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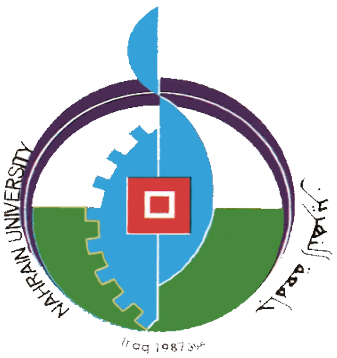
Al-Nahrain University
College of Science
Department of Biotechnology



Production, purification, Characterization and cytotoxic effect of L-asparginase from locally isolated *Pseudomonas aeruginosa*

A Thesis

Submitted to the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

Elaf Ismail AL-khassaki

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Supervised by

Dr. Asmaa A. Hussein

(Assistant professor)

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

((يَا أَيُّهَا الَّذِينَ آمَنُوا لَا تَتَّخِذُوا الْيَهُودَ وَالنَّصَارَىٰ أَوْلِيَاءَ ۘ بَعْضُهُمْ أَوْلِيَاءُ بَعْضٍ ۚ وَمَن يَتَوَلَّهُم مِّنكُمْ فَإِنَّهُ مِنْهُمْ ۗ إِنَّ اللَّهَ لَا يَهْدِي الْقَوْمَ الظَّالِمِينَ))

صدَقَ الله العَظِيم

سورة المائدة – الاية : ٥١

Supervisor Certification

I, certify that this thesis entitled "**Production, purification and Characterization of L-asparginase from *Pseudomonas aeruginosa"*** was prepared by "**Elaf Ismail AL-khassaki"** under my supervision at the College of Science /Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

Signature:
Name: **Dr. Asmaa A. Hussein**
Scientific Degree: Assistant professor
Date:

In view of the available recommendations, I forward this thesis for debate by the examining committee.
Signature:
Name: **Dr. Nidhal Abdulmohaimen Mohammed**
Scientific Degree: Professor
Title: Head of biotechnology department
Date:

Dedication

To the candle that burned to enlighten my way in life

My Mother

To the man who was the best supporter to me in my life

My Father

To the man who was always beside me

My Husband

To my little diamond

Haya

Elaf

Acknowledgment

At the beginning, thanks to creator of the Universe, the great God Allah who gave me the reality and strength to accomplish this work.

I would like to extend my thankful, respection and appreciation to my supervisor Dr. Asmaa A. Hussein who has given me her advices and important guidance throughout this study.

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My thanks are due to the Dean of College of Science as well as head and staff of the Department of Biotechnology.
My thanks are devoted to the colleagues and to all who give me hand in this regards.

Finally, I wish to thank my family for their patience and support asking God to bless them.

Elaf

Summary

In this study, a total of 46 clinical samples were collected for the isolation of *P .aeruginosa*, 14 samples taken from wounds, 20 samples from burns and 12 samples from ear infections. All samples were collected from Al-yarmouk hospital. Among these samples, a total of 29 bacterial isolates were successfully cultured .Six of them were subjected to the identification according to their morphological, cultural, biochemical characteristics and VITEK2 system since they were identified as *P. aeruginosa*.

The ability of these isolates for L-asparginase production was examined. Results showed that all six isolates of *P. aeruginosa* were L-asparginase producers with variable degrees. Among them, the isolate *P. aeruginosa* P4 was the most efficient in L-asparginase production. Its specific activity of L-asparginase in crude filtrate was 0.15U/mg protein. Therefore, *P. aeruginosa* P4 was selected for enzyme characterization and purification.

The optimum conditions for L-asparginase production were studied. Results showed that maximum L-asparginase production was achieved after supplementation of the production medium (pH7) with 0.1% glycerol and 0.1% tryptone , inoculated with a 10^8 cells/ml of fresh bacterial culture and incubation at 37^oC in shaker incubator (150 rpm) for 24h. Under these conditions, the specific activity of L-asparginase produced in culture medium was sharply increased to 0.6 U/mg protein.

L-asparginase produced under the optimum conditions was purified in three purification steps, first by precipitation with 80% saturation of ammonium sulfate, second by ion exchange chromatography using DEAE-Cellulose column, while the gel filtration chromatography throughout Sephadex G-200 column was the third

step. Specific activity of the purified enzyme was increased up to15 U/mg with 25 folds of purification and 60% enzyme recovery.

Some biochemical characteristics of the purified enzyme were studied. It was found that the molecular weight of L-asparginase produced by *P. aeruginosa* P4 was about 120000 Dalton, the highest enzyme activity at pH 7, while that for stability was 8. Also 37^{o}C gave the higher activity, hence the enzyme was stable with full activity at a range of temperatures between 20-37^{o}C.

Enzyme activity was inhibited in the presence of $HgCl_2$, $CaCl_2$, mercaptoethanol, and cysteine that were added individually at different concentrations. Also the enzyme is not affected by EDTA indicating it was not a metalloenzyme.

In this study, crude and purified asparginase were tested for *in-vitro* cytotoxicity activity using MTT assay against MCF7 cancer cell line and the purified asparginase was found to inhibit the growth of cell line with an IC50 of 185.7 Mg\ml in comparison to an IC50 of 355.6 Mg\ml for crude enzyme. On the other hand, the enzyme didn't show significant effect on normal cell that it may have high potential for cytotoxicity on cancer cell.

