Production, Purification and Characterization of β-Lactamase from Clinical Isolate of *Acinetobacter baumannii* A7

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Abstract

Background: *Acinetobacter baumannii* is one of the most important causative bacteria of nosocomial infections. Attention turned toward this Gram-negative bacterium due to its extensive resistance to antibiotics. **Aim and Objective:** Purification and characterization of Purification and characterization of β- lactamases and study the kinetic parameters. **Materials and Methods**: One hundred and fifty samples were collected from different sources from clinical sources (wounds, burns, urine, sputum, and blood) , from Baghdad/Iraq hospitals (Ibn-Al Balady , Medical city and Imamein Kadhimein medical) collected from during the period from beginning of November 2019 to the end of January 2020. **Results:** The results showed that 10 isolates have the ability to produce the enzyme and that isolate A7 has the highest productivity of the enzyme with enzyme activity 14.420U/ml. The *Beta*-lactamase was purified by using three steps including ammonium sulfate precipitation, ion exchange chromatography, and gel filtration, the purified enzyme had fold of purification about 10.545times with 32.44% recovery. The *Beta*-lactamase purified was characterized, the. molecular weight of *B*-lactamase was 40.738KD , the optimum pH for the purified β-lactamase activity was 7, and 7 for stability, the optimum temperature for enzyme activity was 40 ºC and 40 ºC for stability, enzyme showed that β-lactamase lost 50% of its initial activity at 45°C. *β-lactamase* retained 50% of its activity when stored at 4º C for a period of 7 days. The kinetic constant values for purified enzyme was km 0.27mM and Vmax 10.86 mmole/min when using of penicillin G. **Conclusion:** *Acinetobacter baumannii* have been proved to its ability to produce B-lactamase and purified with fold 10.545 and a yield 32.44%.

Keywords: *Beta-lactamase, Acinetobacter baumannii , purification, characterization. Molecular weight*

Introduction

β-lactamases are enzymes (penicillin amid-β-lactam-hydrolyse, EC 3.5.2.6). The purification of beta-lactamases differs according to the type of bacteria, due to the difference in the electrical break-even point and the molecular weight of the enzyme[1]. The purification process may include one step, as Babini *et al*. purified the beta-lactamases produced from both *F. adoratum* and *Aeromonas* spp [2]. Using the Caboxy methyl sephadex G-50 ion exchanger, the column was stabilized using a buffer phosphate solution of 10 mM and pH 8.2, then the enzyme was recovered using a gradient in NaCl concentration (0-0.5 mM) [3]. The first included separation with anion exchange Q Sepharose, and the second included separation with gel filter Sephacryl S-300, while the third included the separation with the ion exchanger Mono Q. Al-Jumaily *et al.,* studied B-lactamase they were purified it from local isolate *Klebsiella pneumonia* by several steps which included precipitation with ammonium sulphate at 20-40% saturation, DEAE-ion exchange chromatography and gel filtration on Sephacryl S-200 column.[4] The obtained purification fold and recovery were 32.66; 47.04% respectively. Arun *et al*. characterize and optimize the production of this

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enzyme by ion exchange chromatographic purification[5]. Also, El-Shora *et al.* using several steps to isolated and purified β-lactamase by included precipitation with ammonium sulphate at 80% saturation, DEAE-Cellulose and gel filtration on Sephadex G-200 column[6]. The step of purifying enzymes comes for the purpose of obtaining the largest amount of the enzyme in a pure manner free from any other large particles that may be associated with it as well as a higher efficacy than the raw extract, so researchers have been using many techniques in purifying enzymes, including beta-lactase, being one of the medically important enzymes.

Objectives

1. Isolation of *Acinetobacter baumannii* from samples like blood, urine , sputum, swab and select the most efficient producing of β- lactamases.

2.Purification and characterization of β- lactamases and study the kinetic parameters.

