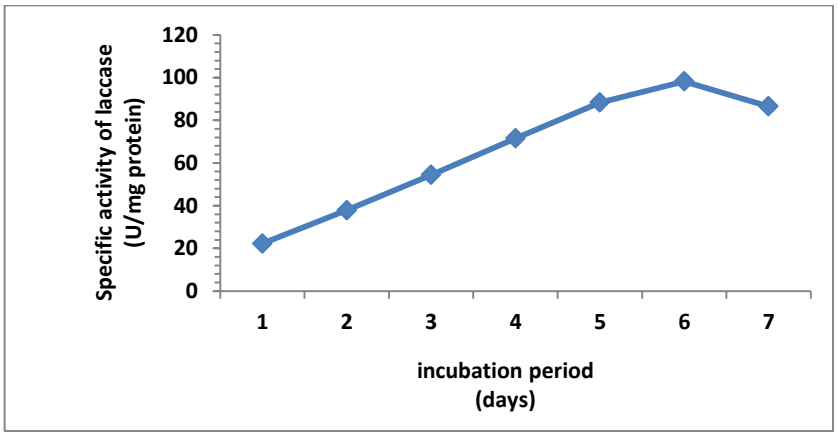

Fig. (5): Effect of incubation period on laccase production by *A. chroococcum*, using Burks medium incubation at pH 7.0 and 30 °C.

The present research demonstrate that the incubation period is a determining factor in the process of laccase production from *A. chroococcum* because the production reaches its maximum value in a relatively gradual manner and then falls. The production decline after attaining the maximum might be due to the depletion of macro-and micronutrients in the fermentation medium with the lapse in time, which stress upon the microorganism's physiology resulting in the inactivation of secretary machinery of the enzymes [25].

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