Original Article

Extraction and Purification of Intracellular Beta-Lactamase from Proteus mirabilis

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Abstract

P. mirabilis was isolated from UTI patient to ensure that the isolate was belonging to P. mirabilis various tests are made such as staining techniques, biochemical assay, morphological and sensitivity test. The gram stain and biochemical test result show rod pink gram negative bacteria and ensure that the isolate was belong to (P. mirabilis). Optimization education for bacterial growth were done by used more than pH and temperature and it was found that the best conditions for the production and increase the number of bacteria at pH 7.7 with bacterial number and Temperature 37°C cell pellet was re-used as a crude enzymatic extract and discard supernatant cell. Purification of Beta-Lactamase enzyme was accomplished by using Ion exchange and gel filtrations chromatographic techniques. The result shows that, gel filtration has optimal specific activity and purification fold at (28.71U/mg), purification fold 2.05 times.

Keywords: Isolation, Identification, Bacteria, Proteus mirabilis, Beta-Lactamase

Introduction

Beta-lactamase Enzymes the most widespread mode of clinical resistance development to B-lactam antibiotics is the expression of beta-lactamases that hydrolyze the antibiotic. It is estimated that $30 billion is the annual economic loss to the US population from disease caused by beta-lactamase producing resistant bacteria 1. Beta-Lactamases hydrolyze the four-membered beta-lactam ring in both penicillin and cephalosporin classes of antibiotics as well as the carbapenem series. They thereby destroy the antibacterial activity by deactivating the chemical properties of the drug molecule, which is the chemically reactive acylating group for modifying the active site serine side-chains in the PBPs 2 .Extended spectrum beta-lactamases (ESBLs) are a group of enzymes produced by certain bacteria that are able to hydrolyze extended spectrum antibiotics belonging to the penicillin and cephalosporin groups and monobactam. ESBLs are found in Gram-negative bacteria, especially in enterobacteriacea 3 . ESBL has generally been defined as transmissible beta-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam, and which are encoded by genes that can be exchanged between bacteria 4.Extended Spectrum Beta-Lactamases Enzymes (ESBLs) are often located on plasmids that are transferable from strain to strain and between bacterial species. Although the prevalence of ESBLs is not known, it is clearly increasing, and in many parts of the world 10–40% of strains of Escherichia coli and Proteus mirabilis express ESBLs. ESBL-producing Enterobacteriaceae have been responsible for numerous outbreaks of infection throughout the world and pose challenging infection control issues 5.

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The aim of the study Isolation and identification of Proteus mirabilis bacteria from persons with urinary tract infection.

Purification of the molecular enzyme by ionc exchange chromatography and gel filtration chromatography.

Materials And Methods

Patients, specimens, collection

Through the period extending from November 2021till December 2021, 40 Clinical specimens comprising from UTI patients were collected as sterilized containers and then test the best isolate for growth by spectrophotometer and was found that the

optimal isolate for enzyme production .

Samples identification

All the bacterial isolates were examined for gram stain ability (6), shape and color of the cells were observed by light microscope using oil emersion, the collected specimens were streak plate technique is used for the isolation into pure culture of the organisms (mostly bacteria), from mixed population. The P. mirabilis was streaked over the agar surface. Some individual bacterial cells are separated and well-spaced from each other. As the original sample is diluted by streaking it over successive quadrants and then incubate at 37°C for 24h., the number of organisms decreases and will show the bacterial morphology. (7)., finally biochemical test was done to ensure that the isolate is belong to Proteus mirabilis . The result deal with (8). As shown in table1.

Optimal temperature and pH for the production of bacteria

The bacterial suspension was cultured once at constant temperature but different pH (5.5, 7 and 9) and once at constant pH but different temperature (32, 35 and 37°C) and measures the absorbance at 556 nm (7). compare the absorbance with McFarland number. As shown in fig(1).

Extraction of enzyme

Taken 500ml of production broth transported it into cooling centrifuge tubes, at

10000 rpm the bacterial cells were centrifuged for 15 minutes at 4°C. The suspension was disrupted by ultra-sonicater in an ice-water bath for 15 min. The disrupted cell suspension was centrifuged at 5000 g at 4ºC for 15 min. The resulting supernatant represented the crude enzyme extract which was stored at -20ºC until use (9).