Methods

β-lactamase was isolated from clinical isolates *Acinetobacter baumannii* . The isolation was carried out according to Hedberg *et al.,*[7] *Beta*-lactamase activity was determined by a micro-iodometric assay according to modification method described by Hassan, [8]. The concentration of protein was estimated with the Bradford's assay and using bovine serum albumin BSA as a standard. The ammonium sulfate was added in different saturation ratios (0- 80%) saturation into crude enzyme. The precipitate obtained was dialyzed and then loaded onto a DEAE-Cellulose anion-exchange column following the method described by Whitaker,[9]. 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). Molecular weight of *B*- lactamase was estimate by using gel filtration on Sephacryl-S-200 (80x1.5cm), using a standard protein by drawing the relationship between the logarithm of a standard protein molecular weight and the size of recovery volume size of Void volume (Ve/V0). 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 effect of pH on β- lactemase stability was examined by adding 1ml of enzyme of to a test tubes containing buffer at different pH (6, 6.5, 7 ,7.5 and 8). The temperature profile of the purified enzyme was studied by measuring the activity at different temperatures (25,30, 35, 40, 45) ˚C . The purified β-lactamase was incubated at different temperature ranged between (25,30, 35, 40, 45)˚C . followed by incubation in ice bath for 30min. The enzyme activity was assayed using penicillin G. The purified enzyme was stored at refrigerator temperature 4^{o} C and freezer temperature -20^{o}C. The remaining enzymatic activity was estimated every week for a month **.** Pre-steady state kinetic analysis was performed using penicillin G at different concentration (8.9 , 10 , 20 , 4.45 ,2.225 mg/ml) in 0.05M of phosphate buffer at pH 7 and estimate the enzyme activity , then the initial velocity (Vo) value was estimated. The relationship between [1/Vo] versus [1/So] was plotted to determine the Km and Vmax values according to Lineweaver-Burk reciprocal plot.

Results and Disussion

Ten isolate of total 33 isolates were β-lactamases producer using rapid iodometric method. Table (1) shown that the *Acinetobacter baumannii* A7 produced the highest amount 14.420 U/ml compared with another isolated . The high incidence of β-lactamases production by this bacteria and other genera may contributed to the increase and widely usage of β-lactam antibiotics that has selected strongly resistance bacteria[10]. Besides, most of β-lactamases genes located on self-transferable genetic factors, and the high level antibiotic resistance is frequently conferred through the transfer of such plasmids which mediated numerous resistance genes, including genes for multiple β-lactamases from different functional classes[11].

Table (1): Screening the *Acinetobacter baumannii* producing *β*-lactamase enzyme.

No.Isolates	Enzyme activity (Unit/ml)	Protein (mg/ml)	Specific activity (unit/ ml)
A1	14.079	0.157	89.675
A2	7.553	0.147	51.381
A3	3.791	0.104	36.452
A4	4.412	0.131	32.681
A5	6.143	0.135	45.504
A6	10.312	0.137	75.270
A7	14.420	0.147	98.095
A8	3.120	0.136	22.941
A9	12.445	0.132	94.280
A10	14.140	0.148	95.540

Crude Extraction:

The *β*-lactamase enzyme was extracted from local isolation of *A. baumannii* after incubated it in 100ml brain heart infusion broth at 37ºC in the shaker incubator with 120rpm at 37°C for 24hrs. and washed with 0.05M sodium phosphate buffer pH 7.0, then lysis, breaking the cell wall of bacteria. After that estimate the enzyme activity in crude supernatant. The enzyme activity is 6.875 U/ml, and specific activity 10.576 U/mg protein, as shown in table (2).