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List of Abbreviation

Abbreviation	Full name
BSA	Bovine Serum Albumin
DEAE	Diethyl aminoethyl
EDTA	Ethylenediaminetetraacetic acid
TCA	Trichloroacetic acid
MDR	Multidrug risistance
OFAT	Optimization by one factor at a time
D.W	Distilled water
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]
IC_{50}	Inhibitor concentration
ID-GNB cards	Identification card
RPMI	Roswell Park Memorial Institute medium
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
ELIZA	enzyme-linked immunosorbent assay
UV-VIS	Ultraviolet–visible
Tris	hydroxymethyl

Chapter One

Introduction and Literatures Review

1. Introduction and Literature Review

1.1 Introduction

The recent advances in the field of biocatalysts have enabled the biological processes to compete successfully with conventional chemical processing. Combination of chemical and biocatalytic systems are being developed there by utilizing the most attractive features of biocatalysts i.e. enzymes, namely high specificity with less side or waste products and higher yields, mild reaction conditions and usually low environmental impacts (Maurice *et al*., 2013).

L-asparginase or L-asparagine aminohydrolase E.C.3.5.1.1 breaks down L-asparagine into aspartic acid and ammonia. L-asparginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings (Savitri *et al*., 2003). Besides its clinical uses, L-asparginase has its application in food industries where it is used to significantly reduce the amount of acrylamide – a potent carcinogen formed in food stuffs by Maillard reaction (Anese *et al*., 2011). It is applied in pharmaceutical, food, and other clinical trials of tumor therapy in combination with chemotherapy (Verma *et al*., 2007). Production of L-asparginase enzyme by submerged fermentation has been reported of low yield as compared to solid state fermentation (Basha *et al.*, 2009). Optimization of growth parameters increases the yield of enzyme activity in fermentation. Optimization by one factor at a time (OFAT) is a well-studied method. This method is time consuming but it helps in finding high and low values of process variables. Use of statistical experimental designs for optimization of the process variables is well suited to study the interactive effects of the variables (Sangita *et al.*, 2013).

L-asparginase enzyme was produced and purified from *P. pseudoalcaligenes*. Apart from ammonium sulphate precipitation method for the partial purification of L-asparginase, other steps of purification were used (ion exchange and gel filtration), various characteristic parameters like optimum pH, and temperature were also identified, which gave an idea about the characteristicof L-asparginase isolated from *P .aeruginosa* (Arastoo *et al*.,2016)

According to those mentioned above, this study was aimed for the production, purification and characterization of L-asparginase from locally isolated *P .aeruginosa* and test some of its biological activity. This was achieved by:

1. Isolation and identification of *P. aeruginosa* from clinical samples.
2. Detection the ability of bacterial isolates in L-asparginase production.
3. Selecting the most efficient isolate in L-asparginase production.
4. Studying the optimum conditions for enzyme production.
5. Purificationof the enzyme using applicable chromatographic technique.
6. Characterization of purified L-asparginase.
7. Testing the possible cytotoxic activity of L-asparginase against MCF-7 cell line.

1.2 Literature Review:

1.2.1 Genus *Pseudomonas*

The genus *Pseudomonas* is one of the most complex bacterial genera and is currently of Gram-negative bacteria with the largest number of species; in fact, the number of species in the genus has increased every year (10 additional species in 2013 and six in 2014). The current number of recognized and validly published species is 144, including 10 subspecies; these species are present in the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2014).

The taxonomy of the genus has evolved simultaneously with the available methodologies since its first description. In 1894, Migula firstly described the genus *Pseudomonas*, which characterized as straight or slightly bent Gram negative rods with one or more polar flagella, not forming spores. Also,its chemoorganotrophic(Fuchs *et al*., 2001).*Pseudomonas* "sensu stricto" group I is the largest of the groups, and includes both fluorescent and non fluorescent ones and the most important fluorescent species are *P. aeruginosa*, *P. fluorescens, P. putida* and plant pathogenic species *P. syringae* (Scarpellini *et al*., 2004)

The characterization of genus *Pseudomonas* is faced with difficulties based on their genetic heterogeneity (Massart and Jijakli, 2007). However the development of molecular techniques has yielded innovative alternative tools for demonstrating the mechanisms underlying biocontrol properties and understanding the role of these bacteria in bioremediation, plant spoilage and pathogenicity (Ravi *et al*., 2011).

1.2.1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a common Gram-negativerod-shapedbacterium that can cause disease in plants and animals, including humans. A species of considerable medical importance, *P. aeruginosa* is a prototypical "multidrug resistant (MDR) pathogen" recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses – especially nosocomial infections such as ventilator-associated pneumonia and various sepsis syndromes. The organism is considered opportunistic insofar as serious infection is often superimposed upon acute or chronic morbidity – most notably cystic fibrosis and traumatic burns – or found in immunocompromised individuals, (Fuchs *et al*., 2001).

Mehri *et al*., (2013) stated that this bacterium is citrate, catalase, and oxidase positive. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, thus has colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, its versatility enables the organism to infect damaged tissues or those with reduced immunity.

1.2.2 Enzymes

Enzymes are macromolecular biological catalysts. Enzymes accelerate, or catalyze, chemical reactions. The molecules at the beginning of the process are called substrates and the enzyme converts these into different molecules, called products. Almost all metabolic processes in the cell need enzymes in order to occur at rates fast enough to sustain life (Stryer and Berg, 2002).Schomburg *et al*.,(2013)reported thatthe set of enzymes made in a cell determines which metabolic pathways occur in that cell.However most enzymes are proteins, although a few are catalytic RNA moleculesand enzymes' specificity comes from their unique

three-dimensional structures. Since it catalyze more than 5,000 biochemical reaction types.

