ORIGINAL ARTICLE

Antibacterial Effect of Purified Protease Extracted from *Acinetobacter baumannii*

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ABSTRACT

The aim of the study is to investigate purified protease produced by *Acinetobacter baumannii* as an antibacterial agent against several gram-positive and gram-negative pathogenic bacteria. In this study**,** two hundred-thirty samples were collected from many hospitals in Baghdad from September 2019 to December 2019; Specimens were collected from numerous sources. Out of 230 clinical specimens, 50 (21.7%) were identified as *Acinetobacter baumannii* which was isolated from C.S, F 18(36%); blood specimens constituted 15(30%); burn and wounds specimens formed 10(20%); sputum specimens achieved 7(14%). A total of 50 isolates were used in screening for protease production on skim milk agar, 30/50 isolates being positive for protease by forming a clear zone around the colonies in different degrees. After that, the strongest protease producer *A. baumannii* was taken to study the optimum condition for its production and purification protease.
Keywords: Antibacterial, Purified Protease, *Acinetobacter baumannii*

INTRODUCTION

Acinetobacter baumannii is an opportunistic, coccobacillus, Gram-negative bacteria that were formerly considered to be the predominant cause of infections mostly in hospitals as well as the community. It avoids numerous types of antibiotics and could also survive throughout difficult circumstances in healthcare settings for such a long time[1]. *Acinetobacter baumannii* causes several different nosocomial infections, occurring in patients throughout the inte(2.

Different genomic, as well as phenotypic analyses of virulence factors have been found, involving external porins, phospholipases, proteases, lipopolysaccharides (LPS), capsular polysaccharides, protein secretion, and iron-chelating systems[3]. Various aspects are being linked to the mechanism of pathogenesis and the success of *A. baumannii*:- (i) The capacity to generate biofilms as well as to prevent drying over abiotic surfaces[4]. (ii) Its capability to bind to human epithelial cells, colonize as well as invade, Adherence with human bronchial epithelial cells via *Acinetobacter baumannii*[5]. (iii) Its antibiotic resistance spectrum can be upgraded as quickly as possible; (iv) its capability to obtain foreign genes via lateral gene transitions to support both its survival against antibiotic as well as host stresses (6). *Acinetobacter baumannii* is an important source of protease that catalyzes and cleavages peptide bonds of proteins and also are essential for cell growth and differentiation[7].

Proteases represent a group of industrialized enzymes, forming separately around 60% of the overall supply of enzymes (8). Protease has been the form of enzymes that occupies the leading role throughout physiological as well as market implementations[9]. Microbial proteases are essential to biotechnological applications between all of the proteases because it seems to be ecofriendly, cost- effective as well as secure catalysts[10]. Microbial proteases have broad variation and specificity being frequently utilized for therapy and diagnosis. Bacterial and fungal proteases may exceedingly contribute to the development of successful therapeutic substances including clot-dissolving, anti-cancer, antimicrobial, anti-inflammatory, etc[11].

Several works have been done on the antimicrobial properties of protease against several pathogenic organisms. However, no knowledge is available on the anti-bacterial effect of purified protease produced from *Acinetobacter baumannii*. Therefore, this work was carried out for isolation of the clinical *Acinetobacter baumannii* and extraction of protease enzyme and investigated the optimum conditions to improve its production, after that purifying protease and use it as an anti-bacterial agent against several types of pathogenic bacteria including (*Escherichia coli, staphylococcus aureus, Salmonella typhi*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*).

MATERIALS AND METHODS

Specimen's collection: Two hundred-thirty samples were collected from many hospitals in Baghdad from September 2019 to December 2019. Specimens were collected from numerous sources: blood, CSF, burn and wounds, sputum. Each swab was placed in tubes containing ready- transfer media and then at the laboratory immediately inoculated.

Isolation and identification of *Acinetobacter baumannii* isolates: In the laboratory under aseptic conditions, the collected specimens were streaked directly on blood agar and MacConkey agar, incubated for 24 hours at37ºC. then non-hemolytic and non-fermenting lactose isolates were streaked on blood agar supplemented with D-glucose as selective media and test its ability to grow at 44 °C. Identification of *A. baumannii* isolates according to morphological and cultural characteristics as well as conventional biochemical tests and after that confirmed by API 20E system.