Ammonium Sulfate Precipitation

Precipitation of enzyme by ammonium sulfate is a useful method of concentration and is ideal as an initial step in purification[12]. The crude β-lactamase was carried out by adding of ammonium sulphate up to 80% saturation at 4ºC. The results of specific activity of supernatant , as its specific activity was (33.50U/mg).The precipitated protein was dissolved in a 0.05M phosphate buffer (pH 7.0) and stored for further purification at 4ºC. El-Shora *et al*.,(2017)[13] found that 75% saturation of ammonium sulfate when total activity and specific activity 1400 unit , 2.6 Unit/mg protein respectly from *Staphylococcus sciuri* ,1200 unit and 3.5 Unit/mg protein from *K. pneumoniae*.

Ionic Exchange Chromatography

After ammonium sulphate precipitation, the concentration of both the enzyme and low molecular weight impurities increases. The dialysate was passed through the ion exchange column DEAE-Cellulose. *β*-lactamase enzyme was obtained by using buffer solution (0.05M) sodium phosphate buffer. 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 two peaks in gradient elution represented enzymic activity (tubes 9-16). Those fractions pooled and tested for specific activity (33.70U/mg) a fold purification of (3.187) and enzymic yield of (50%) in parts. Figure (1) and this result agree with El-Shora *et al.*[13]; Abdul-Hussein *et al*.[13]. Hassan *et al*. used CMC calluses showed that there are two peaks appeared in washing step, while two protein peaks appeared by the gradient concentration of

sodium chloride.[14] Result showed peak in washed protein after concentration by sucrose 57.2 U/ml, while peaks eluted proteins after concentration by sucrose 36.33 U/ml. The results were shown that there are two forms of enzyme (isozymes) which were appeared through the separation techniques.

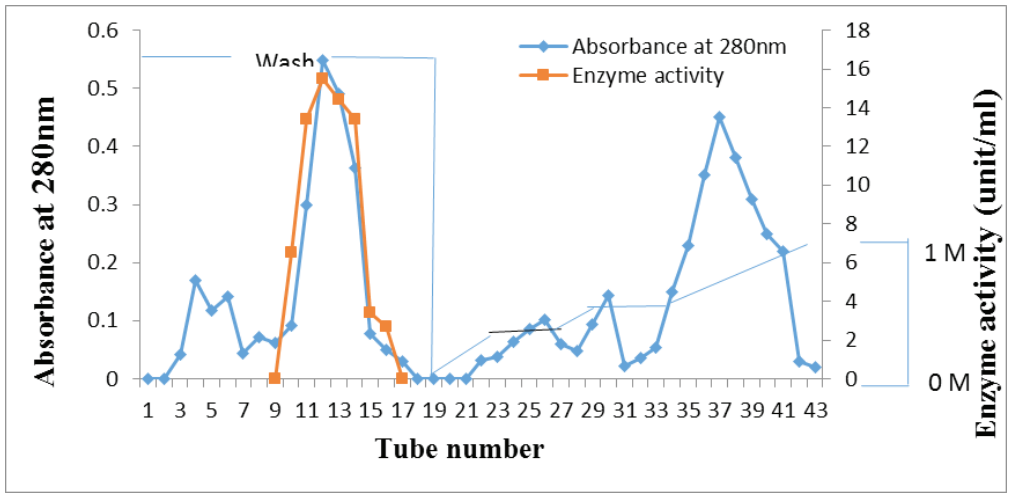

Figure (1): Ion Exchange Chromatatgraphy for *β*- lactamaese from *Acinetobacter boumannii* through DEAE-cellulose (2.5 X 20) cm. The column was washed with Buffer and Eluted with same buffer containing NaCl. Flow rate 60 ml/ hr and 5 ml Fraction.

