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EXTRACTION, PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR PULLULANASE BY *KLEBSIELLA PNEUMONIAE* ISOLATED FROM SOIL

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ABSTRACT

Many soil samples collected from different locations in Mosul city in Iraq showed existence of bacterial isolates produce pullulanases, seven isolates were obtained and the most prominent one was denoted No5 in terms of the decomposition halo diameter, which reached 8.46 mm, and when the isolates subjected to primary screening by cultured on pullulan-supported medium it was identified as Klebsiella pneumonia after a number of diagnostic and biochemical tests. A number of sequenced steps for enzyme purification included precipitation with 75% saturation of ammonium sulphate which record enzyme activity 93.05 U/ml, followed by dialysis step for 24 hours. The purification step by using Sephadex-G100 gave 19.013% as enzymatic yield with 74.581 folds. It is cleared by using electrophoresis, that the molecular weight of the enzyme was 94 KD. By studying some enzyme characteristics, the results showed that optimum pH for activity was 6 while the optimum pH for stability was 6-7, and the optimum temperature was 60ºC, while 95% of enzyme activity was retained at 50-60ºC.

Keywords: *characterization, Extraction, Klebsiella* pneumoniae, *pullulanase, purification.*

INTRODUCTION

Pullulanase (pullulan-6-glucanohydrolase, EC 3.2. 1.41) is acting on branched substrates, it could be used in glycogen hydrolysis and amylopectin, by cleaving α-1,6-glucosidic linkages in amylaceous polysaccharides (Kahar, et al., 2014). Many kinds of bacteria such as *Aerobacter sp.*, *Bacillus sp.* and *Klebsiella sp.* could produce pullulanase, also yeasts, fungi, plants and animals and most pullulanase are type II pullulanases (Kim, et al., 1993). A few types I pullulanases were investigated in gene level, such as *Bacillus flavocaldarius KP 1228*. Because of their specific action on α- 1, 6 linkages, microbial pullulanase attracts more interest, they have a great significance for their applications. It degrades starch to glucose and maltose. For that these enzymes are used on a large scale in glucose and maltose syrup industries (Khabade, et al., 2016). The importance of this enzyme is hydrolyzing the branch points in the amylopectin, while glucoamylase or β-amylase has only to hydrolyze the linear α-1,4-glucosidic linkages, so using both enzymes in combination during saccharification process would allow for more efficient and rapid conversion reactions.

The type I of pullulanase has been characterized from *Aerobacter aerogenes* bacteria (Ohba and Ueda,1982), and from *Bacillus acidopullulyticus*, *Klebsiella pneumonia* and *Streptomyces sp.* on the other hand it has been extracted from moderate thermophilic gram positive bacteria such as *Bacillus flavocaldarius*, *Bacillus thermoleovorans*, *Clostridium sp*. Pullulanase type I has also been reported from *Fervidobacterium pennavorans* (Khabade, et al., 2016; Yao, et al., 2013).

MATERIALS AND METHODS

Samples collection: Soil samples were collected from 5 cm depth from different locations in Mosul city, stored at 4ºC. The bacteria were isolated by taking 1 gram of each sample and preparing five serial dilutions and using the method of pour plates which were incubated at 37°C for 24 h. Then the isolates were sub cultured to obtain pure culture.

Primary screening of pullulanase producing bacteria: The isolated bacteria were screened by cultivate them in nutrient broth at 37°C for 24 hr. and then plating 5µl from each on pullulan agar medium (g/l) which contain: 10 g pullulan, 2g NaCl, 0.1g $MgSO_4.7H_2O$, 0.17g K_2HPO_4, 0.12g $KH_2PO_4.7H_2O$ and 15g agar, pH 7.5 and then the plates were incubated at 37°C for 48 h. Presence of clear zone around the colonies after 48 h of incubation indicates presence of pullulanase. The zone diameter (H) in mm and colony diameter (C) in mm were measured. Degradation efficiency of all the pullulanase producer isolates was calculated using the formula (H-C)/C (%) (Waleed, et al., 2015). The bacterial isolates which showed efficiency above 50% were selected and subjected to secondary screening. The plates were 2 flooded with Gram's iodine solution (0.1% I and 1% KI), then incubated at 30°C for 24 hour and choose the colonies with the largest zone for further investigation.

