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Production and Purification of Laccase Enzyme by Klebsiella pneumoniae K7

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Abstract
Sixty-four isolate were *klebsiella pneumoniae.* Fourteen bacteria isolates "*Kelbsiella* species" were taken from soil and water hospital in the period between October to December 2018, those isolated were cultured on a blood agar to test their ability to hydrolytic due to formation the inhibition zone . Twenty one isolates of *K. pneumoniae* were selected to be cultured in mineral salt agar for testing their efficiency to produce laccase enzyme .The efficient isolate was diagnosed depending on phenotypic, microscopic and biochemical tests to be *Klebsiella pneumoniae* K7. Laccases (benzenediol: oxygen oxidoreductases; EC: 1.10.3.2) are subfamily of multicopper oxidases (MCOs) from *Klebsiella pneumoniae* K7 has been partially characterized by many researchers. In this paper, we purified laccase to homogeneity from *Klebsiella pneumoniae* K7 with about 10.82 ; 5.12 purification fold and a 34.14; 21.46% recovery by ion-exchange and gel-filtration chromatographic respectively. The molecular weight of the Laccase as determined by gel filtration chromatography using Sephacryl S-200 gel was 120 KDalton.
Keywords: Laccases , *Klebsiella pneumoniae,* molecular weight, purification.
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INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases ; EC: 1.10.3.2) are subfamily of multicopper oxidases (MCOs), which catalyze the one electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water. Laccase was first described from the sap of the Japanese lacquer tree Rhus vernicifera. Also laccases are wide spread in fungi, bacteria and insects (Rezaei *et al.*, 2017)[1]. Laccases have a good potential for industrial and environmental applications (Viswanath *et al.*, 2014)[2].

The utilization of bacterial laccases are growing rapidly and became outstanding biocatalysts that applied in different fields. This is because they have many features included: the bacterial systems are easier to handle than fungal ones , work in a broad range of temperature and pH with enormous stability against various inhibitory agents (Guan *et al.* , 2018)[3].Liu *et al.* , (2017)[4] purified thermostable and pH-stable laccase from *K.pneumoniae* which isolated from soil, the laccase cloned and expressed in *E. coli*., rLac purified with the 6-His tag, the cells were harvested by centrifugation , the supernatant was collected by centrifugation and applied to a nickel-nitrilotriacetic acid (Ni-NTA) agarose gel column. The purity and apparent molecular mass of the rLac were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Laccases are exist in a different forms: monomeric, dimeric and tetrameric glycoprotein with molecular mass ranges from 50 to 140 kDa, depending on the organism (Jaiswal *et al.* , 2015)[5] , The molecular weight of bacterial laccases are within the range 60 to 112 kDa (monomer was 32 to 44kDa). The bacterial laccases were found to be thermostable and alkaline stable (Sharma *et al.,* 2007)[6]. Baldrian (2006)[7] suggested that the location of laccase depends on the substrate and its physiological function. Therefore, the aim of the study were to isolate and identify a local laccase producing strain of *Klebsiella pneumoniae*, and to purify and characterize the laccase produced by the bacterium.

MATERIALS AND METHODS

Isolation and identification of bacteria

The Samples of soil and water were collected from different sites in Baghdad city: Tigris , Al Rustamiya Sewage Station, Dora Refinery, Gas station, Electricity generators, Gardens and Water pools at Baghdad University and Car repair places. The isolation of bacteria was based on serial dilution method technique. In this, one gram soil or 1ml of water was added to a tube containing 9ml of PBS solution and mixed vigorously for 30s. The suspension was serially diluted from 10^{-1} to 10^{-8} dilution factors and 0.1ml of each dilution was spread on the surface of MacConkey medium by spread plate method, then the plates were incubated at 37°C for (24-48) hrs and then examined for bacterial growth. After the growth the bacteria isolates, they subculture by streaking on MacConkey agar, incubated at 37°C for 24hrs and take colony for identifying it.

Screening of laccase producing bacteria

The screening of bacteria that have ability to produce laccase were done by using:
Trace Element Solution -1 : 0.10g $ZnSO_4.7H_2O$, 0.03g $MnCl_2.4H_2O$, 0.30g H_3BO_3, 0.20g $CoCl_2.6H_2O$, 0.01g

$CuCl_2.2H_2O$, 0.02g $NiCl_2.6H_2O$, 0.03g $Na_2MnO_4.2H_2O$ dissolved in 1000ml Distilled water.
Trace Element Solution -2: 0.10g EDTA, 0.03g $FeSO_4.7H_2O$, 100 ml Trace Element Solution-1 dissolved in 1000ml Distilled water.
Mineral salt media 3.5g $Na_2HPO4.2H_2O$, 1.0g KH_2PO_4, 0.5g $(NH_4)_2SO_4$, 0.1g $MgCl_2.6H_2O$, 0.05g $Ca(NO_3)_2.4H_2O$ and 1 ml Trace Element Solution-2

These components were dissolved in 900ml of distilled water (D.W) , pH was adjusted to (7±0.2) , and the volume completed to 1000ml . The media then sterilized by autoclave at 121ºC and 15 psi for 15 min.