Detection of protease enzyme production[12]: Skim milk agar medium (10%) was used to determine the ability of protease production from *Acinetobacter baumannii*. The ability of protease production was measured based on the presence of a clear halo zone around colonies and the isolate which showed the clear zone would be selected.

Optimum conditions for protease production: In our

study, we investigate the optimal conditions for protease production to obtain the best results about protease activity.

A. Optimum inoculum size: The optimum inoculum size of selected *A. baumannii* was studied by inoculating (0.5ml, 1ml, 2ml) of bacteria with 100 ml of production medium, then enzyme activity was detected as mentioned above.

B. Production medium for protease production:-The optimum production medium of selected *A. baumannii* was studied by using (nutrient broth, PDB which include peptone 2% + yeast extract 1% + dextran 2%[13], and tryptic soy broth) then enzyme activity was detected as in above.

C. The incubation period in shaker incubator:- Effect of the incubation period was studied by incubating the production medium at different periods (24, 48) hours, then enzyme activity was detected as in above.

D. Agitation in shaker incubator: Different number of rounds per minute (130, 150, 170, 200, and 220) were tested, then enzyme activity was detected as above.

E. Optimum incubation temperature:- the production medium was incubated at different incubation temperatures (28, 30, 35, 37 °C), then enzyme activity was detected as above.

Production and Extraction of protease[14]

we extract the crude enzyme from *Acinetobacter baumannii* isolate under optimal conditions to determine the enzyme activity and specific activity as following:- taking a single colony of *Acinetobacter baumannii* and inoculate it in brain heart infusion broth to activate it for 24 hours at 37 °C, then taking 0.5 ml of bacteria suspension inoculated into the productive medium nutrient broth and incubated in shaker incubator under optimal conditions (37 °C, 200 rpm, 24 hours), after that the supernatant was centrifuged by cooling centrifuge at the circumstances (4 °C, 6000 rpm, 20 minutes) and filtered, then used for determining protease activity by adding 0.2ml from supernatant to 0.8 ml of 1% casein solution and incubated for 30 min then the reaction was stopped by adding 1 ml of 5% TCA. The absorbance for the supernatant was measured at 280 nm using a UV spectrophotometer. The enzyme activity was estimated depending on the degradation of casein protein to small peptides and soluble amino acids. Protein concentration was carried out using method described by Bradford[15].

$$Enzyme\ Activity = \frac{Absorbance\ at\ 280\ nm}{0.01 * 30 * 0.2}$$

Protease purification: Protease produced by the selected isolate of *A. baumannii* was purified as follows:- first step was precipitation of crude enzyme by several concentrations of ammonium sulfate (50%, 60%, 70%, and 80%), and the mixture was mixed gently on a magnetic stirrer at 4°C for 20 minutes, then centrifuged at 6000 rpm for 20 minutes at 4°C. The precipitated proteins were dissolved in 50 ml of 0.05 M Tris- HCl buffer then dialyzed overnight using the same buffer. In the second step, the dialyzed enzyme was applied to an Ion-exchange chromatography column packed with DEAE-Cellulose equilibrated previously with 0.05M Tris-HCl buffer. the last step includes the volume of concentrated protease got from the ion exchange step was applied onto the Sephadex G-200, equilibrated previously with 0.01 M Tris- HCl buffer pH8.0. Protein concentration was carried out using (15) method. The specific activity of protease was estimated according to the following equation[16] :

$$Specific\ Activity\ \left(\frac{unit}{mg}\right) = \frac{Enzyme\ activity\ \left(\frac{unit}{ml}\right)\ protein}{Concentration\ \left(\frac{mg}{ml}\right)}$$

Antibacterial activity of crude and purified protease enzyme by *Acinetobacter baumannii in vitro:* The activity of protease as an antibacterial agent was tested according to the Agar-well diffusion method (17), Muller-Hinton agar plates were inoculated with indicator isolates (*Escherichia coli, staphylococcus aureus, Salmonella typhi*, *Klebsiella pneumonia,* and *Pseudomonas aeruginosa*) by using few colonies from overnight culture transfer to five ml of normal saline to prepare the bacterial suspension which was adjusted to 0.5 Macfarland turbidity equal to (1.5×108) CFU/ml. Spread 0.1 ml of bacterial suspension on the surface of plate Muller-Hinton agar, add 100µl of protease enzyme (concentration 100 µg/ml) into each well. A control well was made in the center with the distilled water. The plates were incubated overnight at 37ºC in an inverted position and the diameter of any resulting zones of inhibition was measured in millimeters.