Detection of β-lactamase activity

Beta-lactamase activity was determined by a micro-iodometric assay according to the modification method described by (10)(11). The reaction mixture (total volume (1325µl)) consisted of:

a. Starch-iodine reagent 1000 µl was added to.

b. Penicillin-G 25 µl .

c. Crude or pure enzyme 100 µl .

d. Starch solution 200 µl .

These components were mixed in small test tubes, blue color developed immediately due to the reaction of iodine with starch. Rapid decolorization indicated β-lactamase production. Control solution was made up by replacing the enzyme in a phosphate buffer solution, and the absorbance was read spectrophotometrically at 620 nm.

International Unit (IU): Is the amount of enzyme needed to hydrolyze 1µmol of penicillin G per minute at 25 ºC and pH 7.0 .

$$Enzyme\ activity(U\backslash ml) = \frac{\Delta E \times 121.9}{\Delta t \times 1}$$

$$\Delta E : change\ between\ blank\ and\ test.$$

$$\Delta t : change\ the\ time\ of\ reaction$$

Determination of protein concentration

Protein concentration was determined according to the method of(12), the protein concentration determined. A 20µl of GTF crude was mixed with 50µl of 1 M NaOH with shaking for 2-3 minutes then 1 ml of Bradford solution, was added with shaking. The absorbance was measured at 595 nm by spectrophotometer (13).

Determination of specific activity

The Specific Activity of an enzyme was calculated as the following :

$$specific\ Activity(Unit\backslash mgprotein) = \frac{Enzymeactivity(U\backslash ML)}{Proteinconcentration(mg\backslash ml)}$$

Beta_lactamase enzyme produced by Proteus mirabilis in the cultures broth was subjected to a purification protocol. After that estimate the enzyme activity in crude supernatant. The enzyme activity 10.50 unit/ml and specific activity 14.00 unit/mg. The purification involved by ion exchange and gel filtration

Separation of enzyme through ion exchange resin (DEAE Cellulose)

The exchanger DEAE cellulose was prepared and packed into column following the method described by (15) .

Twenty five gram of DEAE-cellulose was suspended in 1000 ml of distilled water and left for a time to precipitate. The floating flakes were removed away from the solution until the upper layer was cleared, then filtrated by using a vacuum pump. The precipitate was suspended in 250ml of (0.25M NaCl , 0.25M NaOH) solution, filtered and washed several times with distilled water then washed with 250ml of (0.25M) HCl. The column was mobilized in an ionic exchanger DEAE cellulose to get a dimensions (2.5x20)cm and was balanced with phosphate buffer solution .

The dialysate was loaded onto anion exchange DEAE-cellulose column with a diameter of (2.5x20) cm. Equilibration was done with 0.05M phosphate buffer pH 7.0. The enzyme loaded column was washed with 500ml of the same buffer to remove unbound sample components.

Partially purified concentrated Beta-lactemase enzyme were separately passed after loaded onto the column carefully. Then, (100ml) of (0.05mM) phosphate buffer pH (7.0) was added. Proteins were eluted by using (200 ml) of a stepwise salt concentration from (0.125-1.0 M) in 0.05mM phosphate buffer (pH 7.0). Fractions of (5ml) were collected and absorbency was monitored at (280nm). The presence of the Beta _lactamase enzyme was estimated from each fraction of the major peaks then protein concentrations were determined.

Enzyme separation through Sephacryal S-200 Column

Sephacryl–S200 column (1.5x80cm) was prepared and packed according to the instructions of the manufacturing company (pharmica Sweden). The column was equilibrated with phosphate buffer (pH-7.0; 0.05M). Enzymes fraction from DEAE-cellulose was concerted by sucrose then pooled and passed through gel filtration column. A (5ml) sample of each concentrated enzyme was added to the column by carefully using pasture pipette. For the elution of phosphate , 5ml fraction was collected of each enzyme activity and estimated by measuring the absorbance at 620nm.

Effect of pH on enzyme activity

The effect of pH on the activity of the β- lactamase was determined at 37°C in 0.05M phosphate buffer (pH 6,6.5,7 ,7.5 and 8).