1.2.2.1 L-asparginase

The enzymeL-asparginase belongs to a group of hydrolysis enzymes; it works in the presence of water on the L-asparagine amino acid analysis to L-aspartic acidand ammonia (Figure 1-1).

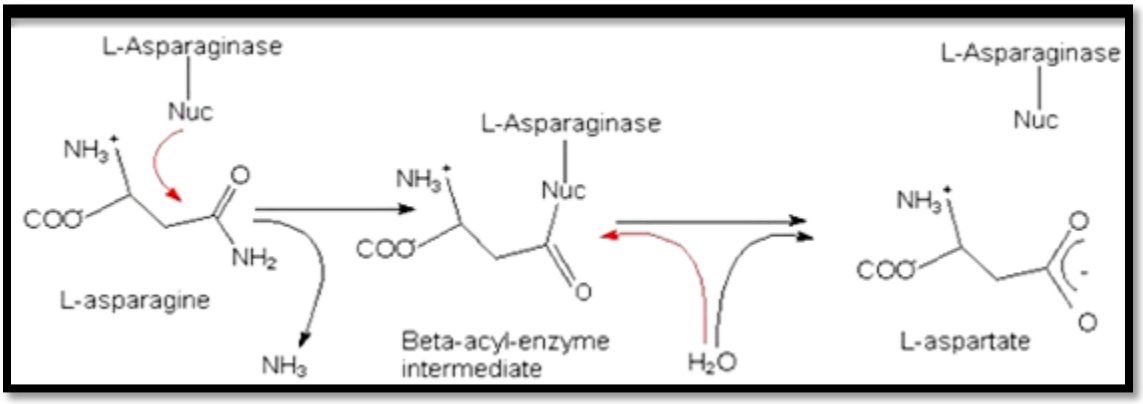

Figure (1-1) Biochemical reaction of L-asparginase (Swain *et al*., 1993).

L-asparginase has been produced throughout the world by both submerged and solid-state fermentations hydrolysis process through an attack nucleophile (Giovanni *et al*., 1973).

Swain *et al*., (1993) discussed the construction engineering for the constituent units of L-asparginase purified from *E. coli*since all under the unit consists of two areas, the first representing the end zone amino consists of 4 units of type beta (β-Sheet) and 4 units of the helix alpha (α -Helix) as well as two units of the type Anti-parallel β-strand outside the end zone acids to form what lookslike a hairpin (β-hairpin) and linked to the end zone amino carboxylic end zone with a series of 21 amino acid (Figure 1- 2).

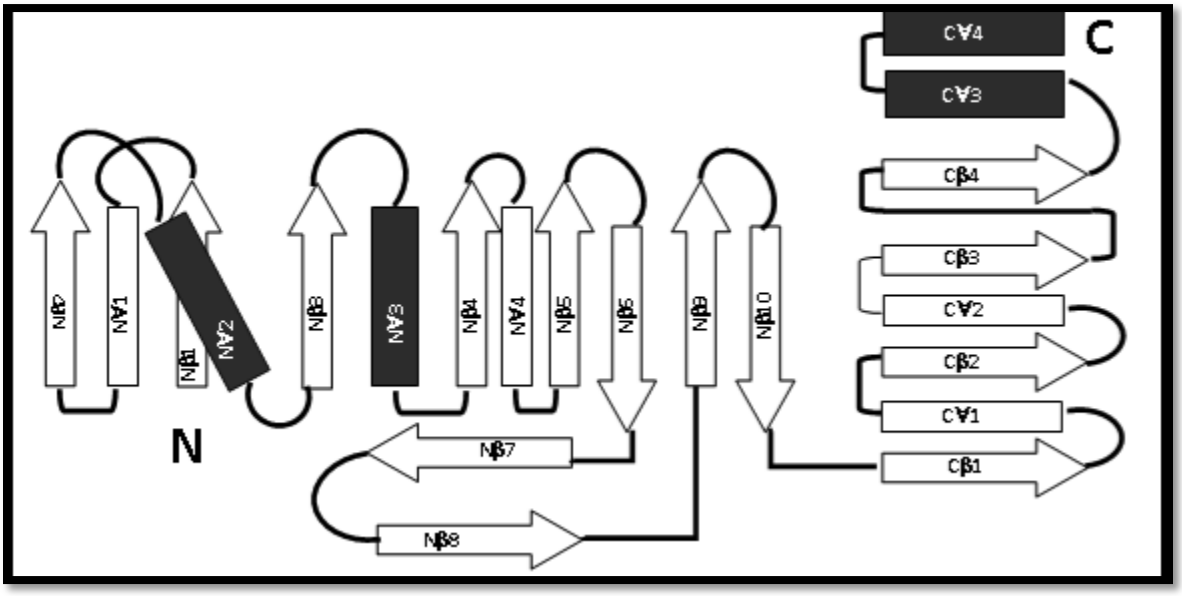

Figure (1-2): Topological form for under one unit of enzyme L-asparginase II in *E. coli* cell. Arrows indicate beta chains (β-strands), while rectangles indicate alpha- helices (α-helices), effective the site is located at the turning point (Switch point) between the end carboxylic Nβ1 and Nβ3(Swain *et al*., 1993).