RESULTS AND DISCUSSION

Isolation and identification of Acinetobacter baumannii isolates: Out of 230 clinical specimens, 50(21.7%) were identified as *A. baumannii* which was isolated from C.S,F 18(36%); blood specimens constituted 15(30%); burn and wounds specimens formed 10(20%); sputum specimens achieved 7(14%) as listed in table (3-1). All isolates show smooth, rounded, mucoid, small, light pink color, and non-lactose fermented on McConkey agar. on blood, agar appears white to grey small colonies and non-blood hemolysis. All isolates show Growth ability at 44 oC. cultured on blood agar supplement with glucose, to indicate the ability of isolates to change the color of the medium around the colony to brown color. Microscopic identification appears gram-negative Coccobacilli bacteria, arranged as diplococci. Biochemical tests as listed in the table (3-2) show positive to citrate and catalase, negative to oxidase and indole production. API 20E system confirmed the results of morphological and biochemical tests.

Screening and selection of the protease producing *Acinetobacter baumannii* isolates

Screening the protease producing isolates: Total 50 isolates that gave typical and morphological characteristics and biochemical tests related to *A. baumannii* were used in screening for protease production skim milk agar. The results of primary screening were 20/50 isolates negative for protease production, 30/50 isolates positive for protease production according to form a halos zone of hydrolysis around the colonies with variable degrees as shown in table 3 and Figure 1.

Selection of maximum protease producing *Acinetobacter baumannii* isolate: Additional screening was made for protease production isolates depending on the clear zone diameter to determine the degree of protease production according to three classes (weak, moderate, strong). Then we take the isolate with the widest diameter of a clear zone (18mm) to study the optimum condition and purification of protease enzyme as listed in table 4, figure 2.

Optimum conditions for protease production by *A. baumannii:*

A. Optimum inoculum size: Three inoculum sizes were used (0.5, 1, 2) ml inoculating to 100 ml of the production medium. It was found that protease production increased with inoculum size to 0.5 ml which clear zone being (22 mm) as shown in Figure (3-3), this result associated with the density of cells, competitions on nutrients, and rising toxic secondary metabolites which suppress growth cells, causing a drop in the production of an enzyme [18].

C. Optimum temperature in shaker incubator: The different in incubation temperatures (28,30, 35, and 37) °C were used. Results showed the optimal temperature at 37oC with (20 mm) clear zone as shown in Figure 5.

B. Optimum production medium for protease production: The media used (nutrient broth, PDB which included peptone 2% & yeast extract 1% a& dextran 2% broth, and TSB). The results showed that nutrient broth was the best one for protease production with (19 mm) clear zone as shown in Figure 4.

D. The optimum incubation period in the shaker incubator : Two times of incubation period (24, 48) were demonstrated, results showed that 24 hours was the best when compared with 48 hours with (19 mm) clear zone as shown in Figure 6.

E. Agitation: Five different rounds per day (130, 150, 170, 200, 220), 200 and 220 rpm showed the best with (20 mm) clear zone as shown in Figure (7). Agitation plays an important role in the transfer rate of nutrients, the solubility of oxygen, cell dispersion, and thereby enhanced aerobic metabolism of microbes [19].

Extraction of the crude enzyme: The selected protease producing isolate was grown through the optimal condition noted above to obtain the protease enzyme, 0.5ml inoculum size inoculated into 100ml of nutrient broth as a productive medium and incubated at 37°C for 24hrs in shaking incubator (200 rpm), protease activity and specific activity was 30.8unit/ml and 236.92unit/mg protein respectively, and yield concentration of protein was 0.130 mg/ml.

