Study of Production and Characterization of Laccase Enzyme from Klebsiellapneumoniae K7 Isolate

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Abstract

Sixty four local isolated of *Klebsiella* spp. have been isolated from environment samples (soil and water). These isolates were identified and diagnosis according to phenotype and biochemical tests. These isolates were subjected to primary and secondary screening, to select the isolate with the highest laccase activity. Fifteen isolates chosen from primary screening for screening their enzyme activity in secondary screening. It has been found the *Klebsiella*(K7) has the highest productivity of the enzyme (12 Unit/ml).*Klebsiella*(K7) isolate was diagnosis by Vitak 2 system, it was identified as*K. pneumonia.* The laccase purified was characterization, the experiments showed that: The molecular weight of laccase was 120KD and the optimum pH for the purified laccase activity was 4.5 and forstability was 6.5. the optimum temperature for enzyme activity was 40ºC,the enzyme showed that laccase lost 22, 23 and 20% of its initial activity at 20, 25 and 35°C, respectively.

Keywords: *Klebsiella (K7, laccase enzyme, Enzyme characterization, Enzyme kinetic.*

Introduction

Laccase (EC 1.10.3.2) is one of the important of multicopper oxidasesenzymes, these enzymes have the ability to oxidize a wide range of organic, and inorganic compounds, including diphenols, polyphenols, substituted phenols, diamines and aromatic amines, this reaction would reduction of molecular oxygen to water[1]. Bacteria are become a featured source of laccase production, the used of bacterial laccase in different applications are growing rapidly, this is due to many reasons such as bacteria are easy to handle, the word in different environmental conditions and the bacterial laccase activity and stability at high temperatureand natural pH[2]. *Klebsiella* spp. is one of the interesting laccase source[3]. The *Klebsiella*is a genus of Gram-negative, non-motile,facultative anaerobic, rod-shaped (ranging from 0.3 to 1.0 μm in width to 0.6-6.0 μm in length), generally with polysaccharide-based capsule bacteria. Unlike other enzymes of oxidoreductases and peroxidase[4], The bacterial laccases were found to be thermostable and alkaline stable by Sharma *et al.*[5]. Laccase activity vary by changing pH, this variation may be due tothe reaction caused by the binding of enzyme to substrate,ionization of the substrate, oxygen, or the enzyme itself[6]. The most thermos table laccases have been isolated frombacteria; the half-life of *Bacillus subtilis* CotA was 112 min at 80°C[7] and that of *Streptomyces lavendulae* laccase was 100 min at 70°C[8]. These properties of laccase make it has many applications in industrial, environmental (paper, pulp, textiles and bioremediation) and in medical fields (synthesis complex medical products such as antibiotics and anticancer drugs)[9]. The aim of this study is an attempt to screening of laccase producing *Klebsiella*, and select the isolate with the highest enzyme activity and characterization of laccase enzyme.

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Materials and Method

ABTS plate screen assay: The isolates showing methyl orange degradation were further screened for enzyme laccase, on this media which prepared by adding 0.2 mM ABTS and 0.1 mM $CuSO_4$ (after filtering them) in Mineral Salt Agar. Blue green oxidation zone around the bacteria colony indicated the presence of laccase.

Laccase assay: Laccase activity was monitored by measuring the maximum absorption of oxidation of ABTS at 25ºC as a substrate. The reaction mixture containing 1ml of crude enzyme and 1ml of ABTS (0.2mM). The oxidation of ABTS was determined by measuring the absorbance at 420 nm. The blank mixture containing 1ml of sodium acetate buffer (0.1mM, pH5) and 1ml of ABTS . Laccase activity was calculated as follows:

$$\text{Laccase activity } \left(\frac{U}{ml}\right) = \frac{A \times V \times 106}{€\ ABTS \times t \times v}$$

Where: A: Absorbance at 420nm; V: Total volume of reaction mixture in (ml); € : molar extinction coefficient of ABTS =36000$M^{-1}cm^{-1}$; t:incubation time (1min) and v: volume of enzyme used in (ml)[10]. Protein concentration was assayed by the method of Bradford[11].

Effect of pH on Enzyme Activity: The effect of pH on the activity of the laccase was determined at 37°C in 0.05M Tric-HCl buffer (pH 3,3.5,4,4.5,5,5.5, 6) 0.05 M sodium phosphate buffer (pH 6.5,7,7.5,8) and 0.05M Tris-base buffer (pH 8) . The laccase activity was determined at different pH (4-9) by mixing 1ml of ABTS with 1ml of purified enzyme and the activity was measured with different buffers.

