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EXTRACTION AND PURIFICATION OF L-ASPARAGINASE PRODUCED *ACINETOBACTER BAUMANNII* AND THEIR ANTIBIOFILM ACTIVITY AGAINST SO PATHOGENIC BACTERIA

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ABSTRACT

L-asparaginase is an enzyme catalyzing the hydrolysis of L-asparagine and formation of L-aspartate and ammonia and widely used as anticancer drug in pharmaceutical and food industry. This amino acid (L-asparagine) has an important role for development of cancerous, in contrast the normal cells don't need this amino acid. Eight Acinetobacter baumannii isolates of were isolated from different blood and sputum samples and it was found that high isolation rate of Acinetobacter baumannii isolates from sputum was consisted with their association with lower respiratory tract infections. All these eight isolates were screened for higher L-asparaginase production and found that among all these isolates, Acinetobacter baumannii Sp9 gave higher asparaginase activity of 7.32 U/ml. L-asparaginase was purified to homogeneity by sequential chromatographic steps involved ammonium sulfate at 45% saturation followed by DEAE-cellulose ion exchange chromatography and sephadex G-100 gel filtration chromatography with a recovery yield of 68% and 22.65 fold of purification. L-asparaginase had antibiofilm activity against all tested biofilm forming pathogenic bacteria (Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus and Acinetobacter baumannii) after using Congo red agar and Microtitration plates methods for detecting biofilm formation ability. Highly antibiofilm of L-asparaginase recorded against Klebsiella pneumoniae followed by Pseudomonas aeruginosa with reduction of biofilm formation ratio to 32 and 41% ,respectively compared with (100)% of control. Thus we can conclude that L-asparaginase has promising benefit as antibiofilm agent against biofilm forming pathogenic bacteria that have multidrug resistance.

Keywords: *Acinetobacter baumannii*, L-asparaginase, Extraction, Purification, Pathogenic bacteria and antibiofilm activity

1. INTRODUCTION

Acinetobacter baumannii can be an opportunistic pathogen in humans, affecting people with compromised immune systems and is becoming increasingly important as a hospital derived infection (nosocomial) (Ahmad *et al.*, 2012). The predominant site of colonization, in hospitalized patients is the skin, but respiratory tract or digestive systems may also be colonized (Bergogne and Tower, 1996). Most of the *Acinetobacter baumannii* infections resulted in pneumonia, urinary tract, blood stream and surgical wound infections (Henwood *et al.*, 2002; Muslim *et al.*, 2015). *Acinetobacter baumannii* are able to survive on moist and dry surfaces, are found on fruits and vegetables and on the health human skin (Patricia *et al.*, 2010) so that the environment, soil, water and animals are the natural habitats for it Fournier and Richet (2006). Non specific nutritional requirements, resistance to desiccation and the ability to form biofilm permit *Acinetobacter baumannii* to grow in various environment, and therefore initial contact with the pathogen preceding infection can be made in a variety of ways (Patricia *et al.*, 2010; Espinal *et al.*, 2012)

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L-asparaginase is an enzyme (L-asparaginase amidohydrolase, EC 3.5.1.1) that lead to hydrolysis of L-asparagine and formation of L-aspartate and ammonia. This amino acid has an important role for development of cancerous, in contrast the normal cells don't need this amino acid (Ebrahiminezhad *et al*., 2011; Ahmad *et al*., 2012). The cancer cells deprived of an important growth factor In the presence of L-asparaginase so that these cells cannot grow, therefore; L-asparaginase may be used as anticancer drug (Ahmad *et al*., 2012). L-asparaginase is used as therapeutic agents in the treatment of Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosarcoma (Verma, 2007; Ahmad *et al*., 2012).

L-asparaginase produces by bacteria and plants also found in the rodent·s surum,. Microorganisms can produce of L-asparaginase frequently, because they can be easily cultured and the procedures of purification of L-asparaginase from them are convenientand suitable for the production of enzyme at the industrial scale (Ahmad *et al*., 2012).

Biofilms are defined as communities of microbial cells growing on a surface and embedded in a self-synthesized matrix composed of extracellular polymeric substances (David *et al*., 2002). The major components of these exopolysaccharides (EPSs) are considered the key component of extracellular matrix, but nucleic acid, proteins, lipids and other components released from cell can constitute a significant rate in the biofilm matrix and act as a physical substratum to maintain biofilm structure and functions (Dowrkin, 1993). Exopolysaccharides have been found to play an important role in the initial attachment to a surface (David *et al*., 2002). There are numerous examples of biofilms formed by pathogenic strains which pose serious problems to human health, such as lung infections, ear and eye infections, dental diseases, and urinary tract infections. Deleterious biofilms are also problematic in industry because they cause corrosion in heat exchangers and pipelines for transferring oil and service water. Biofilm cells survive highly resistant to antibiotics and lead to medical treatment failure (Ren *et al*., 2004). Therefore, this study aimed to screen the production of L-asparaginase by *Acinetobacter baumannii* besides to purify this enzyme and investigating the antibiofilm activity of L-asparaginase against some biofilm forming pathogenic bacteria.