Secondary screening of pullulanase producing bacteria: A loop full of selected bacterial isolates was transferred in 50 ml of pullulan medium broth (inoculation medium) for the preparation of

inoculum. The flask was incubated in a shaker incubator at a speed of 200 rpm at 37°C for 24 h. the production medium was inoculated with 10^7 cfu of bacterial inoculum. The flask was incubated in a shaker incubator at a speed of 200 rpm at 37°C for 48h. After incubation, the production was centrifuged at 7000 rpm for 10 min in a cooling centrifuge. Supernatant was collected and used for the pullulanase assay (Hii, et al., 2012).

Identification of Bacteria: The morphological and biochemical parameters for the selected bacterial isolate that show high pullulanase activity was determined as described in the Bergey's manual of systematic bacteriology (Bergey, 2010).

Production medium: Extracellular pullulanase was obtained by *K. aerogenes NCIM 2239* in a modified mineral Czapek medium according to Hii, et al., (2009) with maltose as a carbon source.

Pullulanase assay: For determination enzyme activity, 0.5 ml of enzyme sample was added into 0.5ml of 1% (w/v) pullulan in 0.02 M sodium phosphate buffer with pH 6.9. The reaction mixture was incubated at 40°C for 30min and immediately allowed to cool, adding 3 ml of DNS solution to stop the reaction followed by heating for 10minutes. The OD was taken at 540nm. Standard curve was made by taking OD of sample containing 1 ml of glucose solution in the range of 50 micro moles to 400 micro moles, 0.5 ml of pullulan in water and 0.5ml pullulan in phosphate buffer. One unit of pullulanase activity is defined as the amount of enzyme required to produce 1.0µmol reducing sugar (equivalent to glucose) /min under the assay conditions.

Protein determination: The protein concentration was measured according to Bradford method with bovine serum albumin as a standard curve at 280nm.

Pullulanase purification: Many steps were followed; first step was ammonium sulfate precipitation by added ammonium sulfate up to 80% saturation then centrifuged at 20,000 rpm for 5 min and the pellet was suspended and dissolved in 0.02 M sodium phosphate buffer, pH 6.9. The second step was dialyzed the sample against the same buffer until complete removal of ammonium salts was achieved. All steps of enzyme purification were carried out at or below 4ºC.

Third step was gel filtration by using Sephadex G-100 (1.5cm x 70 cm), which was prepared according to Pharmacia Fine Chemicals Company. Three ml of concentrated enzyme solution was added to the column equilibrated previously with 0.02M phosphate buffer pH 6. And eluted using the same buffer with flow rate of 30 ml/hour (5ml for each fraction). Absorbance of each fraction was measured at 280 nm. Enzyme activity and protein concentration was also determined in each fraction, were pooled and kept at 4°C for further studies.

Determination of molecular mass by SDS-PAGE: The molecular mass of the purified pullulanase was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Staining was done by Coomassie blue and then de-stained using a mixture of methanol, glacial acetic acid and distilled water. The molecular weights of the proteins were determined using the standard protein mixture of 220, 97, 66, 45, 30, 20 and 14 KD.

Effect of pH: Optimum pH for pullulanase activity was studied at pH 1-10 by using pullulan as a substrate. The pH stability of the enzyme was studied by incubating the enzyme in buffers at differrent pH for 24 h at 40°C. The buffer systems were: 0.02 M potassium chloride-HCl buffer for pH 1.0-2.0; 0.02M glycine-HCl buffer for pH 3; 0.02M acetate buffer for pH 4-5; 0.02M phosphate buffer for pH 6-8 and 0.02 M glycine-NaOH buffer for pH 9-10.