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 0.05M sodium phosphate buffer and then the sample passed, fractions were collected up to 39 fractions. The fractionation yielded one protein peaks as absorbance reading at 280nm, only one peak appeared when reading absorbance at wavelength of 280 nm and when determined for enzyme activity in resulting parts enzyme activity recorded in (13-19) the specific activity reached (111.525U/mg), fold of (10.545) and a yield (32.44%) as mentioned in Table (4-7) and Figure (4-8) and this result agrees with (Al-Jumaily *et.al*.[4] who mentioned *Klebsiella pneumonia β*-lactamase has specific activity (23.75U/mg protein). Omeiri *et al*. reported a specific activity of 24.1 mg/protein from *S. aureus* with purification fold of 102.3 and yield of 58.74%.[15] Many studies used ammonium sulfate, ionic exchange and gel filtration chromatography to purify β-lactamase enzyme. [16, 17]

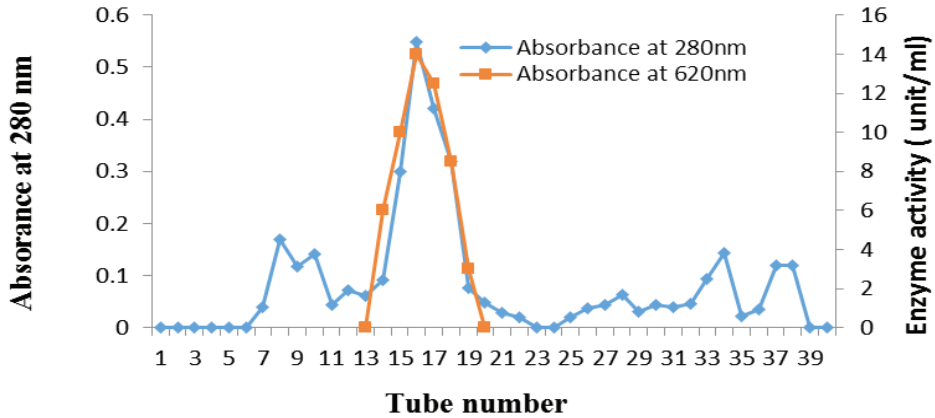

Figure (2): Gel-filtration chromatography for purification B- lactamaese from *Acinetobacter boumannii* by using Sephacryl S-200 column (1.5 X80) cm. The column was calibrated with (0.05M) sodium phosphate buffer pH 7; flow rate 60 ml/hrs and 5 ml/fraction.

(Table 2): Purification steps of *β*-lactamases produced from *A. baumannii*

Steps	Volume (ml)	Enzyme activity (U/ml)	Total activity (Units)	Protein Conc. (mg/ml)	Specific activity (U/mg)	Fold of putrefaction	Yield (%)
Crud extract	40	14.200	568	0.650	21.846	1	100
Ammonium sulfate Precipitation 80%	10	33.125	331.250	0.489	27.080	1.240	58.32
DEAE cellulose Ion-exchange	20	14.200	284	0.204	69.61	3.187	50
Gel-filtration Sephacryl-S200	20	9.213	184.259	0.040	230.325	10.545	32.44

Enzyme characterization

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 [17]. Sephacryl S-200 (1.5 x 80) cm was used for estimation the molecular weight of purified β-lactamase from *Acinetobacter bumannii*. According to the logarithm molecular weight and elution volume/void volume (Ve/Vo) Table (3) standard curve the calculated molecular weight of the β-lactamase found to be (40.738) KD.

Table (3): Molecular weight of standard proteins.

Proteins Strandard	Ve/Vo
Pepsin (34.5 KD)	2.75
Bovine Serum albumin (67 KD)	2.15
Alkaine phosphatase (80KD)	1.6
Catalase (232KD)	1.2
Arginine Deaminase (143.548 KD)	1.558
B-Lactamase from (40.738KD)	2.58

There are many studies like Issa *et al.* [16] showed that molecular weight of β-lactamase in *staphylococcus aureus* the was~ 30kDa that estimate by SDS-poly acryl amide gel electrophoresis. Furthermore, Al-Jumaily *et al*. [4] recoded a molecular weight of 40 kDa for *K. pneumoniae* β-lactamase. Ranade *et al*. reported a higher molecular weight between 100 to 150 kDa for *E.coli* β-lactamase [18] . El-Shora *et al*. used SDS-PAGE estimate the molecular weight in *Staphylococcus sciuri* and *Klebsiella pneumoniae* was 30 kDa, 28 kDa respectively [17]. Also Abdul-Hussein *et al*. found the enzyme purified from *A. baumannii* the molecular weight was 44.668KDa.[13]