Methyl orange plate screen assay

This prepared in by adding 0.1g/l of Methyl Orange (after filtering it) in sterilized Mineral Salt Agar. Drop 5µL of overnight culture onto the plate (when this dries it will form a little circle), incubated at 37ºC for 24hrs. A clear zone of methyl orange hydrolysis around the drop was an indication of laccase secretion.

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 using a spectrophotometer . The blank mixture containing 1ml of sodium acetate buffer (0.1mM, pH5) and 1ml of ABTS . Laccase activity was calculated as follows:

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

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)

One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of substrate per minute. Protein concentration was assayed by the method of Bradford (1976)[8] and Stoscheck (1990)[9], with bovine serum albumin as standard.

Crude enzyme

The isolate , that have the highest enzyme activity, was incubated into fifty hundred milliliter of Luria bertani broth in sterile Erlenmeyer flasks at 37°C for 24hrs on a shaker incubator with 120 rpm. After 24hrs, the broth culture was filtrate by using cooling centrifuge at 15000rpm and 4°C for 5min to obtain supernatant, this supernatant used as crude laccase, which stored at 4˚C for further purification steps. The laccase activity in the supernatant was determined spectrophotometrically with the ABTS assay.

Ammonium Sulfate Precipitation

The ammonium sulfate was added in different saturation ratios (0-100%) into crude enzyme. To reach the optimum ratio of ammonium sulfate, the amount of salt was add gradually to crude enzyme solution in ice bath with gently magnetic stirrer , to avoid foaming, for 1hr. The resulting solution was kept stationary overnight and centrifuged at 10000 rpm for 20 min, dropped the supernatant and take the precipitate , dissolved it in 5ml Tric-HCl buffer (0.05M, pH=8.0), and calculated the activity and protein concentration. The supernatant was then subjected to ammonium sulfate precipitation. The precipitate obtained was dialyzed and then loaded onto a DEAE-Cellulose anion-exchange column.

Ion exchange chromatography

The exchanger DEAE-cellulose was prepared and packed into column following the method described by Whitaker, (1972)[10]. A column with a diameter of (7.5 x 2.5 cm) was equilibration with 0.05 mM Tris-HCl buffer pH 8.0. Partially purified concentrated laccase enzyme (12 ml) were separately passed after loaded onto the column carefully. Then (100 ml) of (0.05mM) Tris-HCl buffer pH (8.0) was added. Proteins were eluted by using (200 ml) of a stepwise salt concentration from (0.125-1.0 M) in 0.05mM Tris-HCl buffer (pH 8.0). Fractions of (5 ml) were collected and absorbency was monitored at (280 nm). The presence of the Laccase enzyme were estimated from each fraction of the major peaks then protein concentration and specific activities were determined for the collected active fractions.

Gel filtration chromatography

Sephacryl S-200 column (60x1.5cm) was prepared and packed according to the instruction of the manufacturing

company. The column was equilibrated with (0.05 mM) Tris-HCl buffer (pH-8.0) at a flow rate of (50 ml/hour). A (5 ml) sample of each concentrated partially purified laccase enzyme was added to the column, carefully using pasture pipette. A (5 ml) fraction were collected for each laccase then protein contents were estimated by measuring the absorbance at (280 nm). Laccase activity was determined for each fraction of the major peaks. Protein concentrations and specific activities were also determined for the collected fractions of the major peaks of the Laccase enzyme.

Determination of the molecular weight

Molecular weight was determined by gel filtration chromatography by using Sepharose 6B, (Laue and Rhodes, 1990)[11] and the standard proteins used for the standard curve and their relevant molecular weights were (Catalase, 230000; Arginine deaminase , 125000; Alkaline phosphatase, 80000; Bovine serum albumin, 67000; Pepsin 34000).

RESULTS AND DISCUSSION

Isolation and Identification of the Bacteria :

Fifty eight samples that collected of soil and water samples were used for isolated *klebsiella*. , several morphological, physiological and biochemical tests were made to identify the isolates. The results of bacterial subculture on MacConkey and biochemical test showed that 64 isolate were *k. pneumoniae.* (Table 1).