2. MATERIAL AND METHODS

2.1. Sample Collection

The study included a total of 60 samples of which 30 were from sputum and 30 were from blood collected from patients at different hospitals in Baghdad city. The collected microbial sources were transported to the laboratory. Samples were plated primarily on to blood agar and MacConkey agar which was incubated at 37 ºC for 48 h.

2.2. Bacteriological Analysis

The pure culture isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of systemic bacteriology. Suspicious isolates were presumptively identified by using colony morphology, negative oxidase test, positive catalase test inability to motile coccobacilli (Constantnu *et al*., 2004). In addition *Acinetobacter baumannii* isolate was confirmed by using Vitek 2 system by using Vitek GPI card (bio Merieux, France) according to the manufacturer's instructions.

2.3. Screening for L-Asparaginase Production

All bacterial isolates were evaluated for their ability to produce L-asparaginase by culturing in M9 medium containing L-asparagine with phenol red as indicator and incubating at 30ºC for 24h. L-asparaginase producing colonies were selected on the basis of formation of pink zone around the colonies of the medium (Kamble *et al*.,

2012). All active isolates were cultivated in M9 broth supplemented with L-asparagine and L-asparaginase activity in the cells was assayed after centrifugation at 3000 rpm for 20 min.

2.4. L-Asparaginase Purification

Acinetobacter baumannii L-asparaginase was purified by a modification of the method (Boyd and Phillips, 1997). Cells harvested after 24 h of incubation in M9 medium containing L-asparagine with phenol red as indicator were washed twice with 0.05M tris-HCl buffer, pH 7.2 and suspended in two volumes of cold buffer . The sonically disrupted suspension was centrifuged at 10000xg for 20 min . To the supernatant fluid, 0.05ml of 1.0M $MnCl_2$ was added for removal of nucleic acids. The mixture was then stirred for 2 hours and recentrifuged. To the supernatant L-asparaginase activity was assayed. The sample was subjected to ammonium sulfate fractionation at ratio of saturation 20-60%. The mixture was centrifuged, the preipitate was resuspended with0.05M tris-HCl buffer, pH 7.2, then L-asparaginase activity was assayed. The supernatant was dialazed against distilled water and the L-asparaginase activity was assayed. The supernatant was loaded on chromatographic column (2.5 by 20 cm) containing 100 ml of diethyl aminoethyl (DEAE)-cellulose which had been equilibrated with 0.05M tris-HCl buffer, pH 7.2. The column was washed with the same buffer. A gradiual elution was then run ranging from 0.1M KCl to 0.4M KCl. Fractions (3ml) were collected and assayed for Lasparaginase enzyme activity .The Fractions that shown L-asparaginase activity were loaded on Sephadix G-100 column (1.5 by 80 cm) containing 100 ml of Sephadix G-100 which had been equilibrated and washed with 0.2 M phosphate buffer and the elution done by the same buffer . The fractions (2ml) were collected and assayed for L-asparaginase activity.

2.5. L- Asparaginase Assay

L-asparaginase activity was measured following method of Boyd and Phillips (1997). This method utilizes the determination of ammonia liberated from L-asparagine in the enzyme reaction by the Nessler's reaction. Reaction was started by adding 0.5 ml supernatant into 0.5 ml of 0.04 M L-asparagine and 0.5ml of 0.05M tris-HCl buffer, pH 7.2 and incubated at 35ºC for 30 min. The reaction was stopped by the addition of 0.5ml of 1.5 M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined calorimetrically by adding 0.2 ml Nessler's reagent (45.5 g HgI_2 and 35.0 g KI in 1 liter distilled water containing 112g of KOH) into tubes and incubated at room temperature for 15 min. After vortexing, the absorbance was measured at 500nm. A standard curve was drawn with various concentrations of ammonia.

2.6. Protein Determination

Total protein was determined by using bovine serum albumin as protein standard and according to the method of Bradford (1976).