1.2.2.2 Classification of Enzyme

L-asparginase has two types, L-asparginase I and L-asparginase II. Whereas L-asparginase I is a constitutive cytoplasmic enzyme and its synthesis is almost unaffected by the growth conditions, L-asparginase II is an inducible Periplasmic enzyme, and its amount in the bacterial cell varies greatly depending on the growth conditions (Lupescu *et al*., 2000).

1.2.2.3 Sources of L-asparginase

The L-asparginases are enzymes expressed and produced by different microorganisms **(**Geckil and Gencer, 2004*)*. The production of L-asparginase has been studied in *Serratia marcescens* (Khan *et al*., 1970), *Erwinia carotovora* (Maladkar *et al*., 1993), *Escherichia coli* (Wei and Liu, 1998), *Enterobacter aerogenes* (Mukherjee *et al*., 2000), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002), *Bacillus subtilis* (Fisher and Wray, 2002), *Aspergillus tamari*,

Aspergillus niger, and *Aspergillus terreus*(Mishra, 2007) with various carbon and nitrogen sources under both aerobic and fermentative conditions. L-asparginase has also been found in variety of fungi, yeasts and algae, (Table 1-1).

Table (1-1): Microorganisms producing L-asparginase (Geckil and Gencer, 2004).

Bacteria	
Escherichia coli F-221	*Streptomycescalifornicus* IFO3386
Proteusvulgaris IFO3167	*Streptomycesglobiformis* IFO12208
Serratiaindica IFO3759	*Streptomycesgriseoflavus* IFO3428
Serratiapiscatarum IFO12527	*Streptomycesnetropis* IFO3723
Alcaligensfaecalis IFO12624	*Streptomycesolivochromogenes* IFO3178
Pseudomonas fluorescens IFO3461	*Streptomycesrimosus* IFO3226
Pseudomonas aureofaciens IFO3522	*Streptomycesroseochromogenes* IFO3363
Pseudomonas schuylkilliensis IFO12055	
Micrococcus cereficans IFO12522	
Bacillus pumilus IFO 12093	
Fungus	
Ascomycetes	**Fungi Imperfect**
Anixiella reticulate IFO5814	*Verticilliummalthoasei*IFO6624
*Microascusdesmosporus*IFO7021	*Penicillium urticae*IFO4633
*Dichotomycesalbus*var. *spinosus*IFO8655	*Penicillium claviforme*IFO4676
*Nectriahaematococca*IFO6891	**Ascomycetes**

*Nectriaelegans*IFO7187	*Penicillium expansum*IFO5453
*Hypomycessolani*IFO7707	*Penicillium granulatum*IFO5737
*Hypomyceshaematococcus*IFO5980	
Yeasts	
Hansenula petersonii	*Saccharomyces peka*
Pichiaetchelsii	*Debaryo myceshanseni*
Candida utilis	*Sporobolo mycesruber*
Bullera alba	*Trichosporon cutaneum*
Rhadotorula maceran	

1.2.3 Optimization of L-asparginase

Optimization was done to examine the impact of environmental parameters on enzyme production to increases the yield of enzyme activity in fermentation. Optimization by one factor at a time (OFAT) is a well-studied method. This method is time consuming but it helps in finding high and low values of process variables (Sangita *et al.*, 2013).

1.2.3.1 Optimum Culture Medium

The optimization process plays a vital role in industrial production of any metabolites. It was submitted byuse different Carbone and nitrogen sources were used to optimize L-asparginase production from *Bacillus* sp (Makky *et al*.,

2013).In another research, glucose was proven to be the best carbon source and maximum enzyme production was recorded from *Bacillus licheniformis* in the presence of both glucose and lactose (Renuka and Nikhilesh, 2016). Kumar*et al*., (2012) stated that various alternative nitrogen sources [peptone, tryptone, yeast extract, malt extract and beef extract (1% w/v)] were substituted singly and later in various combinations in the medium used for optimize L-asparginase from *Enterobacter aerugenes*. While (Geckil and Gencer, 2004) founded that glucose showed a slightly inhibitory effect on L-asparginase synthesis by *P.aeruginosa*.

1.2.3.2 Optimum pH for L-asparginase Production

Microbial enzyme activity is greatly influenced by the surface charges present on the amino acids. Enzyme activity can be either enhanced or inhibited depending on the change in the pH, and hence can influence the growth of microorganisms (Jalgaonwala and Mahajan, 2014).

Interestingly, A pH of 5.0 was found to be optimal for L-asparginase production by *F. oxysporum* (Niharika and Supriya ,2014),the results are in accordance with that obtained with *E. coli* (Liboshi *et al*.,1999) *Pseudomonas aeruginosa* 10145 (Roberts *et al*.,1968) and other microbial asparginase activities (Balcao*et al*., 2001).

Maximum L-asparginase production at pH 7.0 by *Fusarium equiseti* (Hosamani *et al*., 2011) and *Aspergillus terreus (*Chandrasekhar, 2012) has been reported. Whereasa pH of 6.0 was the optimal pH for L-asparginase production in *Penicillium* sp. (Mohsin *et al*., 2012).Also L-asparginase produced by *Aspergillus terreus* KLS2 was found to be highest at pH 4.5 with L-asparginase activity of 5.210 IU after 72 h of submerged fermentation process (Siddalingeshwara and Lingappa, 2010).