Effect of pH on Enzyme Stability: The effect of pH on laccase stability was examined by adding 1ml of enzyme of to a test tubes containing buffer at different pH (3, 3.5, 4,4.5, 5,5.5,6, 6.5,7,7.5,8), and incubated 30min at 37ºC in a water bath, then added the ABTS to mixer and measured the activity with different buffers.

Effect of Temperature on Enzyme Activity: The temperature profile of the purified enzyme was studied by measuring the activity at different temperatures (25,30, 35, 40, 45, 50, 55, 60) ˚C . The purified laccase solution was incubated with ABTS in different temperatures for 10 min and the activity was determined.

Effect of Temperature on Enzyme Stability: The purified laccase was incubated at different temperature ranged between (25,30, 35, 40, 45, 50, 55, 60, 65,70)˚C . followed by incubation in ice bathfor 30min. The enzyme activity was assayed using ABTS, and the relation between remaining activity (%) toward temperature was plotted to determine the optimum temperature of laccase stability.

Laccase Kinetics :K_m, V_{max} values determination: Pre-steady state kinetic analysis was performed using ABTS at different concentration (0.1, 0.15, 0.2, 0.25,0.3mM) in 0.1mM of sodium acetate buffer at pH 5 and estimate the enzyme activity, then the initial velocity (V_o) value was estimated. The relationship between [1/ V_o] versus [1/S_o] was plotted to determine the K_m and V_{max} values according to Lineweaver-Burk reciprocal plot.

Results and Discussion

Screening of *Klebsiella pneumoniae* Isolates for Laccase Production: Out of sixty four *Klebsiella pneumoniae* isolates, there were thirty isolates showed a clear zone around the colony on methyl orange plate, these isolate further screened for enzyme laccase on ABTs plate, it found that twenty one give a positive results . The activity of the enzyme of the fifteen isolates of *K. pneumoniae*, that have ability to produce laccase, was measured to select the highest isolation activity for choose the isolate that have the highest activity (Table 1). The laccase activity was monitored by measuring the maximum absorption of oxidation of ABTS as a substrate.

Table (1) : Screening the *Klebsiella pneumoniae* producing Laccase enzyme.

***Klebsiella pneumoniae* Isolates**	**Enzyme activity (Unit/ml)**
K1	8.23
K2	7.60
K3	10.23
K4	10.06
K5	9.37
K6	6.57
K7	12
K8	6.78
K9	7.83
K10	9.54
K11	6.97
K12	7.94
K15	7.21

Molecular Weight Determination: The moleculare weight of purifed laccase was determinaed by Gel filtration chromatograph, which depending on the size of the separated molecules with their charge[12], using Sephacryl S-200. The results in Figure (2) shows the relationship between logarithm of molecular weight and (Ve/Vo) of four different standard proteins in gel filtration column include Catalase (232KD), Arginine Deaminase (125.892KD), Bovine Serum albumin (67KD) and Pepsin (34.5KD), these proteins and laccase were eluted individually. The results in indicated that laccase has a molecular weight of (120KD). The molecular weight of laccase was determined which that purified from *c-Proteobacterium* JBBy using ABTS as substrate[13]. Also, Siroosi*et al.*[14] estimated molecular weight of laccase from *Bacillus* sp. WT to be 180KD and Verma and Shirkot[15] purified laccase from *Geobacillusthermocatenulatus*MS5 with molecular weight 42.5KD.By using another different substrates of laccase the molecular weight was estimated.

Effect of pH on Enzyme Activity: The optimum pH for laccase activity was determined with ABTS as substrate in a pH range of (3-7) . As results in Figure (1), it was found that the best pH of laccase activity in ranged between (4,4.5 and 5), with a maximum and sharp decline in enzyme activityat pH 4.5 with enzyme activity (2.8 U/ml).While, observed that enzyme activy was reduced at nearly acidic pH (3,3.5) and at alkaline pH (5.5-7).