Optimum pH for β-lactamase activity:

Table (4) shown the effect of reaction solution pH on the β-lactamase activity and stability. It has been noticed that the optimum pH of β-lactamase activity was 7.0, and the activity began to decrease activity between pH values of 7.5-8.0. This result was compatible with that mentioned by (Ranade *et al*. [18], they found that the optimal pH activity was 7.0. El-Shora *et al*. mentioned that β-lactamase from *Staphylococcus sciuri* had optimum pH for the enzyme activity were around 7 but optimum pH for the enzyme activity was 6.5 from *Klebsiella pneumoniae*.[6] Hassan *et al*. mentioned the maximum enzyme activity was recorded at pH 7.0 produced from *Staphylococcus aureus*.[14]

Also from table (3) illustrates the pH profile for enzyme stability, 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. Issa *et al*. found that *β*-lactamase enzyme was stable at values pH between 6 – 6.5 – 7. [17] Hassan *et al*. found that maximum enzyme stability was recorded at pH range from 6-7.5 for *Staphylococcus aureus*. [15]

Table (4) : Effect of different pH on activity of purified *B*-lactamase from *Acinetobacter baumannii.*

pH	Enzyme activity (unit/ml)	Enzyme stability (unit/ml)
6.0	3.242	1.292
6.5	4.315	1.642
7.0	5.656	4.405
7.5	5.314	3.352
8.0	4.559	0.731

The optimum temperature for *β*-lactemase enzyme activity and stability :

Each enzyme works within a range of temperature specific to the type organism. Purified *β*-lactemase from *A. baumannii* . Showed the highest activity at 40 ºC figure (3). The activity at temperature 30 and 35 was higher than 25 and 45 ºC. These results were compatible with those of El-Shora *et al*. who mentioned that the optimal temperature for enzyme was most active at 40 ºC when *β*-lactamase purified from *Klebsiella pneumonia* while 35 ºC from *Staphloccous sciuri* .[6]

Also, figure (4) explains the influence of temperature on purified *β-lactamase*s stability upon 30 min incubation at different temperature ranged from 25-45ºC. The enzyme showed a maximum stability at 40 ºC as it retained the entire activity, besides the enzyme retained move 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 loss of activity to the breakdown of substrates . [19, 12]