The β- lactamase activity was determined at different pH (6-8) by mixing 1ml of pencillin G with 1ml of purified enzyme and the activity was measured with different buffers .

Effect of pH on enzyme stability

The effect of pH on β- lactemase stability was examined by adding 1ml of enzyme of to test tubes containing buffer at different pH (6, 6.5, 7 ,7.5 and 8), and incubated 30min at 37oC in a water bath , then the pencillin G was added to a 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 and 45) ˚C. The purified β-lactamase solution was incubated with penicillin G in different temperatures for 10 min and the activity was determined.

Effect of temperature on enzyme stability

The purified β-lactamase was incubated at different temperatures ranging between (25,30, 35, 40 and 45)˚C . followed by incubation in an ice bath for 30min.The enzyme activity was assayed using penicillin G , and the relation between the remaining activity (%) toward temperature was plotted to determine the optimum temperature of Beta lactamase stability.

Results And Discussion

Identification of Bacteria

The process of identifying bacteria began with cultivating the samples and incubating them for 24 hours at 37°C,followed by a biochemical test to confirm that the isolate belonged to Strep P.mirabilis. The results obtained are inagreement with the description in(8) (tabl 1).

Ionic Exchange Chromatography

This is one of the most useful methods for protein purification. Depending on the surface molecule charge, the protein and the buffer conditions, the protein will have net a positive or negative charge. Beta-lactamase enzyme was obtained by using phosphate buffer solution (pH=7.0). 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 the NaCl gradient (0.1-1M) used to elute the bounded protein. Ionic exchange chromatography patterns showed one protein peak in wash and one peaks in gradient elution, represented enzymic activity (tubes 17-23). Those fractions pooled and tested for specific activity (18.83 U/mg) a fold purification of (1.3 time) and enzymic yield of (80.71%) in parts. (fig. 2).

Gel filtration chromatograph

Purification of β-lactamase enzyme by gel filtration chromatography was done by passing the sample through sephacryl S-200. Enzymes fraction from DEAE cellulose were pooled and passed through gel filtration column.

Column was eluted by buffer solution (0.05M) sodium phosphate buffer and then the sample passed. The fractionation yielded one protein peak as absorbance reading at 280nm and when determined for the enzyme activity in the resulting parts the enzyme activity recorded in (18-21) the specific activity reached(28.71 U/mg), fold of (2.051 time) and a yield (71.785%).(fig. 3).

Characterization of Beta_lactamase enzyme

pH and temperature optimum

Initial pH of the culture broth is one of the most critical environmental parameters affecting both growth and Beta_lactamase production. The results show that P.mirabilis was able to grow in the pH range of 7.0 to 8 and They found that the optimal pH activity was 7.0 . Thes\\data agree with (9) .The results of this study show that maximum Beta_lactamase activity was detected at 35_40°C. A decrease activity was observed at above 30°C and completely ended after 50°C. (14).(fig. 4)

pH and temperature stability

The pH stability of the Beta_lactamase was determined by the activity retained at different pH from 6 to 8 after 30 min at 37°C of incubation. The obtained results showed a maximum stability at pH 7 as the enzyme retained its entire activity. The enzyme was stable at pH 7.0 and retained its entire activity. The enzyme was stable at pH 7.0 and retained more than 80% of its entire activity, while at pH values 6, 6.5, 7.5 and 8.0 the enzyme lost about 100% of its total activity. This decline could be attributed to the influnce of pH effect on the enzymatic protein structure or irreversible denaturation may occur in a high acidic or basic solution which led to a change in the active site of the enzyme, so loss of the activity was observed (15). purified β-lactamases stability upon 30 min incubation at different temperatures ranging from 25-45oC. The enzyme showed a maximum stability at 40 oC as it retained the entire activity. Besides, the enzyme retained more than 60% of its entire activity at temperatures between 30-35 oC, and 50%of total activity at 45 oC. The gradual loss in enzyme activity could be because of temperature effect on the tertiary structure of the enzyme or, and distortion in the active site of protein due to the loss of activity to the breakdown of substrates (16) (14).(fig. 5)

Table 1: Identification of P. mirabilis .