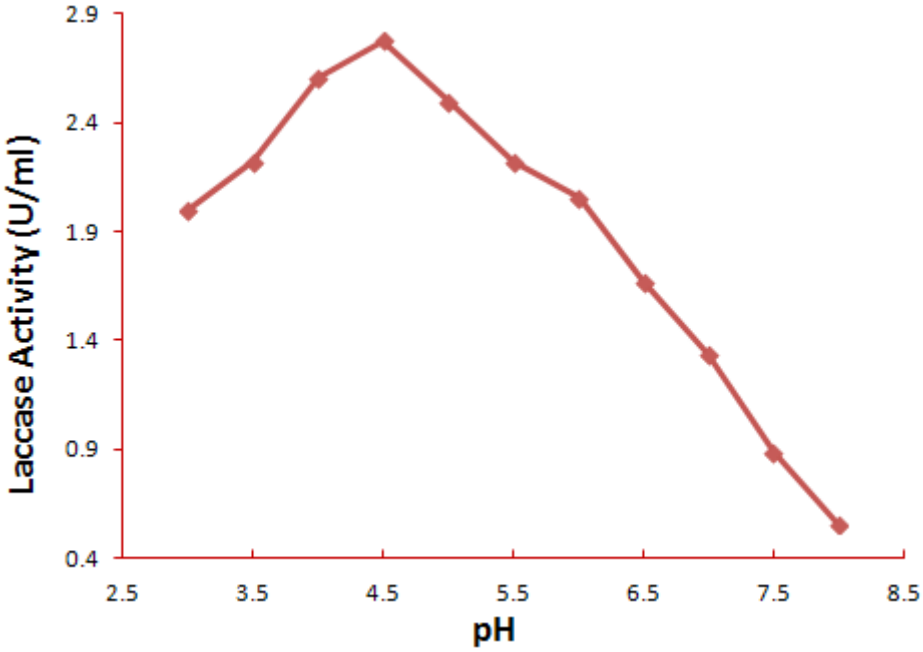

Figure (1): Effect of different pHon Laccase activity.

Verma and Shirkot[15], and Liu *et al.*[16] were purified laccase from *Pseudomonas aeruginosa*, *Ochrobactrum.* sp.531 and *Thermu- thermophilus* SG0.5JP17-16, receptivity by using ABTS as a substrate, found that the optimum pH of laccase activity was at 4.5. The optimum pH on laccase activity was at 4.5, which purified from*Trametes versicolor* .[17]

Compared to phenolic substrates, the redox potential of ABTS does not depend on the pH, oxidation does not involve the protons . So, the effect of pH on the laccase activity towards the different substrates could be related to the balance between the two opposing effects, first the redox potential difference between the substrates and type 1 copper site and second binding of hydroxide anion (OH^-) to the type 2/type 3 copper site[18].

Effect of pH on Enzyme Stability: Figure (2) shown that the optimum pH of laccase stability ranged between 6-7 and the a maximum stability was at pH 6.5, where the enzyme retained 100% of its activity. While, retained 94.42% of its activity at pH 6 and about 94.42% and 82.03% at pH 7.

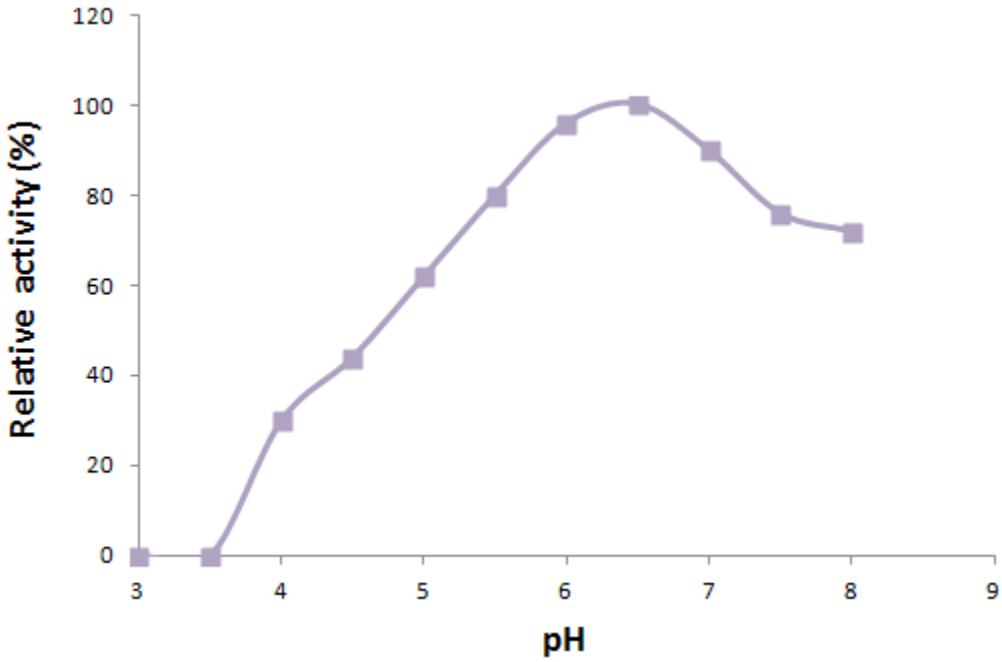

Figure (2): Effect of different pHon Laccase stability

The reason for decreasing in enzyme stability at acidic pH is due to the effect of acidic environment in enzymatic structure which cause ionizing groups in active site. The declining in enzyme activity at pH above the optimum pH may be due to irreversible denaturation of enzyme molecule that leads to change in enzyme structure associated with the formation of enzyme dimerization that leads to enzyme autolysis[19].