Table (1): Biochemical test of *klebsiella*.

Biochemical test	*Klebsiella*
Catalase	+
Oxidase	-
Indole production	-
Voges proskauer	+
Methyl red	-
Simmon's citrate	+
Urease	+

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 twenty one 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 2). The laccase enzyme extraction then the laccase activity was monitored by measuring the maximum absorption of oxidation of ABTS as a substrate.

Table (2) : Screening the *Klebsiella pneumoniae* producing Laccase enzyme.

Klebsiella pneumoniae Isolates	Enzyme activity (Unit/ml)
K1	4.69
K2	6.11
K3	5.54
K4	8.23
K5	7.60
K6	6.57
K7	12
K8	6.78
K9	7.83
K10	9.54
K11	6.97
K12	7.94
K13	10.23
K14	6.50
K15	7.21
K16	9.37
K17	7.08
K18	10.06

Klebsiella pneumoniae Isolates	Enzyme activity (Unit/ml)
K19	8.46
K20	5.26
K21	5.94

Crude Extraction:
The laccase enzyme was extracted from the local isolation of *K. pneumonia* after incubated in Luria broth in the shaker incubator at 37° C for 24 hrs . The production medium contains the laccase enzyme that was excreted outside the cell bacterial to the medium of culture production. After centrifuge , the volume of crude enzyme was 300ml with enzyme activity 146 U/ml and specific enzyme 0.695 U/mg protein, as shown in Table (3).

Ammonium Sulfate Precipitation:
The crude enzyme extract was fractionated with different percentage of ammonium sulphate (0- 100%) . The results of specific activity of supernatant were showed in Table (3) , the higher specific activity was (1.21U/mg) at (60-80%) than other saturation of ammonium sulphate.

Table (3) : Fractionated Laccase produce from *K. pneumonia K7* with different percentage of ammonium sulphate.

Ammonium sulfate (%)	Specific activity (Unit/mg protein)
0-20	**0.12**
20-40	**0.78**
40-60	**1.12**
60-80	**1.21**
80-100	**1.16**

Ionic Exchange Chromatography :
Laccase enzyme was obtained by using buffer solution at concentration of 20mM Tris –base pH 8.3. Absorbance of eluted fractions were measured at 280 nm upon the arrival of absorbance to the line of zero (line base), then same buffer with stepwise of NaCl (0.125-1M) used to elute the bounded protein. Ionic exchange chromatography patterns showed two protein peak in wash elution and two peaks in stepwise elution. Only one peak among the stepwise elution peaks represented enzyme activity (tubes 28- 39). Those fractions pooled and tested for specific activity (3.65 U/mg) a fold purification of (5.12) and enzyme yield of (34.14%) in parts. (Figure 1).