2.7. Biofilm Inhibition Assay by L-Asparaginase

2.7.1. Congo Red Agar Medium Method

Five bacterial isolates included *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Acinetobacter baumannii* were collected from biology department laboratories. These isolates have the ability to form the Biofilms. In order to assess the biofilm formation ability of these bacterial isolates, the Congo-red agar medium containing per liter brain heart infusion broth,37g; sucrose,50g; agar,10g and kongo red stain,0.8g (Freeman *et al.*, 1989) was inoculated with a suspension of bacterial isolate that turbidity is approximately equivalent to the McFarland No. 0.5 turbidity standard. The plates were incubated aerobically for 24-48 hours at

37°C. A positive result was indicated by black colonies with a dry crystalline consistency as blank. Non- slime produces usually remained pink.

The purified L-asparaginase was tested for its potential to prevent biofilm formation of a biofilm producing bacteria that mentioned above by modification the method that described by Christensen *et al.* (1982). 0.1 ml of purified L-asparaginase was spreaded on the surface of Congo-red agar medium and left to dry for 15 min then each type of bacterial isolates was cultured and incubated at the same conditions above. After the incubation period the growing colonies were compared with the blank.

2.7.2. Microtiter Plate Method

Flat bottomed Microtitration plates were used to determine the effect of L-asparaginase in inhibition of biofilm formation. The first well was filled with 200 µl of brain heart infusion broth containing 2% sucrose and bacterial culture of each type of bacterial isolate, this well was used as blank. The second well was filled with 100 µl of the purified L-asparaginase and 100 µl of brain heart infusion broth containing 2% sucrose and bacterial culture of each type of bacterial isolate. The plates were incubated at 37°C for 24hrs., culture supernatants from each well were then decanted and planktonic cells were removed by washing for three times with distilled water. Biofilms were rinsed with water and stained for 1 min with 200 µl of Gram crystal violet. Stained biofilms were rinsed with water and then dried. The amount of crystal violet binding was quantitated by destaining the biofilms for 10 mints. with 200 µl of 33% acetic acid and then the absorbance of the crystal violet solution at 630 nm was measured. The percentage of biofilm formation was calculated from the equation that described by Gudina *et al.* (2010).

Percentage of biofilm formation (%)= (O.D. in presence of purified L asparaginase / O.D of blank)x 100

3. RESULTS AND DISCUSSION

3.1. Isolation of *Acinetobacter Baumannii*

Out of 60 samples collected, 8 (13%) *Acinetobacter baumannii* was isolated. Among which 5/8 (62.5%) were from sputum , 3/8(37.5%) were from blood as shown in figure-1. The high isolation rate of *Acinetobacter baumannii* isolates from sputum is consisted with their association with lower respiratory tract infections (Humphreys and Tower, 1997). A small majority of the isolates belonging to other groups were recovered from blood, indicating that they also cause serious infections, albeit less frequently than isolates of the *Acinetobacter baumannii*, however, this association with blood could also reflect the fact than non- *Acinetobacter baumannii* isolates are ubiquitous members of the normal human skin flora and are therefore prone to cause contamination of blood cultures (Bergogne and Tower, 1996). *Acinetobacter* spp. isolates are mostly implicated in various nosocomial infections like respiratory tract infections, blood stream infections, wound infections and urinary tract infections (CLSI (Clinical and Laboratory Standards Institute), 2011). In addition (Brock *et al.*, 2009) found that *Acinetobacter baumannii* was most frequently isolated from sputum samples (48%) and wound samples (19%), while *Acinetobacter lwoffui* was mainly isolatated from blood samples or intravascular lines (42%).

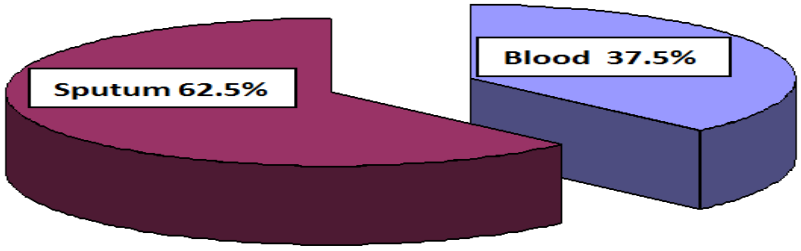

Figure-1. Isolation percentage of *Acinetobacter baumannii* from sputum and blood samples