Characteristic of P. mirabilis	*Test*
Oxidase (-), Catalase (+), Indol (-) , Citrate (+)	Biochemical
the Proteus has ability to change their rod-shaped vegetative cell in to elongated and highly flagellated cell that has swarming motility on planting medium.	Morphology
Pink rod gram stain	Gram stain

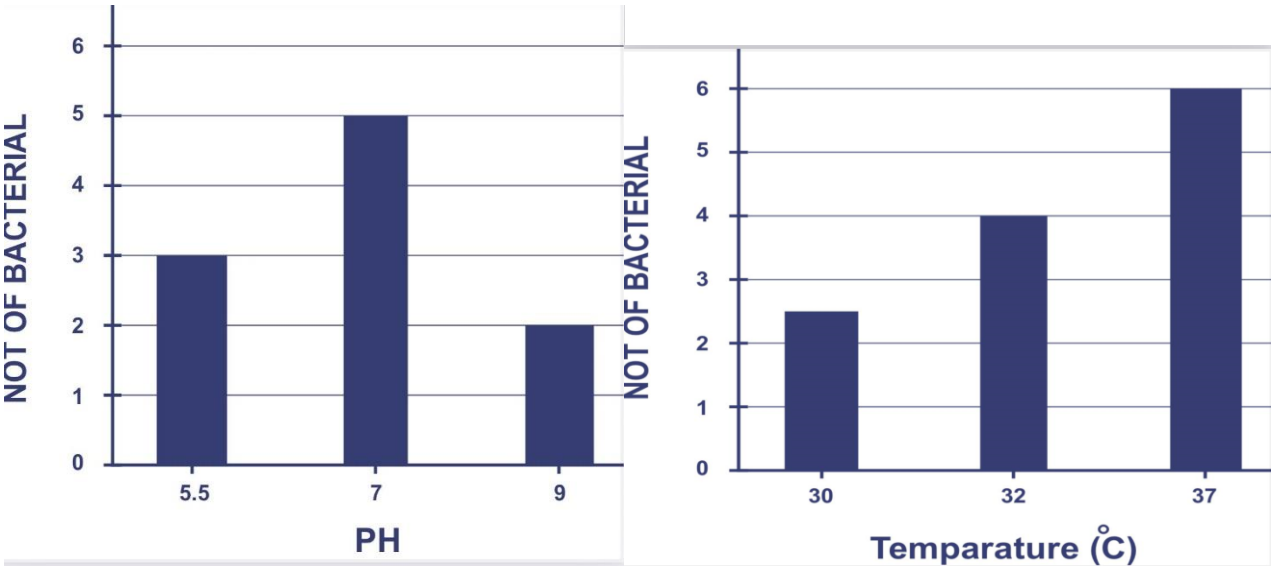