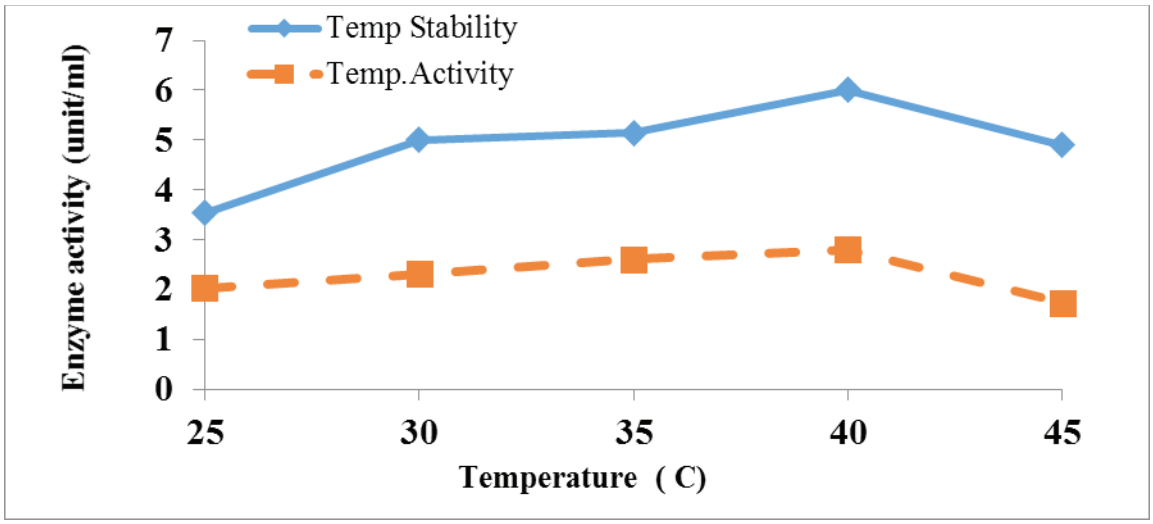

Figure (3) : Effect the temperature © on enzyme stability and enzyme activity of purified B-lactamase from *Acinetobacter baumannii*.

Determination Km, Vmax Values:

The penicillin G was used as substrate to determine the kinetic constants of purified *β-lactamase* , As shown in figure (4) relation between substrate concentration penicillin G and enzyme activity used Lineweaver-Burk, Km was 0.27mM and Vmax was 10.86 mmole/min. El-Shora *et al*. reported that Km value of 175.43 µg /ml and Vmax of 7.69 U/ mg protein for *Staphylococcus sciuri β-lactamase*. [20] On the other hand, a Km value of 222.22 µg/ ml and Vmax of 8.33 U/ mg protein were recorded for *Klebsiella pneumonia β-lactamase*. Also, Omeiri *et al.* reported that the Km and Vmax values of 111 µg /ml and 16.66 U /mg protein for *Staphylococcus aureus β-lactamase*. [15]

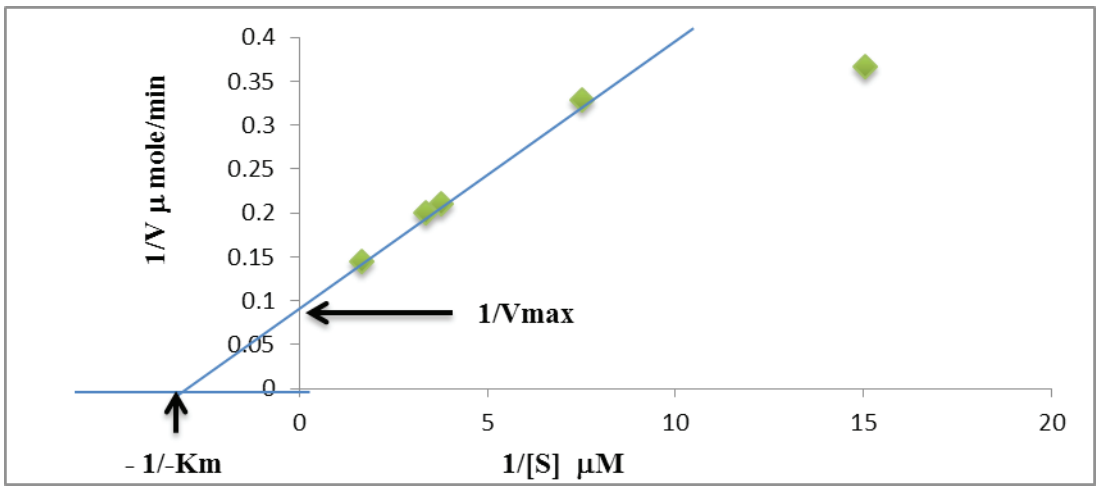

Figure (4): Determination of the Km value of *β-lactamase* with the use of a double resciprocal plot of the enzymic rate (mmole/min) against substrate concentration (mM).

(Vmax = 10.86 mmole/min) (Km = 0.27 mM)

Ethical Clearance : Taken from institutional ethical committee.