Effect of Temperature on Enzyme Activity: The temperature profile of the laccase was studied by measuring the activity in a range of 25–60ºC with 5°C interval. The results in Figure (3) showed an increase in laccase activity towards 40°C with an activity of 3.17 U/ml. After 40°C the activity decrease and the minimum activity was recorded at 60°C which was 2.5 U/ml.

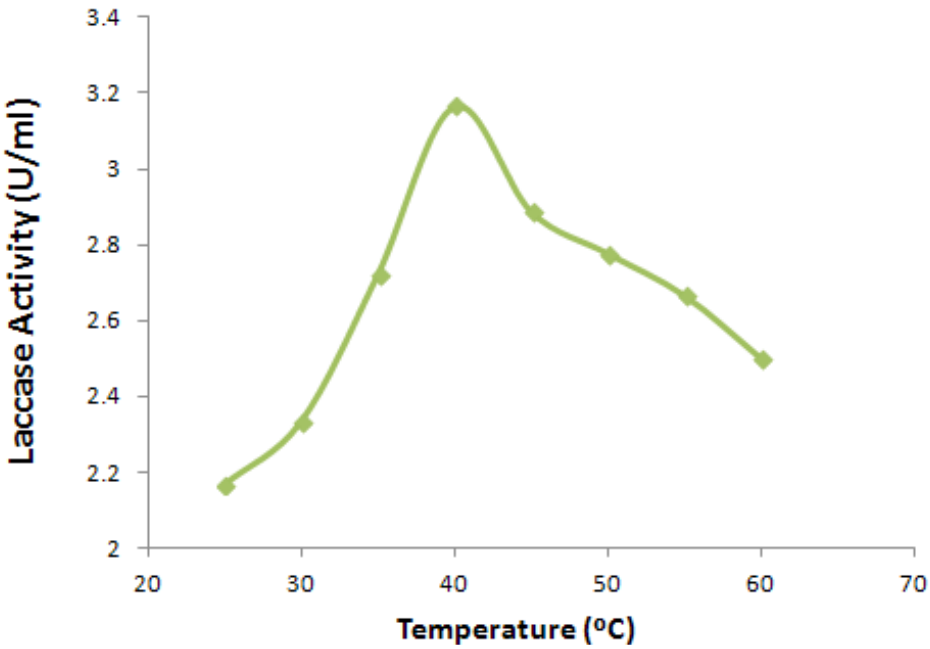

Figure (3): Effect of different temperatures on Laccase activity.

Siroosi*et al.*[20] determined the optimal temperature of laccase activity at 37 °C, which purified from *Bacillus sp. WT*, *Pseudomonas aeruginosa* and *Rhodococcus sp.*, respectively. Demissie and Kumar[21] found the optimal temperature, that purified from *Streptomycetes sp.* was at 35 °C. While, temperature was 35 °C of laccase from *Pseudomonas luridastrain LR5.1* determined by Dhiman and Shirkot[22]. The reason for decreasing of an enzyme activity towards higher temperature is that the speed of enzyme interaction increase with increasing temperature within a certain range due to increased energy kinetics and the collisions between enzyme molecules and substrate, except that the high temperatures within certain limits lead to denaturation of the enzyme and loss of three dimensional structure and then decline in enzyme activity.[23]

Effect of Temperature on Enzyme Stability: The thermostability of laccase was determined at temperatures ranging from 25 to 60°C . The results in Figure (4) showed that laccase lost 22, 23 and 20% of its initial activity at 20, 25 and 35°C, respectively.