Fig. 1: show optimal pH and Temperature of P.mirabilis

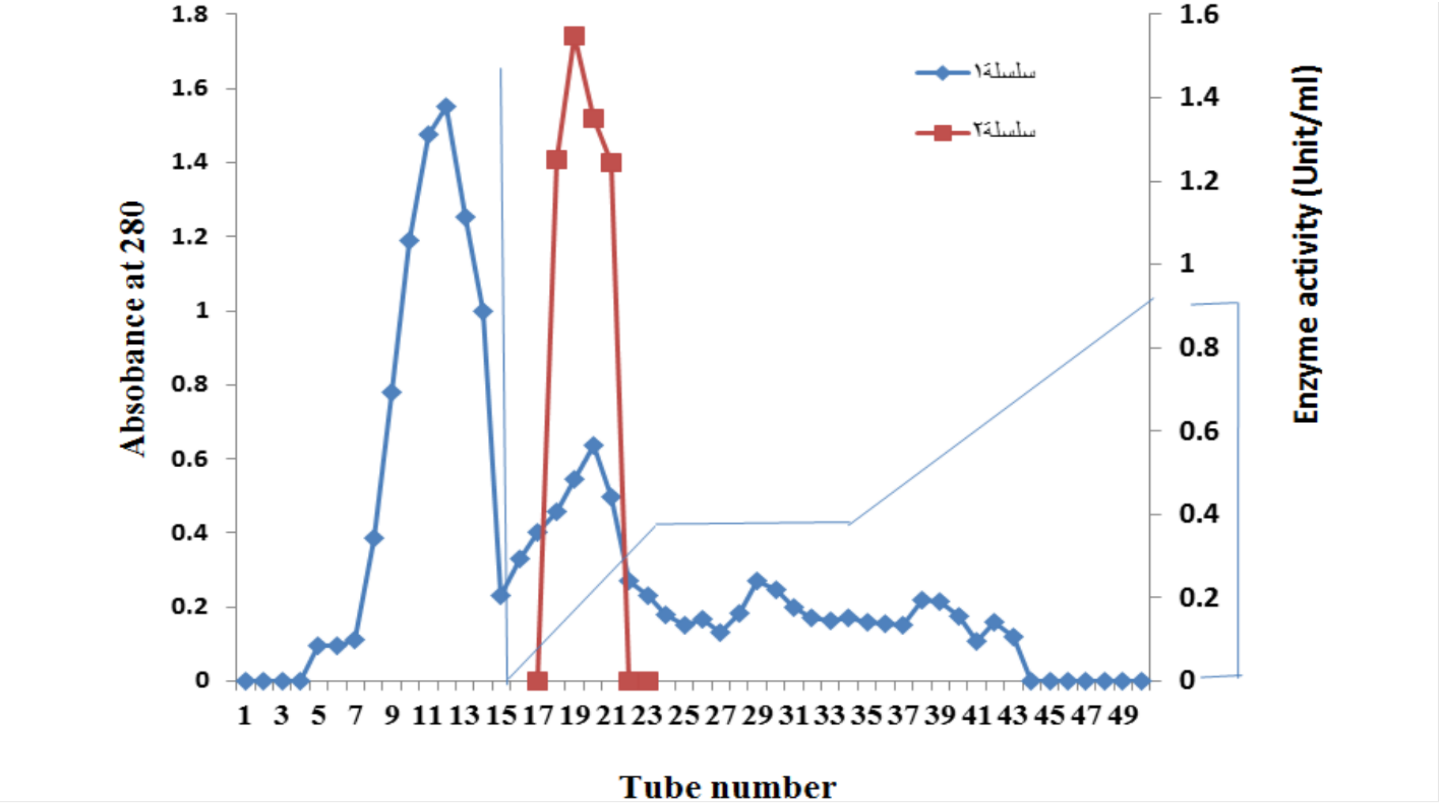

Fig. 2: Ion exchange chromatatgraphy for β- lactamaese from A. baumannii through DEAE-cellulose (2.5 x 20) cm. The column was washed with Sodium phosphate buffer and Eluted with same buffer containing NaCl. Flow rate 60 ml/ hr and 5 ml Fraction.