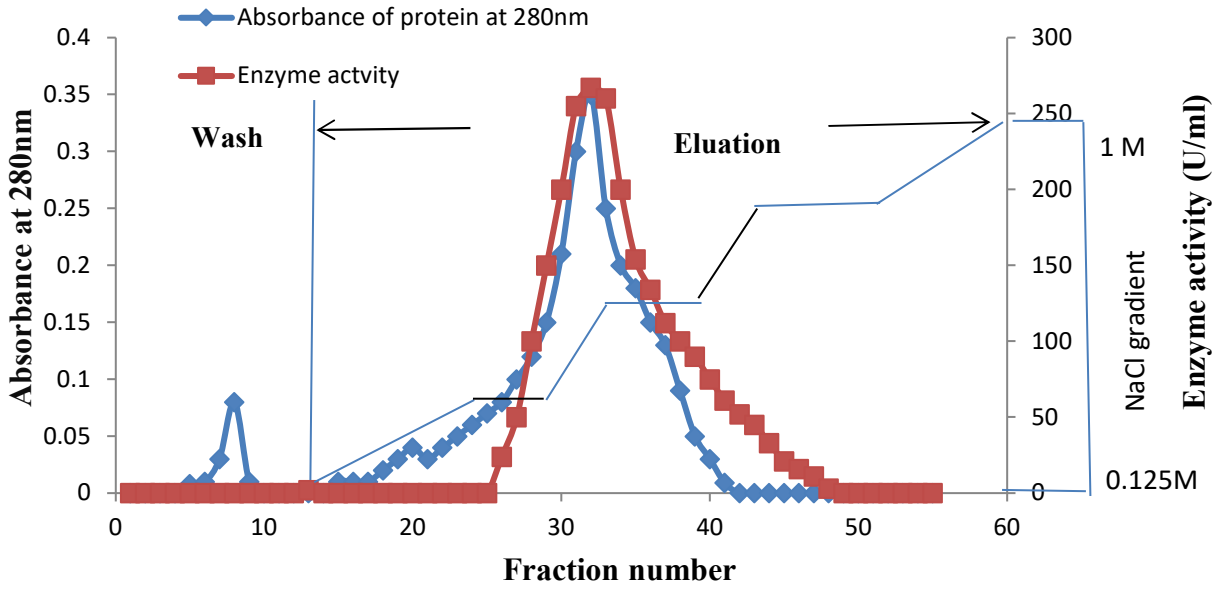

Figure (1): Ionic exchange chromatography for Laccase enyme from K. puomonae K4 through DEAE-cellulose column (2.5 X 7.5 cm). The column was calibrated with 0.05 mM Tris-HCl buffer pH 8.0 flow rate 60ml/hrs and 5 ml fraction, eluted with stepwise (0.125-1 M) NaCl.

Gel filtration Chromatography:

Purification carried out by a gel filtration using Sephacryl S-200. Enzymes fraction from DEAE cellulose were pooled and passed through gel filtration column. The fractionation yielded two protein peaks as absorbance reading at 280nm (wave length), only one peak where appeared when reading absorbance at wavelength of 280 nm and when determined for enzyme activity in resulting parts enzyme activity recorded in 16-20, the specific activity reached 7.52U/mg ,fold of 10.82 and a yield 21.46% as mentioned in table (3) and figure (2). Laccase from *T. versicolour* is purified with Ion Exchange chromatography followed by gel filtration with specific activity of 101UmL−1 and 34.8 fold purification. (Cordi *et al*,, 2007)[12].

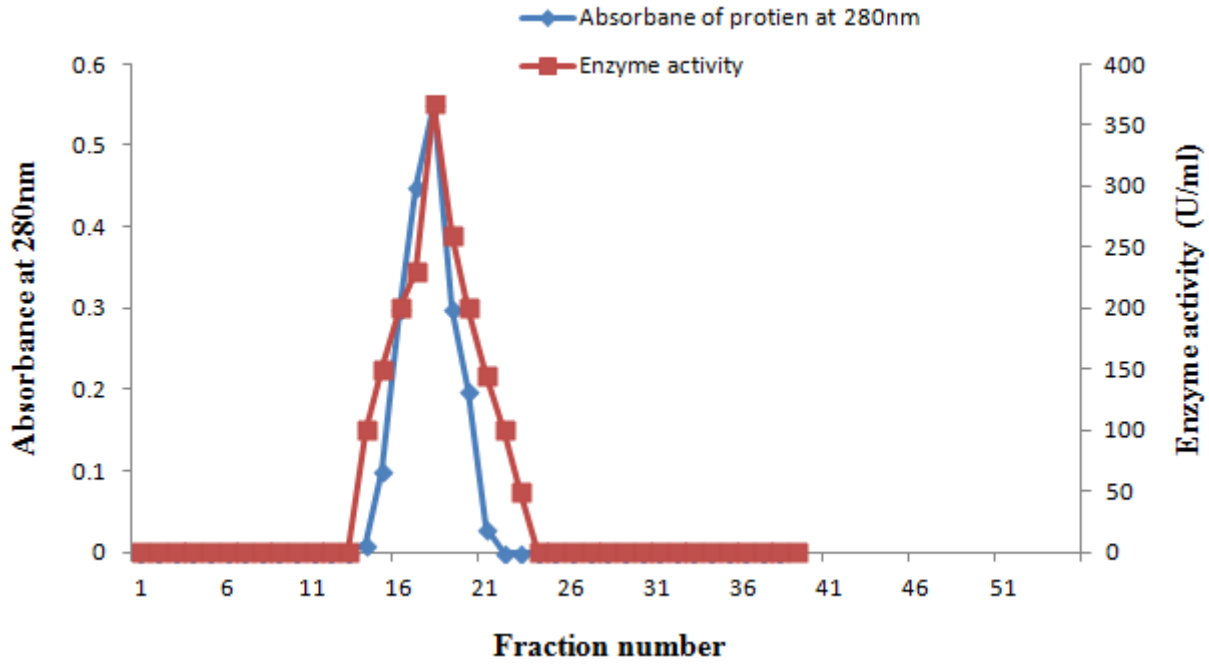

Figure (2): Gel filtration chromatography for purification Laccase from *Klebsiella pneumoniae* K4. using Sephacryl S-200 column (60x2.0cm) cm . The column was calibrated with 0.05 mM Tris-HCl buffer pH 8.0 ; flow rate 50 ml/hrs and 5 ml/fraction.