Purification of protease: Crude protease precipitated by Ammonium sulfate, the best saturation for precipitation was 80 %, the protease activity, and specific activity was (81.75 unit/ml) and (345 unit/mg) protein respectively, and the protein concentration was 0.237mg/ml, with a fold of purification 1.45 and enzymic yield 66.37%. The next step of purification by Ionic exchange chromatography patterns that showed protease activity and specific activity was (52 unit/ml), (1209.3unit/mg) respectively, and a fold of purification (5.1) and enzymatic yield (33.76%). The gel filtration chromatography technique being the last step in the purification of protease produced by *Acinetobacter baumannii*, protease activity (59.3unit/ml), protein concentration (0.0295mg/ml) with specific activity (2010 unit/mg) and the purification fold was (8.48) with a yield of an enzyme (28.87%).

In vitro Anti-bacterial activity of protease: The protease was tested for its' antibacterial effect against several pathogenic bacteria including (*Escherichia coli, staphylococcus aureus, Salmonella typhi*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*), the results listed in the table (3-6) showed that protease has an inhibitory effect against all isolates except *Klebsiella pneumonia.* Also, our results demonstrated the pure protease has a better effect as an antibacterial agent comparing with crude protease. The actual mechanism by which the proteolytic enzymes act on bacteria has not been defined. Since it had been established that these enzymes hydrolyze ester or peptide bonds it seems likely that protease act by affecting the chemical integrity of the bacteria, attacking the proteinaceous components of their cell walls[20].

Table 1: The source of isolated *Acinetobacter baumannii*

Source of isolate	%age	No. of isolates
C.S.F	3	18
BLOOD	30	15
SPUTUM	14	7
Swab (burn, post- surgical) Wound	20	10
Total	100	50

Table 2: Biochemical tests of *about Acinetobacter baumannii*

Biochemical tests	Results
Oxidase production	-
Catalase production	+
Indole production	-
Methyl-red	-
Voges- Proskauer	-
Citrate utilization	+
Lactose fermentation	-
Growth at 44C	+
Hemolysin production	-

(+): positive result; (-): negative result.

Table 3: Screening about protease producer *Acinetobacter baumannii*

No. of isolates	Protease production	Non-protease production
50	30	20
%age	60	40

Fig 1: A. Non-protease producer *Acinetobacter baumannii*
B. Protease producer *Acinetobacter baumannii*

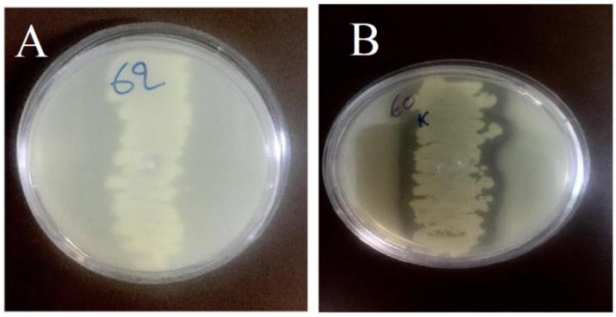

Fig. 2: Protease production on skim milk agar by *Acinetobacter baumannii*

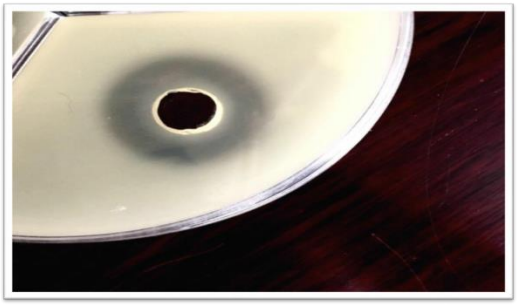

Figure 3: Protease production by Acinetobacter baumannii using different quantity of inoculum size