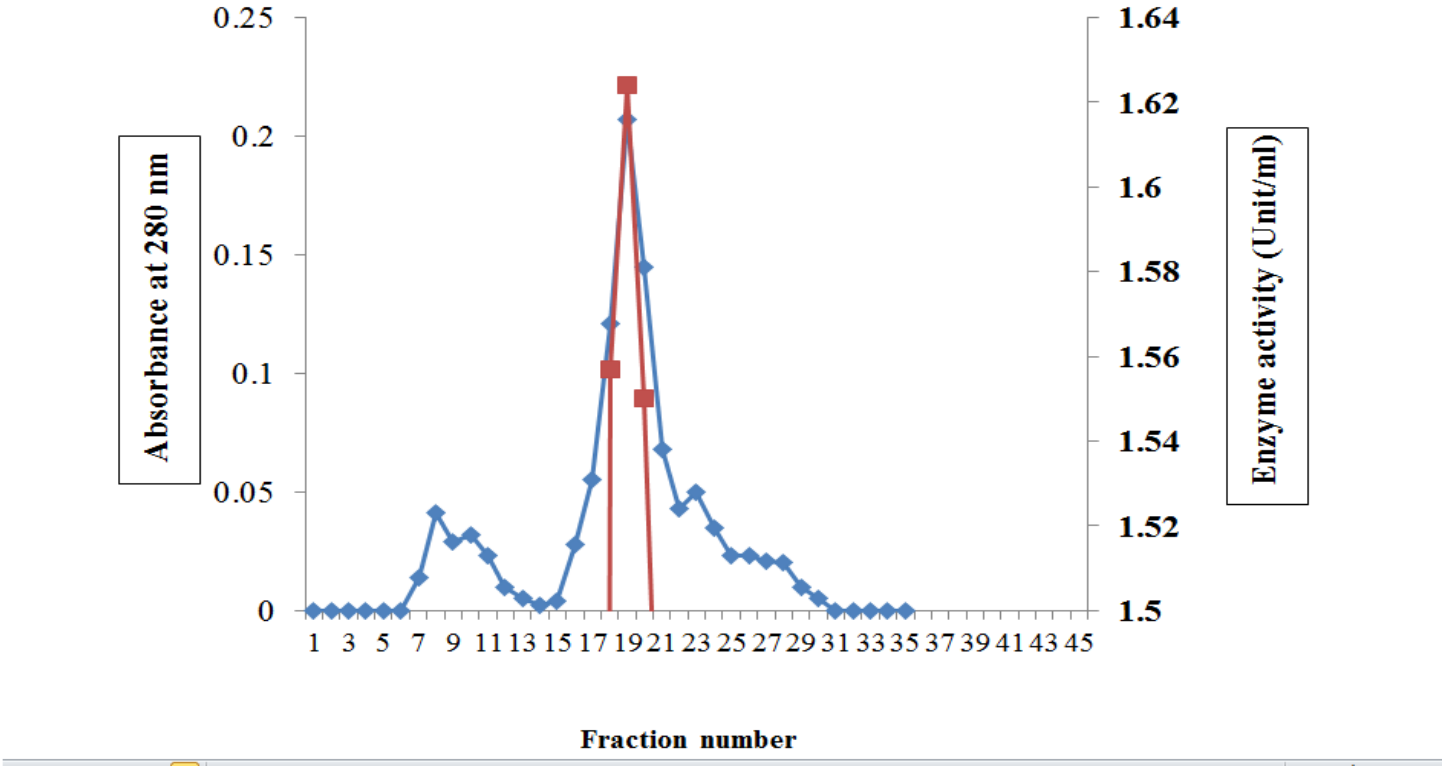

Fig. 3 : Gel-filtration chromatography for purification β- lactamaese from P.mrabilis by using Sephacryl S-200 column (1.5 x80) cm. The column was calibrated with (0.05M) Sodium phosphate pH7; flow rate 60 ml/hrs and 5 ml/fraction.

Table 2: Purification steps of β-lactamases produced from Proteus mirabilis.

Steps	*Volume (ml)*	*Enzyme activity (U/ml)*	*Total activity (Units)*	*Protein Conc. (mg/ml)*	*Specific activity (U/mg)*	*Fold of purification*	*Yield (%)*
Crud extract	20	10.50	210	0.75	14.00	1	100
DEAE cellulose ion-exchange	15	11.30	169.5	0.60	18.83	1.345	80.71
Gel-filtration Sephacryl S-200	15	10.05	150.75	0.35	28.71	2.051	71.785