1.2.3.3 Optimum Temperature

Temperature affects the ionization state for each of the active enzyme and substrate and is reflected on the speed of enzymatic reaction. The effect of heat on the effectiveness of enzymes is related to the nature of the protein enzyme, persistence and concentration of the substrate, enzyme stability and time of heat exposure (Fullbrook, 1983).

The optimum temperature for the enzyme production was determined by incubating the inoculated culture medium for *Bacillus licheniformis* at temperature as 30, 37, 40and 50 °C. After incubation specific enzyme activity of crude extract was calculated by L-asparaginase assay method and the optimum temperature was found to be 37^{o}C (Renuka and Nikhilesh, 2016).

Siddalingeshwara and Lingappa, (2011) founded that the temperature optimum of L-asparginasefrom*Aspergillusterreus*KLS2 is 37°C. Also, it is active at a wide range of temperaturecondition from 30^{o}C to 75^{o}C. And beyond thesetemperaturesthe enzyme becomes unstable.

1.2.3.4 Optimum Oxygen Supply

The importance of oxygen supply for microbial growth and product formation by microorganisms is well known in sub-merged culture which utilizes oxygen dissolved in the fermentation medium, a critical oxygen transfer rate is essential for microbial biosynthesis of specific end products.In this regard, although L-asparginase is considered as the product of cultures in anaerobiosis state, a critical low oxygen level is the requirement for its optimal synthesis (Hikm*et al*.,2006).

L-asparginase production normally decreases with increasing oxygen tension (Mukherjee *et al*., 2000). Although,It is well known that the effect of culture medium, culture pH, and oxygen transfer rate on L-asparginase synthesis vary for different organisms (Barnes*et al*.,1977).

1.2.3.5 Optimum Incubation Period for L-asparginase Production

Incubation period is an important parameter which effect on L-asparginase activity. L-asparginase activity, pH and biomass concentration was increased with the increase in incubation period (Priya *et al*., 2011).

Moorthy *et al.*, (2010) approved that the isolated bacterial culture was incubated for different incubation time as 24hrs to 120hrs, maximum enzyme synthesis by *Bacillus* sp. was observed after 24 hrs of incubation, analysis of L-asparginase production was carried out at every 24 hrs interval after inoculation with the maximum activity obtained at 24 h of incubation as 0.68 IU/ml.

Other studies reflect on the shorter incubation period required for L-asparginase production by *Aspergillus* sp. (Siddalingeshwara and Lingappa, 2011; Chandrasekhar, 2012); *Penicillium* sp.(Mushtaq *et al*.,2012). An incubation period of 120 hrs required for the production of L-asparginase enzyme by *Fusarium* sp (Thirunavukkarasu *et al*., 2011), whereas efficient asparginase production by *Aspergillus* sp. ALAA- 2000 was reported on the 6th day of the incubation period by submerged fermentation (Abbas *et al*., 2015).

1.2.4 Purification of Enzyme

Protein isolation is endeavoring to purify a particular protein, from some biological (cellular) material, or from a bioproduct, since proteins are only synthesized by living systems. The objective is to separate the protein of interest from all non-protein material and all other proteins which occur in same material. Removing the other proteins is the difficult part because all proteins are similar in their properties. In an ideal case, where one was able to remove the contaminating proteins, without any loss of the protein of interest, clearly the total amount of protein would decrease while the activity (which defines the particular protein of interest) would remain the same (Clive, 2002). The L-asparginase was purified to

homogeneity from *P. aeruginosa* cells that were grown on solid-state fermentation using different purification steps includes the concentration of crude enzyme (culture filtrate) by precipitation with ammonium sulfate , gel filtration and ion exchange(Kamble *and Khade*., 2012).

Ratikumari,(2015)mentioned that the purification of L-asparginase from *Pseudomonas fluorescens* was done by two methods; first by ammonium sulphate precipitation method which showed the specific activity of 229.16 with 2.34 fold purification which was quite higher than its crude sample with a specific activity of 92.82.

1.2.5 L-asparginase Characterization

1.2.5.1pH Effects on L-asparginase Activity and Stability

Since enzymes are proteins, they are very sensitive to change in pH. Each enzyme has its own optimum range for pH where it will be most active. The result of effect of pH on a combination of factors: (1) the binding of enzyme to substrate, (2) the catalytic activity of the enzyme, (3) the ionization of the substrate, and (4) the variation of protein structure (Clive, 2002).Siddalingeshwara and Lingappa, (2011) studied the optimum pH of the purified L-asparginase over a range of pH(4.0-11.0)with an optimum at pH 9.0. While Dahabi (2000) pointed that the optimal pH of the effectiveness of the enzyme L-asparginase partially purified from a local isolated cells for *E. coli* with 8.4.Also, for L-asparginase isolated from *Enterobacter cloacae*, optimum pH has reached 8.5 (Nawaz *et al*., 1998).

In another study, the maximum enzyme activity was recorded at pH 8 as 0.66 IU/ml/min for L-asparginase from *Staphylococcus aureus* (Kamble *and Khade*., 2012).