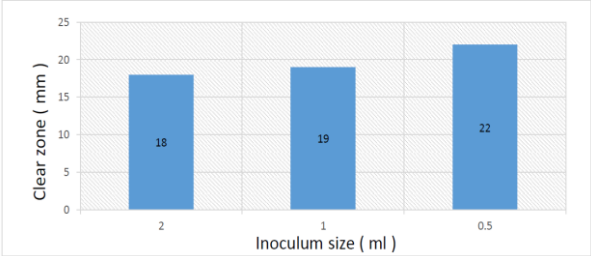

Figure 4: Protease production by *Acinetobacter baumannii* using different types of media

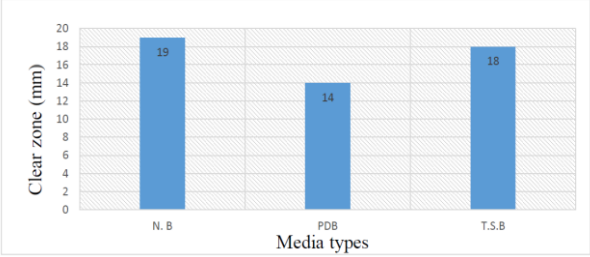

Figure 5: Protease production by *A. baumannii* using a different temperature degree

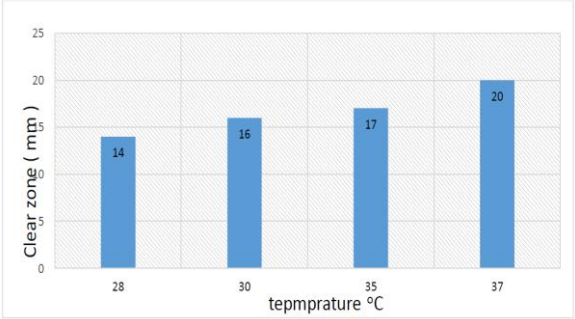

Figure 6: Protease production by *A. baumannii* using two different incubation periods

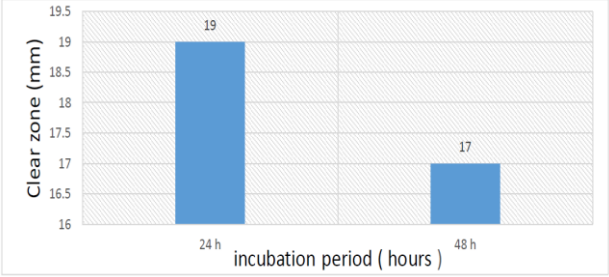

Fig. 7: Protease production by *A. baumannii* testing different agitation

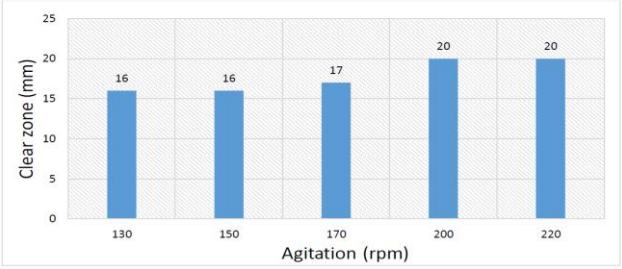

Figure 8: Ion exchange chromatography for purification of protease produced by *Acinetobacter baumannii* using DEAE-Cellulose column (20x2cm) with a flow rate of 30 ml/hour

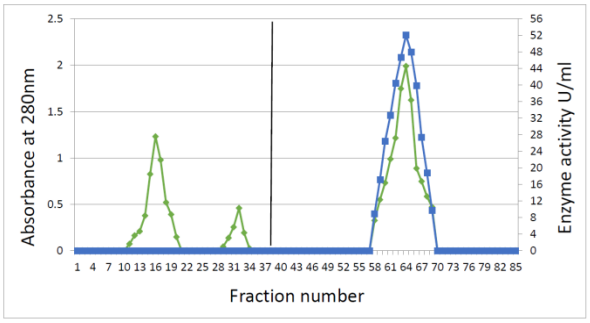

Figure 9: Gel filtration chromatography of protease produced by the *Acinetobacter baumannii* using Sephadex G-200 column (1.5cmx35cm) equilibrated with Tris-HCL buffer pH 8, fraction volume was 3ml at a flow rate of 30 ml/hour.