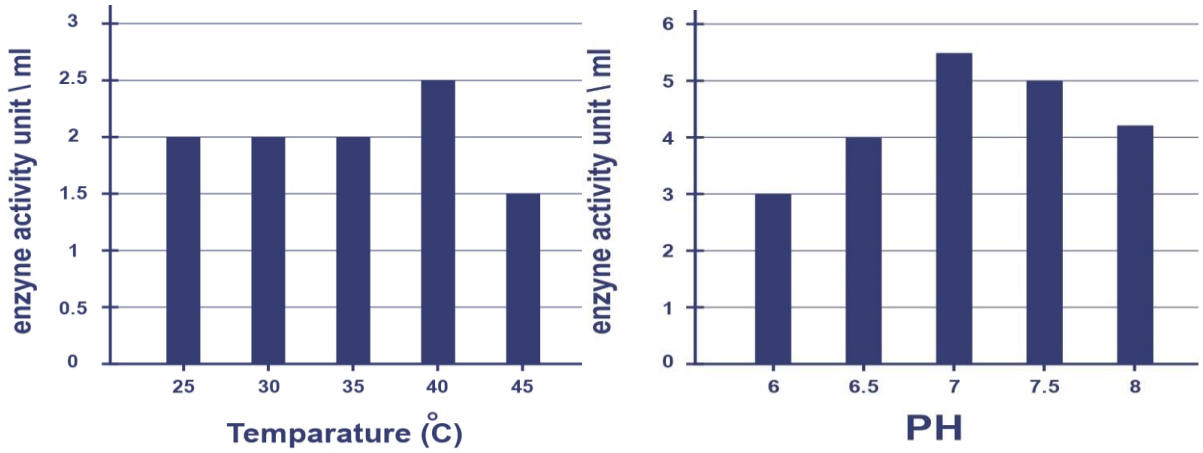

Fig. 4: Effect of different pH and temperatures on activity of purified β-lactamase from P.mirabilis.

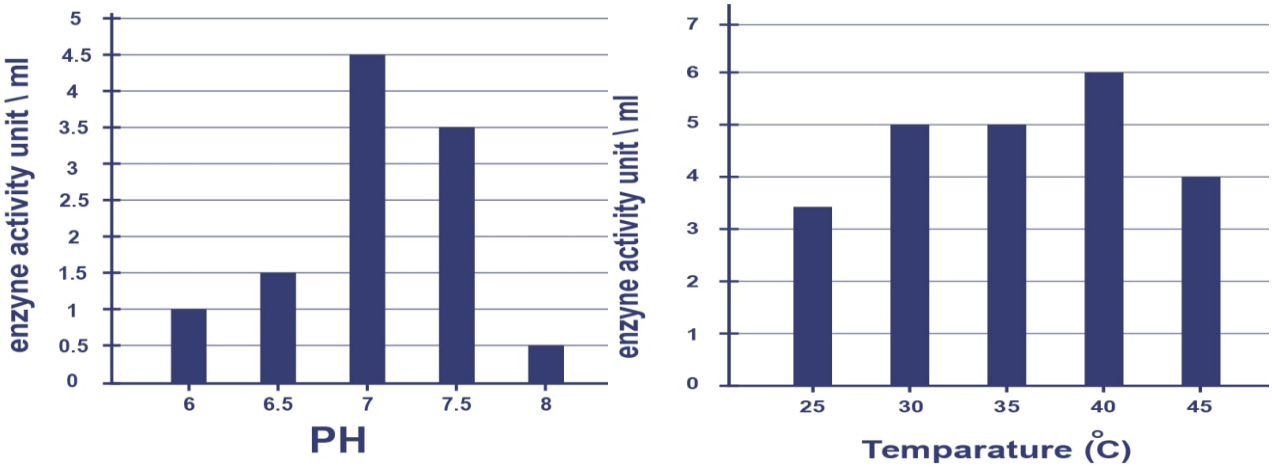

Fig. 5: Effect of different pH and temperatures on enzyme stabitity of purified β- lactamase from P.mirabilis.

The experimental data of this research showed that the intracellular Beta_lactamase enzyme can be easily purified by the protocol used to purify proteins. One of the successful methods used to purify enzymes was the ion exchange chromatography and gel filtration were used, and the results of these two steps reflected the difference between the previous steps in purification. This was due to the fact that the highest values of specific activity and high purity of the enzyme were obtained in these two steps.

Since enzymes can be used for different applications, it is necessary to identify the thermal stability and activity in a wide range of pH values. The results showed that the

optimum temperature for Beta_lactamase activity was 35_40°C and that thermal stability was within the range of 40 °C. the enzyme retained more than 60% of its entire activity at temperatures between 30-35°C, and 50%of total activity at 45°C. The gradual loss in enzyme activity could be because of temperature effect on the tertiary structure of the enzyme or, and distortion in the active site of protein due to the loss of activity to the breakdown of substrates (14) (16).