1.2.5.2 Temperature Effects on L-asparginase Activity and Stability

Functional studies of enzyme temperature linked properties have defined a so-called enzyme "temperature optimum" which is being derived from a complex mixture of both activity and thermal stability effects, and dependent on assay duration. Consequently, it is of limited value for measuring enzyme temperature adaptation (Lee *et al*., 2007). Siddalingeshwara and Lingappa, (2011) indicated that no significant L-asparginase activity was lost when it is pre incubated at 70°C for 30 and 60 min. The residual activity is 100% at this temperature. At 80°C it retains 69% and 60% activity for 30 min and 60 min, respectively. Similar results were reported by Pritsa and Kyriakidis, (2000) they found 100% activity of enzyme at 77°C.Moharam*et al*., (2010) characterize enzyme in terms of temperature which shows maximum activity at 50°C for native enzyme. According to Kushwaha *et al*., (2012) the bacterial L-asparginase showed maximum activity under 37°C at optimized conditions.

1.2.6Application of L-asparginase

1.2.6.1Food Processing

Hendriksen *et al*., (2009) showed that theL-asparginase can effectively reduce the level of acrylamide up to 90% in a range of starchy foods without changing the taste and appearance of the end product.

1.2.6.2 Treatment

One of the major important of this enzyme come from the fact that acute lymphoblastic leukemia cells and some other suspected tumor cells are unable to synthesize the non-essential amino acid asparagine, whereas normal cells are able to make their own asparagine; thus leukemic cells require high amount of asparagine. These leukemic cells depend on circulating asparagine. Asparginase,

however, catalyzes the conversion of L-asparagine to aspartic acid and ammonia. This deprives the leukemic cell of circulating asparagine, which leads to cell death (Narta *et al*., 2007).

The enzyme has also been studied for application in L-asparagine biosensor for leukemia (Verma *et al*., 2007). Muller,(1998)mentioned that it can also be associated with a coagulopathy as it decreases protein synthesis, including synthesis of coagulation factors (e.g. progressive isolated decrease of fibrinogen) and anticoagulant factor (generally anti thrombin III; sometimes protein Cand S as well), leading to bleeding or thrombotic events such as stroke. Ruzzo *et al*., (2013) mentioned thatAsparagine is required for development and function of the brain, Patterson, (2005*)* noted that it's important to protein function.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Apparatus

The following apparatus were used in this study

Apparatus	Company / Origin
Autoclave	Express / Germany
Compound light microscope	Olympus / Germany
Cooling centrifuge	Harrier / UK
Distillator	GFL / Germany
Electrophoresis Unit	Bio-Rad/ USA
Electrophoresis constant power	Pharmacia / Sweden
Eppendorf bench centrifuge	Netherland / Germany
Hot plate with magnetic stirrer	Gallenkamp / UK
Incubator	Gallenkamp/ UK
Laminar air flow	Memmert / Germany
Mircopipette	Brand / Germany
pH-meter	Metter-Tolledo/ UK
Refrigerator	Beko / Turkey
Sensitive balance	GFL/ UK
Shaker incubator	Gyromax/ U.S.A
U.V/ visible spectrophotometer	Shemadzu / Japan
VITEK 2 system	Biomereiux / France

Vortex	Buchi / Switzerland
Co2 incubator	Gallenkamp (England)
Magnetic stirrer	K and K (Korea)
Deep freezer	Thermo Scientific (USA)
Water Bath	Gallenkamp (England)
pH-Meter	Radiometer (Denmark)
Eliza	National (Japan)
96- wall Microtiter Plate	Flow Lab., Irvine (U.K)

2.1.2 Biological and Chemical Materials

The following materials were used in this study

Material	Company \ Origin
Ninhydrine, Laspargine, L-glutamine, D-L-aspartic acid.	Fluka\USA
Tetramethyl-p-phenylenediamine,Na_2HPO_4–TCA, KH_2PO_4, DPPH, EDTA, DMSO, Trypsin**,** RPMI 1640 media, Phosphate Buffer Saline, RPMI Media, DMEM Media, Fetal bovine Serum.	Sigma\USA
Bovin serum albumin, Comassie brilliant blue-G250, DEAE-Cellulose, Ethanol 96%**,**Glucose**,**Glycerol**,**Hydrogen peroxide**,** Hydrochloride, Acetic acid**,** Ammonium sulfate, KOH, Sodium chloride, Sodium hydroxide, MTTL-asparginase, Absolute Methanol, Ethel acetate	BDH/England
Nitrocellulose membrane	Schleicher&Schuell

Sephadex G-200	Pharmacia Chemical/Sweedan
Sucrose, Tris-Hcl, Tryptone, Yeast extract, Lactose, Maltose, Peptone	Himedia/ India
Cytotoxicity3 Kit, Oxidative stress kit	Thermoscientific /USA

2.1.3 Media

2.1.3.1 Ready- to- use culture media

The following media were used in this study and prepared according to theinstructions on their containers by the manufacturing companies and sterilized byautoclaving.

Medium	Company / origin
Brain heart infusion broth	Himedia/ India
Nutrient agar	Himedia/ India
Nutrient broth	Himedia/ India
Simon citrate agar	Himedia/ India
Cetramide agar	Himedia/ India
Transport medium swabs	Local Market /Iraq
Mackonky agar	Himedia/ India

2.1.3.2 Laboratory-Prepared Media

All media were prepared according to the related references after they were brought to boiling to dissolve the constituents completely, sterilized by autoclaving, then poured in sterile petri dishes and incubated at 37 ℃ for 24 hours,pH was adjusted to 7.