Table(3):Purification steps of Laccase produced from *Klebsiella pneumoniae* K4.

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yield (%)
Crude enzyme	300	146	210	0.695	43800	1	100
Ammonium Sulfate Precipitation	132	213	176	1.210	28116	1.74	64.19
Ion exchange	56	267	75	3.56	14952	5.12	34.14
Gel filtration	25	376	50	7.52	9400	10.82	21.46

The molecular weight was estimated by gel filtration depending on the size of the separated molecules with their charge .It was possible that the different methods of estimation may be used.(Segal,1992)[13]. Sephacryl S-200 for gel filtration was used for estimation the molecular weight of Laccase enzyme that purified from *Klebsiella pneumoniae* K4 illustrated in figure (3). The logarithm molecular weight standard protein versus elution volume / void volume ratio (Ve/ V0) was the basis to estimate the molecular weight. By using this relationship, the molecular weight of the enzyme was estimated with 120000 Dalton.

This result, compared with Singh *et al.* (2008)[14] determined the molecular weight of laccase that purified from *c-Proteobacterium* JB By using ABTS as substrate .Also, Siroosi *et al.* (2016)[15] estimated molecular weight of laccase from *Bacillus* sp. WT to be 180KD and Verma and Shirkot (2014)[16] purified laccase from *Geobacillus thermocatenulatus* MS5 with molecular weight 42.5KD.

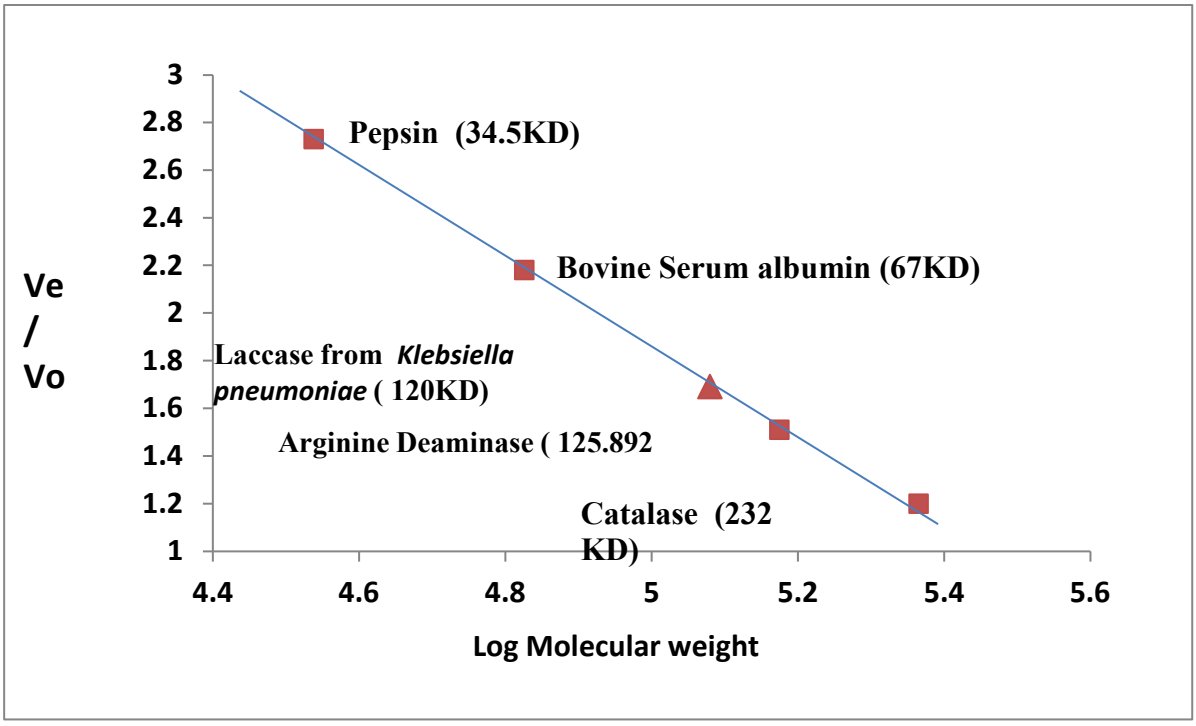

Figure (3): Standard curve to estimate molecular weight of Laccase enzyme produced by *Klebsiella pneumoniae* K4 using gel filtration by using Sephacryl S-200.

Conflict of Interest

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

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