In this study, the optimal pH for enzyme activity and stability was determined; accordingly, the findings revealed that the optimum pH for Beta_lactamase activity was 7.0. This is consistent with the results of the study conducted by. (9) . The enzyme was stable at pH 7.0 and retained more than 80% of its entire activity, while at pH values 6, 6.5, 7.5 and 8.0 the enzyme lost about 100% of its total activity. This decline could be attributed to the influnce of pH effect on the enzymatic protein structure or irreversible denaturation may occur in a high acidic or basic solution which led to a change in the active site of the enzyme, so loss of the activity was observed (15).

Compliance with Ethical Standards statements

Ethical approval: Department of Biotechnology, College of Science, University of Technology, Baghdad, Iraq certifies the ethical approval, Funding details (In case of Funding): I am responsible for paying the financing, Conflict of interest: There is no conflict of interest, Informed Consent: Department of Biotechnology, College of Science, University of Technology, Baghdad, Iraq Agreed

Reference

Walsh, C. (2003). Antibiotics, Action, Origins and Resistance. Washington, District of Columbia: ASM Press.

Livermore, D. and Woodford, N. (2000). Carbapenemases: a Problem in Waiting? Curr Opin Microbiol, 3:489–495.

Ejaz, H.; ul-Haq, I.; Mahmood, S.; Zafar, A. and Mohsin, M. (2013). Detection of extended-spectrum β-lactamases in Klebsiella pneumoniae: Comparison of phenotypic characterization methods. Pakistan Journal of Medical Science, 29: 3.

Shaikh, S.; Fatima, J.; Shakil, S.; Mohd, S.; Rizvi, D. and Kamal, M. (2014). Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi Journal of Biological Sciences, 22:90–101 .

Rupp, M. and Fey, P. (2003). Extended Spectrum β-Lactamase (ESBL)-Producing Enterobacteriaceae: Considerations for Diagnosis, Prevention and Drug Treatment. Journal Drugs, 63:4..

Leboffe, M.J. and B.E. Pierce (2012). Microbiology: laboratory theory and application. Morton Publishing Company., 1(12): 105.

Antoni RÓ, Agnieszka T, Magdalena M, Iwona k, Agnieszka M, kingaostrowska, Dominika D, Agnieszka Z, Agata P, Małgorzata S, Paweł S. 2012. Proteus sp. an opportunistic bacterial pathogen classification, swarming growth, clinical significance and virulence factors. Folia Biologica et Oecologica 8, 1–17.

C.M. O'Hara, F.W. Brenner, and J. Michael. Clinical Microbiology Reviews, 2000: 13(4), 534-546.

El-Shora, H.M.; Al-Hayanni, H.S. and El-Shobaky, A.M. (2017). Characterization of β-Lactamase from Two Pathogenic Bacteria. International Journal of Current Microbiology and Applied Sciences ISSN: 2319-7706, 6(6): 927-941.

Novick , R . P . (1962) . Micro-iodometric assay for penicillinase . Biochem . J . 83:236-240.

Moland ES, Hanson ND, Black JA, Hossain A, Song W, Thomson KS. 2006. Prevalence of newer β-lactamases in Gram-negative clinical isolates collected in the United States from 2001 to 2002. Journal of Clinical Microbiology 44, 3318–24.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72:248-254.

Baron, E.J., J.M. Miller, M.P. Weinstein, S.S. Richter, P.H. Gilligan, J.R.R.B. Thomson, P. Bourbeau, K.C. Carroll, S.C. Kehl, W.M. Dunne and B. Robinson-Dunn (2013). A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM) a. Clinical infectious diseases., 57(4): 22-121.

Bhatt, S. M.(2011).Enzymology and Enzymatic Technology. S. Chand Higher Acadamic. chapter 4,p 92.

Whitaker, J. R. (1972). Principles of Enzymology for the Food Science. (ed. Oen, R. F.) Marcel Dekker INC. ; New York.

Whitaker, J.R. and R.A. Bernhard (1972). Experiments for anIntroduction to Enzymology. Whiber Press., 1(19): 1-9.

Wenk, M.R and Fernandis, A.Z.(2007).A manual for Biochemistry Protocols .Vol.3.editor Jan-Thorston Schantz.British library cataloginig-in-publication data.p2-7.