Effect of temperature: The reaction mixture was incubated at different temperatures for 30 min with and without the present of Ca^{2+} ion in order to determine optimum temp. Thermal stability was determined by incubating the enzyme sample in different temperatures for 30 min and then residual pullulanase activities were assayed.

RESULTS AND DISCUSSION

Primary Screening: The results of detection pullulanase produced bacterial isolates we found three out of seven isolates that produced the enzyme when they cultured on detective media by determination the clear zone dimeter as shown in table 1, the highest clear zone diameter (analyzing ability) was observed for the isolate no.5 which was 8.46 mm followed by the isolate no.4 (7.15 mm), other isolates were varied and the lowest one was no.7 which was 6.82mm. These differences between isolates could be due to the way of consumption carbon source by bacterial strains which catalyze the production of enzyme.

Table 1: Screening of bacterial isolates from soil for pullulanase activity

No. of isolates	Zone of Hydrolysis (mm)
1	2.54
2	4.62
3	5.20
4	7.15
5	8.46
6	5.95
7	6.82

Secondary screening: The three selected isolates for their pullulanase production on plates were re-cultured in liquid media for pullulanase production by submerged culturing for choosing the best one in pullulanase production. As shown in table 2, the isolate no.5 gives the highest pullulanase activity which was 87.21U/ml., while the others no. 4, no.7 were 68.99, 54.54U/ml, respectively.

Table 2: Production of pullulanase by submerged cultures

No. of isolates	Activity of pullulanase (Unit/ml)
4	68.99
5	87.21
7	54.54

Identification of isolated bacteria: The selected isolate no.5 as a best isolate for pullulanase production was then identified by microscopic test and bio-chemical tests. Results showed that the colony is pink, shiny and mucus, under microscope it is G-ve, single or double or short chains. The results of biochemical tests are shown in table 3, it is non motile, oxidase negative, indole negative, methyl red negative, catalase positive, Nitrate oxidase positive, Voges proskauer positive, citrate consumption positive, carbohydrate fermentation positive and urease test positive, according to these results this isolate is *Klebsiella pneumonia*.

Table 3: Biochemical characteristics of Isolate No.5

Biochemical Tests	Result
Gram stain	-
Oxidase	-
Catalase	+
Nitrate oxidase	+
Voges Proskauer	+
Methyl red	-
Indol	-
Citrate	+
Carbohydrate fermentation	+
Motile	-
Urease	+

Purification of pullulanase: The purification steps of pullulanase are shown in table 4, first step is using ammonium sulphate (40-80) % saturation and the specific activity was 8.720 U/mg which raised in next dialysis step, it was 18.472 U/mg with 6.566 folds, this indicates to release a large amount of the salts. This result agreed with Khalaf and Aldeen (2014) who mentioned to rising of specific activity and folds after using ammonium sulphate concentration. The final step of purification by gel filtration achieved 74.581 folds and it was clear there was one peak had the activity as shown in figure 1. This method used later in molecular weight determination.

Some researcher purified pullulanase from *K. pneumonia* gained 1884.8U/mg with7.8 yield and 52.6 folds by using Sephadex G-100 (Yoo and Yu,1997). Others found that the pullulanase from B. lichenoformis BS18 by using Sephacryl S-200 gained specific activity 247.3U/mg with 37.6% yield and 7.92 folds (Khalaf and Aldeen, 2014).