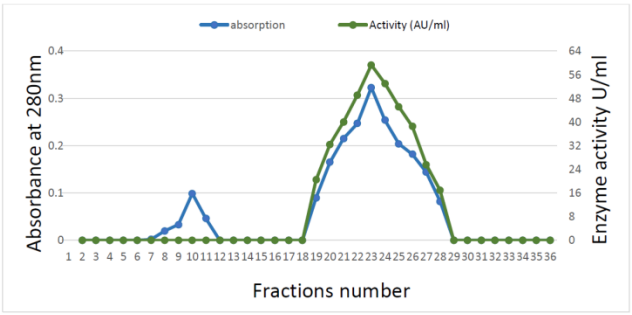

Figure 10: antibacterial effect of crude and pure protease produced by *Acinetobacter baumannii* against different types of pathogenic bacteria symbol (c) represent control, (1) pure protease, (2) crude protease against A) *Salmonella typhi,* B) *Staphylococcus aureus* C) *Klebsiella pneumonia* D) *Pseudomonas aeruginosa* E) *Escherichia coli*

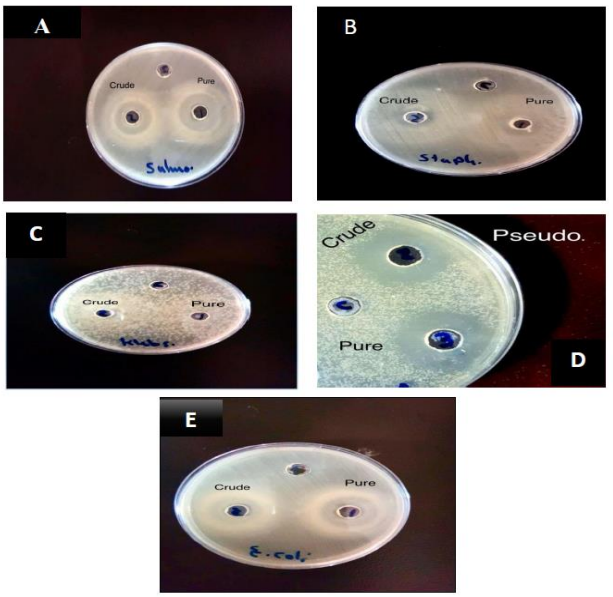

Table 4: Protease production degree from *acinetobacter baumannii*

Productive isolate	Degree of protease production	The diameter of a clear zone(mm)	Productive isolate	Degree of protease production	The diameter of a clear zone(mm)
1	+ +	12	16	+ + +	15
2	+ +	13	17	+ + +	16
3	++	14	18	+++	17
4	++	12	19	+++	17
5	++	13	20	+++	15
6	+	9	21	+++	18
7	+	9	22	++	13
8	+	10	23	++	14
9	++	14	24	++	12
10	++	12	25	+++	16
11	+	10	26	+++	17
12	++	13	27	+++	16
13	++	14	28	+++	16
14	+	11	29	++	13
15	++	12	30	+	11

Table 5: Purification steps for Protease produced by *A. baumannii.*

	Vol. (ml)	Conc.	Enzyme activity U/ml	Specific Activity (U/mg)	Total Enzyme activity	Fold	Yield%
Crude	100	0.130	30.8	236.92	3080	1	100
Ammonium Sulphate%	25	0.237	81.78	345	2044.5	1.45	66.37
Ion –exchange	20	0.043	52	1209.3	1040	5.1	33.76
Gel filtration	15	0.0295	59.3	2010	889.5	8.48	28.87

Table 6: Antibacterial effect of crude and pure protease against pathogenic bacteria

Bacterial isolate name	Type of enzyme	The diameter of the inhibition zone(mm)
Escherichia coli	Crude enzyme	12 mm
	Pure enzyme	15 mm
Staphylococcus aureus	Crude enzyme	10 mm
	Pure enzyme	13 mm
Salmonella Typhi	Crude enzyme	13 mm
	Pure enzyme	16 mm
Klebsiella pneumonia	Crude enzyme	No effect
	Pure enzyme	No effect
Pseudomonas aeruginosa	Crude enzyme	18 mm
	Pure enzyme	20 mm

Conflict of interest: No conflict of interest
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