3.2. Screening for L-Asparaginase Production

The results showed that all *Acinetobacter baumannii* isolates could grow on M9- asparagine agar plates with phenol red. The pink zones around *Acinetobacter baumannii* colony indicate the pH alteration which originated from ammonia accumulation in the medium. *Acinetobacter baumannii* Sp_3 exhibited the large zone red when compared with their colony diameter (figure-2). All active isolates were cultivated in M9 broth supplemented with L-asparagine. It was found that the range of L-asparaginase activity 2.76 to 7.32 U/ml (figure-2). *Acinetobacter baumannii* Sp_3 was demonstrated higher asparaginase activity of 7.32 U/ml. Shah *et al.* (2010) revealed that by using the optimized fermentation parameters, the enzymatic activity was at lowest values in the log phase and increasing in the exponential phase, at 26 hours it reached to the maximum values and in the early stationary phase (up to 30h.) of the growth cycle the activity was stable and continued in decreasing at late stationary phase. L-asparagine as carbon source in the culture lead to induce of L-asparaginase production by the tested organism (Amena *et al.*, 2010). Some microorganisms such as *E. coli*, *Aeromonas* spp., *Proteus* spp., *Acinetobacter baumannii*, *Serratia* spp., *Pseudomonas aeroginosa and Bacillus* spp. were screened for L-asparaginase production and revealed an ability to reduce this enzyme (Kamble *et al.*, 2012).

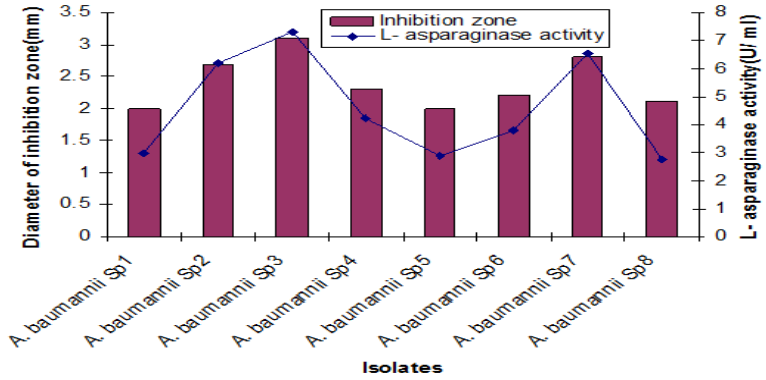

Figure-2. Detection of L-asparaginase production for *Acinetobacter baumannii* isolates

3.3. Purification of L-Asparaginase

L-asparaginase purification from *Acinetobacter baumannii* Sp_3 is summarized in table-1. L-asparaginase yielded with a 22.65-fold purification and 68% recovery. The precipitation step with ammonium sulfate at 45% saturation to the crude extract led to rise in the enzyme activity. The precipitate was dialyzed against distilled water to remove the remain of ammonium sulfate and loaded on DEAE- cellulose column(2.5 by 20 cm). The typical elution profile from DEAE column is shown in figure-3. since the elution with KCl solution led to presence three protein peaks and L-asparaginase activity was located in the third protein peak. Additional purification was done on Sephadix G-100 column (1.5x 80cm), the column was washed with several column volumes of 0.2 M phosphate buffer, and finally eluted with the same buffer. Fractions containing high specific activity were pooled and used for further studies as shown in figure-4.

L-asparaginase was purified from *Acinetobacter baumannii* isolated from cucumber and lettuce plant rhizosphere soils to homogeneity with a recovery yield of 77% (Muslim, 2014). L-asparaginase was purified from *Pseudomonas aeroginosa* by gel filtration on sephadex G-100 column (Amena *et al.*, 2010). Also in a study reported by Baumann (1996) found that L-asparaginase was purified from *Erwinia carotovora* by sulphopropyl sephadex chromatography. In addition, *E. coli* L-asparaginase was partially purified with ammonium sulphate precipitation at 20-40% saturation (El-Bessoumy *et al.*, 2004).

Table-1. A summary of treatments used for the purification of L-asparaginase from *Acinetobacter baumannii* Sp3

Purification step	Size(ml)	L-asparaginase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/ mg)	Total activity	Purification fold	Yield (%)
Crude extract	150	7.32	2.18	3.35	1098	1	100
$(NH_4)_2SO_4$ precipitation	65	16.31	1.65	10.45	1060	3.11	96.5
DEAE-cellulose	20	39.20	1.02	38.43	784	11.47	71.4
Sephadex G-100	12	62.25	0.82	75.91	747	22.65	68.0