2.1.3.2. A culture Medium For Qualitative and Quantitative Detection of L-asparginase (Cedar and Schwartz, 1968)

It was prepared by dissolving20% sucrose, 10 mM L-asparagine in distilled water with 2%agar, PH was adjusted to 7**.**

2.1.3.2. B Minimal Salt Medium (Makky*et al*., 2013)

This medium consist of the following components

Components	Concentration(g/L)
Yeast extract	8
Na_2HPO_4	10.75
K_2HPO_4	3.55
$MgSO_4$	0.025
$MnCl_2.4H_2O$	0.0025
$FeSO_4.7H_2O$	0.0027
$CaCl_2.6H_2O$	0.015

2.1.3.2. C Blood agar (Collee *et al,* 1996)

It was prepared by adding of 5% human blood to warm autoclaved blood agar base under aseptic condition, mixed and poured into plate and kept at 4 °C until use for isolationand identification of *Pseudomonas spp.*

All components were dissolved in 1L of distilled water and sterilized by autoclaving.

2.1.3.2. D Peptone water (Collee *et al*., 1996)

Peptone water medium was prepared by dissolving 20 g peptone and 5 g NaCl in 900 ml of D.W, pH was adjusted to 8.9, mixed thoroughly, and then volume was completed with D.W to 1000 ml, distributed into test tubes and sterilized by autoclaving.

2.1.4 Reagents and Dyes

2.1.4.1 Catalase reagent (Atlas *et al.*, 1995)

Catalase reagent was prepared to be consisting of 3% hydrogen peroxide .

2.1.4.2 Oxidase test reagent (Atlas *et al*., 1995)

This reagent was freshly prepared by dissolving 1 g of tetramethyl-p - Phenylenediaminedihydrochloride in 100 ml D.W. and stored in dark bottle.

2.1.4.3Kovac's reagent (Atlas *et al*., 1995):

It was prepared by dissolved 10 g ρ-Dimethyl-aminobenzaldehyde in 150 ml of isoamyl alcohol with heating in a water bath at 50℃ and concentrated HCl 50 ml was added slowly. The reagent was prepared in small quantities and stored in refrigerator.

2.1.4.4Gram Stain Solution

It was prepared according to Atlas *et al.*, (1995).

2.1.5 Buffers and Solutions

2.1.5.1 L-asparginase Assay(Amena*et al*.,2009)

A. Ninhydrin reagent

It was freshly prepeared by dissolving 0.5 gm of ninhydrin in100 ml of aceton and kept in dark container.

B. Nessler's reagent (Cedar & Schwartz, 1968)

This solution is prepared according to manufacturing company.

C. Ammonium sulphate stock solution

It was prepared by dissolving 64 gm of ammonium sulphate in 100 ml of D.W.

D. Tris buffer (0.1M Tris-HCl, pH 8.6)

It was prepared by dissolving 1.21 gram of Tris in 50 ml of D.W, pH was adjusted to 8.6 using hydrochloric acid 1 mM , the volume was completed to 100 ml with distilled water and sterilized by autoclave.

E. Substrate stock solution (0.1mM L-asparagine, pH 8.6)

It was prepared by dissolving 0.66 mg of the amino acid (L-asparagine) in 50 ml of tris buffer which prepared in paragraph (2.1.5.4) and sterilized by autoclaving.

F. L-asparagine (5mM, pH 8.6)

It was prepared by dissolving 33.27 mg of asparagine in 50 ml of tris buffer (2.1.5.4).

G. Trichloro acetic acid (5% TCA)

It was prepared by dissolving 5 g of TCA in 100 of distilled water.

2.1.5.2 Protein assay solution (Bradford, 1976)

A. Bovine serum albumin stock solution (BSA)

It was prepared by dissolving 1 gram of bovine serum albumin in 10 ml of D.W.

B. Comassie brilliant blue dye (G-250)

This dye was prepared by dissolving 0.1 grams of comassiebrilliant blue G-250 in 50 ml of ethyl alcohol, then 100 ml of phosphoric acid was added ,mixed well and filtered using filter paper (Whatman No. 1) and saved in dark bottle.

C. Destaining solution

It was consist of 40% methanol and 10% acetic acid.

2.1.5.3 L-asparginase Purification Assay Solution (Amena *et al*., 2009)

A. Sodium hydroxide solution (5N NaOH):

It was prepared by dissolving 20 grams of sodium hydroxide in 50 ml of D.W and the volume was completed to 100 ml of D.W.

B. Sodium phosphate buffer (25mM Na_2HPO_4, pH 8.0)

It was prepared by dissolving 3.54 grams of sodium phosphate in 500 ml of D.W, pH was adjusted to 8.0, the volume was completed to one liter of D.W and sterilized by autoclave.

C. Potassium phosphate solution (0.02M K_2HPO_4, pH 8.0)

It was prepared by dissolving0.27 gm of potassium phosphate in 50ml of D.W, and then the pH was adjusted to 8.0and the volume was completed to 100ml of D.W and sterilized by autoclave.

D. Hydrochloric acid solution (0.25N HCl)

It was prepared by transferring 5.2 ml of concentrated hydrochloric acid (11.6 mM) to a volumetric flask and the volume was completed to 250 ml of D.W.

E. Ammonium persulfate (1.5%)

It was freshly prepared by dissolving 1.5 grams of ammonium persulfate in 100 ml of D.W.

F. Sodium acetate buffer

It was prepared by dissolving 0.49 gm of potassium acetate in 30 ml of D.W,pH was adjusted to 4 and the volume was completed to 50 ml with D.W.