Table 4: Purification of pullulanase produced by K. pneumonia

Purification step	Volume (ml)	Enzyme activity (u/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity	Yield (%)	Purification folds
Crude enzyme	100	64.85	23.05	2.813	6.485	100	1
Ammonium sulfate precipitation (75%)	52	93.05	10.67	8.720	4.838	74.602	3.099
Dialysis	35	120.44	6.52	18.472	4.215	64.996	6.566
Gel Filtration Sephadex G-100	7	176.23	0.84	209.797	1.233	19.013	74.581

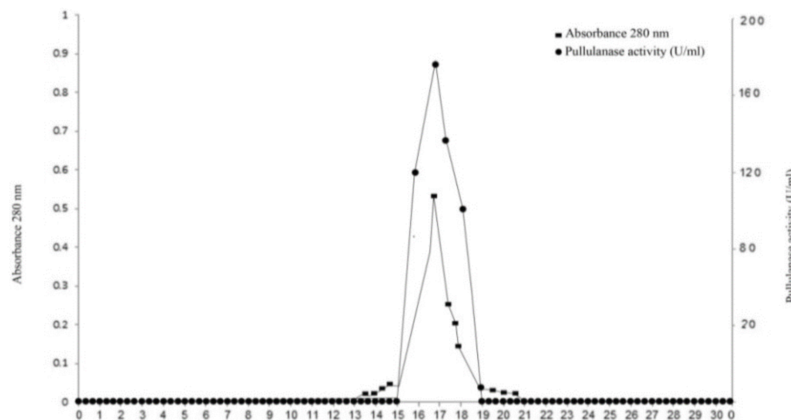

Fig. 1: Purification of pullulanase enzyme produced by K. pneumonia by gel filtration method Sephadex G-100 (1.5 cm x 70 cm) equilibrated by phosphate buffer (0.02M), pH (6) with flow rate 30ml/hr.

Molecular weight for purified enzyme: The purified enzyme from K. pneumonia was added on acrylamide gel with SDS, there was one band with 94 KD according to the bands of standard proteins as shown in figure 2.

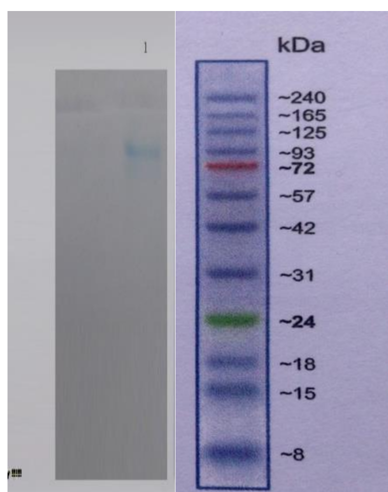

Fig. 2: Purified enzyme from *K. pneumonia* on acrylamide gel with SDS

Effect of pH on enzyme activity and stability: The results of studying the optimum pH for pullulanase activity showed that the best enzyme activity was 140.22 U/ml at pH 6.0 as shown in figure 3, while it was decreased to 126.66 U/ml at pH 7.0, and that because of changing in the active site of the enzyme or may be the ionic groups affected by changing pH. This result agreed with Shehata (2016) who found that the best pH for pullulanase activity from white edible mushroom was 6.0 and with Khabade, et al., (2016) who found that the best pH for pullulanase activity from *Bacillus sp.* was 6-7, while other research found that the optimum pH for pullulanase activity from *K. pneumonia* was 5.0 (Yoo and Yu, 1997).

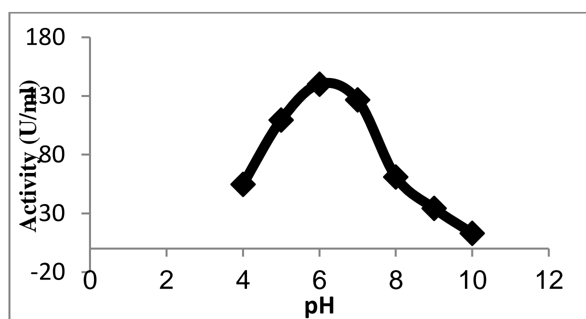

Fig. 3: The optimum pH for pullulanase activity from *K. pneumonia*.