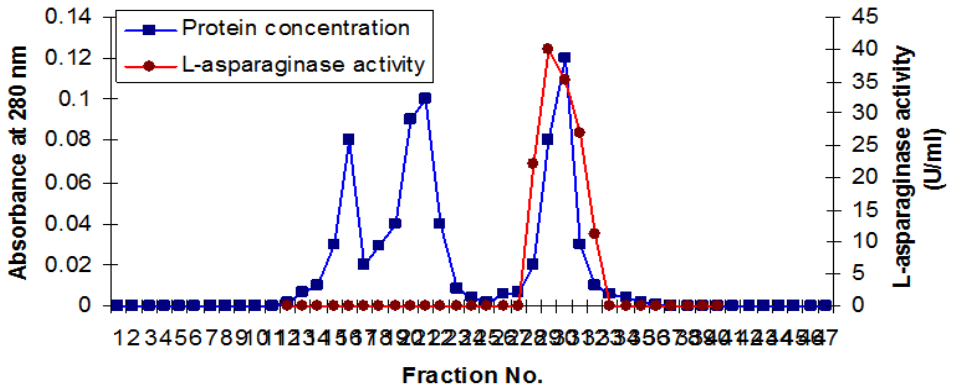

Figure-3. Purification of L-asparaginase from *Acinetobacter baumannii* Sp_3 by using ion exchange chromatography on DEAE-cellulose column

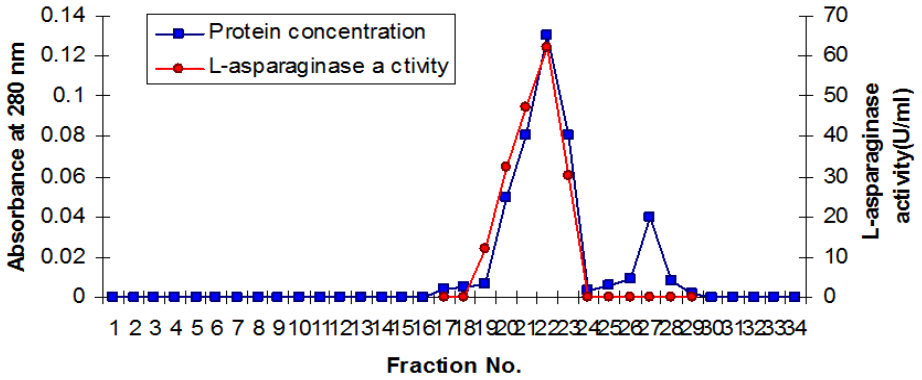

Figure-4. Purification of L-asparaginase from *Acinetobacter baumannii* Sp_3 by using gel filtration chromatography on Sephadex G-100 column

3.4. Biofilm Inhibition Assay by L-Asparaginase

3.4.1. Congo Red Agar Medium Method

The results showed that the color of growing colonies was changed from black colonies with dry crystalline consistency to pink or pale grey colonies with no change in the color of the medium according to the type of bacteria. The colonies of *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* appeared as pink colonies, while *Pseudomonas aeruginosa* and *Acinetobacter baumannii* appeared as pale grey colonies so that these results refer to inhibit of biofilm formation by the purified L-asparaginase. The presence of pink or pale grey colonies on Congo red agar medium refer to lose the ability to form bio films and non-slime layer on the surface of the medium.

3.4.2. Microtiter Plate Method

The purified L-asparaginase in microtiter plate method showed varying proportions in biofilm inhibition according to the tested bacteria. The higher inhibition rate by L-asparaginase against *Klebsiella pneumoniae* since the biofilm was formed with 32% followed by *Pseudomonas aeruginosa* with 41%. In contrast, the lower inhibition

rates by L-asparaginase against *Staphylococcus aureus* and *Acinetobacter baumannii* with 79 and 86%, respectively (table-2). According to these results we can conclude that L-asparaginase has an ability to inhibit biofilm formation by pathogenic bacteria. There are three modes of action for most antibiofilm polysaccharides ,the first they may be act as surfactant molecules that modify the physical characteristics of bacterial cells and abiotic surfaces. or they act as signalling molecules that modified gene expression of biofilm forming bacteria, or a competitive inhibition of multivalent carbohydrate–protein interactions (Rendueles *et al*., 2012).

Table-2. Percentage of biofilm formation by pathogenic bacteria in presence of purified L-asparaginase from *Acinetobacter baumannii*

Pathogenic bacteria	Optical density		Percentage of biofilm formation (%)
	Blank	Purified L-asparaginase at(µg/ml)	
Staphylococcus aureus	0.152	0.109	75
Klebsiella pneumoniae	0.265	0.085	32
Pseudomonas aeruginosa	0.233	0.096	41
Acinetobacter baumannii	0.135	0.112	83
Escherichia coli	0.235	0.133	56

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