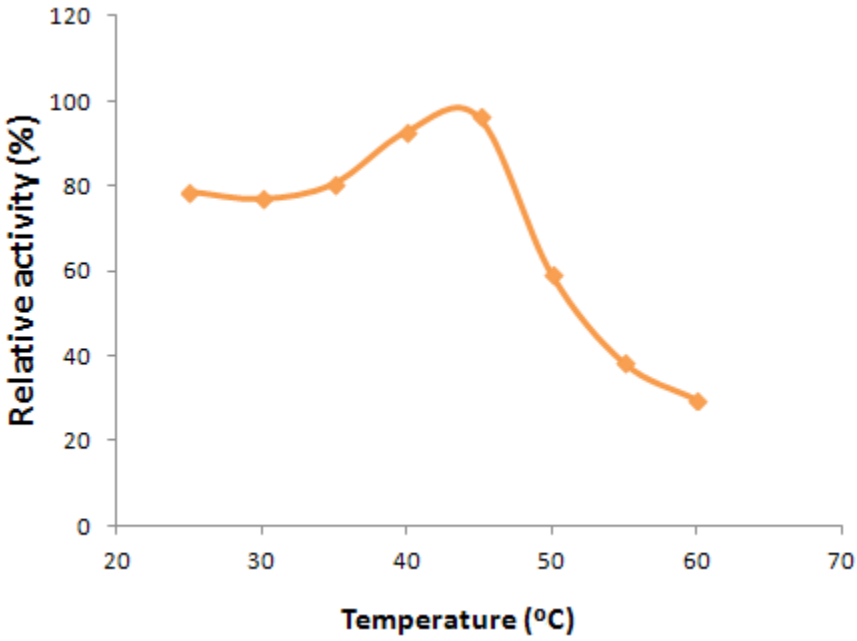

Figure (4): Effect of different temperatureson Laccase stability.

Most cold-adapted enzymes exhibit poor thermal stability at temperatures above 40°C, and their activity decreases as temperature increases.[24]

The reduction of activity above 40°C is due to sensitivity to high temperature, reflecting the effect of temperature on the three dimensional structure of protein by damaging R-groups of amino acids which lead to denaturation of protein and losing its activity. In general, laccases are stable at 30-50ºC and rapidly lose activity at temperatures above 60ºC[25].

Determination Km, Vmax Values: As shown in Figure (5) relation between substrate concentration ABTS and enzyme activity. Activity was measured at different ABTS concentrations (0.1, 0.15, 0.2, 0.25 and 0.3mM) in 0.1mM of sodium acetate buffer at pH 5.

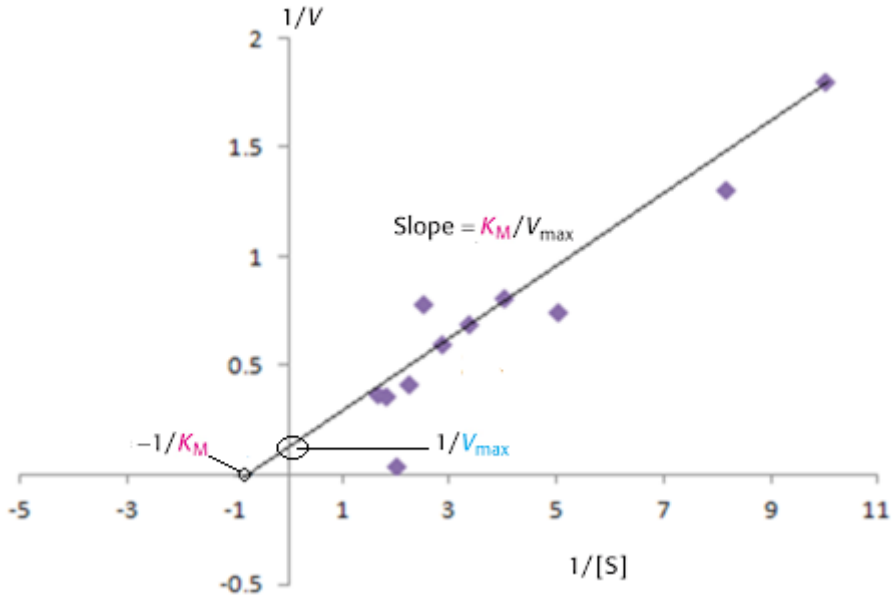

Figure (5): Lineweaver-Burk double reciprocal plot of (1/Vo) versus the [So] for the purified laccase.

Singhal *et al.*[34] characterized laccase enzyme activity produced by *Cryptococcus albidus*, ABTS was used as a substrate. By Michaelis–Menten kinetics the K_m was 0.8158 mM and V_{max} was 1527.74 U/mg. The Km of laccase purified *Bacillus subtilis*CotA by Martins *et al*[7] was 0.106mM. Laccase purified from *K. pneumoniae* by using ABTS as substrate. The K_m values were 0.467, 3.97 and 0.38mM for NITW715076, NITW715076_1 and NITW715076_2 *K. pneumoniae* strains, respectively[26].

Conclusion

K. pneumonia have been proved to its ability to produce laccase. And The molecular weight of laccase was determined at 120KD. The optimum pH of enzyme activity and stability is 4.5 and 6.5, receptivity. The optimum temperature for laccase activity is 40ºC while forstability is (20- 35ºC).

Conflict of Interest: The author knows of no financial interest or any conflict of interest relative to this article.

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Ethical Clearance: Not required

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