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Conflict of Interest : None

References

1. Kesado, T.; Lindqvist, L.; Hedberg, M.; Tunér, K. & Nord, C. E. Purification and characterization of a new beta-lactamase from Clostridium butyricum. *J. Antimicrobial agents and chemotherapy. 1989 ;*

33(8):1302-1307.

2. Babini, G. S.; Yuan, M. & Livermore, D. M. Interactions of β-lactamases with sanfetrinem (GV 104326) compared to those with imipenem and with oral β-lactams. *Antimicrobial agents and chemotherapy*. *1998*, *42*(5):1168-1175.

3-Abdul-Hussein, T. M.; Almaali, H. M. A.; Al-Jumaily, E. F.; Saadedin, S. M. & Al-Wattar, W. M. Extraction, Purification and Genotyping of β-Lactamase from Local Isolate of *Acinetobacter baumannii*. *Indian Journal of Public Health Research & Development. 2019*, *10*(10): 2013-2018.

4. Al-Jumaily, E.F.; Al-Taeei, Z.A.S. & Al-Safar, M.A. Purification and Characterization *β*-lactamase produce from local isolate Klebsiella pneumonia. *Baghdad Science Journal*, 2009, *6*(1): 50-60.

5. Arun, K.; Jayata, C. & Priyanka, D. Thermostable alkaline *β*-lactamase isolation and purification fromthermophilic bacterial strain Geobacillus stearothermophilus "AAP7919". *Indianjournals. com, Adv. Appl.Res.* 2014, *6*(2): 169- 177.

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

7. Hedberg, M.; Lindqvist, L.; Bergman, T.& Nord, C. E. Purification and characterization of a new beta-lactamase from Bacteroides uniformis. Antimicrobial agents and chemotherapy. 1995, *39*(7):1458-1461.

8. Hassan, R.M. Molecular and biochemical characterization of *β*-lactamase produced from local isolate of *Staphylococcus aureus*, M.Sc. Thesis. College of Biotechnology. *Al Nahrain University. 2019.*

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

10. Livermore, D. M. & Woodford, N. The β-lactamase threat in Enterobacteriaceae,Pseudomonas and Acinetobacter. *Trend s in Microbiol. 2006,* 14 (9): 413-420.

11. Bush, K. & Jacoby, G. A.Updated functional classification of β-lactamases. *Antimicrobial agents and chemotherapy,2010,* 54(3): 969-976.

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

13. Abdul-Hussein, T. M.; Saadedin, S. M.; Almaali, H. M. A.; & Al-Wattar, W. M. Evaluation of the phenotypic and genotypic detection of *Acinetobacter baumannii* isolated from Baghdad hospitals. *Plant Archives*, 2019 *19*(2): 3801-3804.

14. Hassan, R. M.; Abdullah, M. H.; Aziz, G. M. & Al-Sa'edy, A. J. Molecular and Biochemical Characterizations of Staphylococcusaureus ß-Lactamase Recovered from Iraqi Patients with UTI. *Indian Journal of Public Health, 2020*, *11*(04):1673.

15. Omeiri, M.; Holail, H. & Olama, Z. Purification and characterization of Staphylococcus *aureus* beta -lactamase from Lebanese community. *Int. J. Curr. Microbiol. App. Sci. 2014, 3* (2): 527-535.

16. Issa, A.H.; Saeed, E.A.& Sucker, D.K. The isolated and purified β-lactamase from local isolate of Staphylococcus aureus. *Al-Qadisiya J. Vet. Med. Sci., 2010*, *9*(1):11-20.

17. Segel, I.H. Biochemical calculations ,John Wiely and sons.NewYork.2nd edition. *1976*, pp344.

18. Ranade, Y. A.; Dharmadhikari, S. M. & Wadegaonkar, P. A. Screening, production, purification and characterization of beta-lactamase from uropathogenic E. coli. *Eur. J. Exp. Biol, 2013, 3*(1): 434-442.

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

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