Pullulanase showed stability at pH 6.0-7.0 (Fig. 4) it kept about 90% of its activity, in another hand it lost 52% of its activity at pH 4.0. The enzymatic activity seemed be more stable at neutral or semi-neutral pH, while it's negatively affected at lower or higher pH. This result agreed with Khabade, et al., (2016) who found that the pullulanase enzyme from *Bacillus sp.* was stable at 6-7, while Yoo and Yu (1997) found that the enzyme from *K. pneumonia* was kept 80% of its activity at pH range 4-7. In another hand Rajaei, et al., (2014) found that the pullulanase enzyme from *Exiguobacterium sp.* was stable at pH range 7.0-8.0.

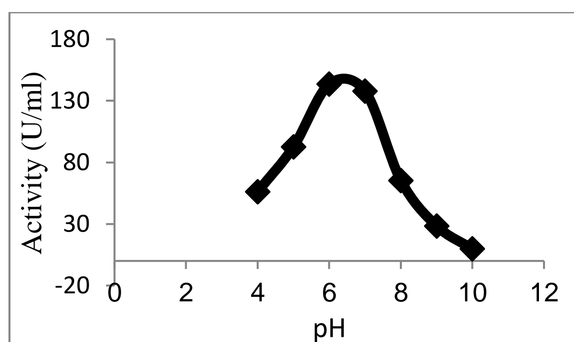

Fig. 4: The pH stability for pullulanase activity from *K. pneumonia*.

Thermal influence on enzyme activity: The enzymatic activity affected by temperatures, it is raising as the temperature increased until reaching 60ºC which gave the highest activity 125.73 U/ml as shown in fig. 5. The increasing in temperatures caused decreasing in enzyme activity because the tertiary structure of protein was changed, and this performs to denature the active site of the enzyme. This result agreed with Yoo and Yu (1997) who found that the enzyme from *K. pneumonia* had the optimum temp. at 60ºC, while Khabade, et al., (2016) found that the pullulanase enzyme from *Bacillus sp.* had the optimum temp. at 37 ºC, in another research it found that the optimum temperature was 50ºC for pullulanase enzyme activity from *Streptococcus sp* (Malakar, et al., 2012).

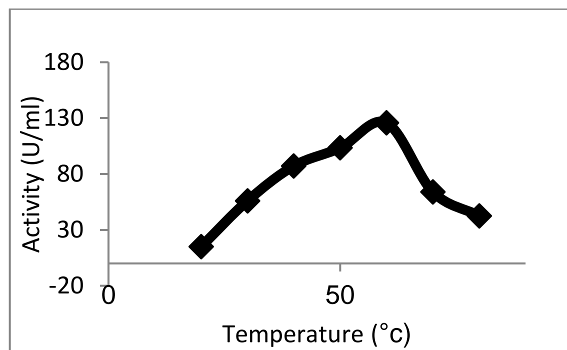

Fig. 5: The optimum temperature for pullulanase activity from *K. pneumonia*.

The enzyme was stable at 50-60 ºC when it kept 95% of its activity, as shown in fig.6. This agrees with Ara, et al., (1992) who said that the enzyme was stable at 50 ºC, and Malakar, et al., (2012) found that the enzyme was stable at 40-50 ºC.

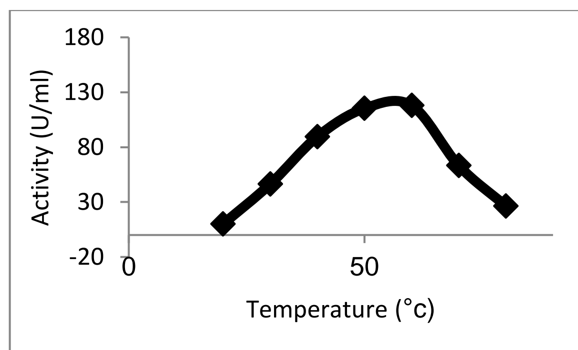

Fig. 5: The temperature stability for pullulanase activity from *K. pneumonia*.

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