2.1.5.4 Cytotoxicity Assay Solution (Manish and Thankamani,2015)

A. PBS Solution

It was prepared by adding 450 ml of distilled water to 50 mL of Phosphate Buffer Saline (PBS) stock solution.

B. Trypsin-EDTA Solution

0.25% (w:v) Trypsin- 0.2% (w:v) EDTA stock solution was prepared then added to 90 ml of PBS).

2.1.6 Sterilization Methods (Baily *et al.*, 1990)

Three methods of sterilization were used:

(A) Moist Heat Sterilization (Autoclaving)

Media and solutions were sterilized by the autoclave at 121˚C (15 Ib/in2) for 15 min, unless indicated.

(B) Dry Heat Sterilization

Electric oven was used to sterilize glasswares and others at 160-180 ˚C for 2-3 hr.

(C) Membrane Sterilization (Filtration)

Millipore filtration was used to sterilize heat sensitive solutions using millipore filters (0.22 and 0.45 µm).

2.2 Methods

The main steps of the research plan were summarized in scheme (2-1).

Scheme (2-1): Methodology of the research project

2.2.1 Specimen Collection

A total of 46 specimens were collected from the burns, ear infections and wounds from Al-yarmok hospital in Baghdad during the period from 1/12/2015 to 1/2/2016. Swab specimens were aseptically transferred under cooling conditions to the laboratory for analysis.

2.2.2 Isolation of *Pseudomonas aeruginosa.*

According to Macfadden, (2000), swabs were taken carefully from the site of infection and placed in tubes containing transferred medium to maintain the swab wet during transferring to laboratory. Each specimen was inoculated on *Pseudomonas* isolation Mackoncky agar. All plates were incubated aerobically in incubator at 37ºC for 24 hrs.

2.2.3 Identification of *Pseudomonasaeruginosa*

2.2.3.1Cultural Examination

Morphological characteristics of colonies were studied on cetramide agar .Color; size and edge of colonies were recorded after 24 hrs of incubation at 37˚C.

2.2.3.2 Microscopic Examination

A single colony of each isolate was fixed on a clean slide to study gram stain , under light microscope (Atlas *et al.* , 1995).

2.2.3.3 Biochemical tests

The suspected isolates were subjected to the biochemical tests as mentioned by Macfadden (2000), and as follow:

2.2.3.3.A Catalase test

A single colony of each bacterial isolatewas taken and smeared on clean glass slide , then a drop of hydrogen peroxide (3drops was flooded with 1.0 ml of 3% hydrogen peroxide (Catalase reagent 2.1.4.1).Presence of gaseous bubbles indicate a positive result.

2.2.3.3. B Oxidase test

This test was done using filter papermoistened with few drops of afreshly prepared solution of tetramethyl-ρ-phenylenediaminedihydrochloride. Aseptically, a clump of cells was picked up from the slant growth with a sterilewooden stick and smeared on the moistened paper. Development of a violet orpurple color within 10 seconds indicates a positive result.

2.2.3.3.C Indole test

Colony was inoculated into peptone water broth and incubated at 37°C for 24 hrs in a shaker incubator. After incubation,few drops of Kovac's reagent (2.1.4.18) were added. Presence of pink colored ring indicates a positive result.

2.2.3.3. DCitrate utilization test (Simmon's Citrate slant)

A loopful of colony was streaked onto a simmon citrate agar slant, and then incubated for 24 to 48 hrs at 37°C in incubator. Change in medium color to blue color indicates apositive results.

2.2.3.4 Identification of suspected bacteria by VITEK 2 system (David, 2005)

The VITEK 2 is an automated microbiology system utilizing growth-based technology. Used for bacterial identification.

2.2.4 Maintenance of *Pseudomonas aeruginosa* isolate

Bacterial isolates were maintained according to Johnson*et al.*(1988) as follows:

2.2.4.1Short – term storage (few weeks)

Bacterial isolates were maintained for few weeks by culturing on plates of nutrient agar, and incubated at 37°C for overnight. The plates were then tightly wrapped with parafilm and stored at 4 °C.

2.2.4.2Medium – term storage (1 – 3 months)

Bacterial isolates were maintained as stab culture for few months by inoculated in small screw capped bottles containing (5 – 8) ml of sterile nutrient agar (as slants), then incubated at 37°C for 24 hrs in incubator. The bottles were tightly wrapped with parafilm and stored at 4°C .

2.2.4.3Long time storage

Test tube containing 10 ml sterile brain heart broth was inoculated with the bacterial isolate, and incubated at 37°C for 24 hrs in incubator. After incubation, sterile glycerol (20%) was added and mixed by vortex before**.**

2.2.5Screeningthe local isolates of *P.aeruginosa*for L-asparginase production.

2.2.5.1 Semi quantitative screening (Cedar & Schwartz, 1968)

The isolated *P. aeruginosa* was subjected to rapid screening for L-asparginaseproduction by agar plate assay. Bacterial isolateswere grew on nutrient agar and incubated at 37^{o}C for 18hrs , after incubation period ;the colonies of bacteria were transferred to nitrocellulose membrane that placed on medium (preparedin2.1.3.2.A)and incubated at 37^{o}C for 6 hrs, the nitrocellulose paper then replaced byfilter paper and incubated for two minutes at 37^{o}C , the filter paper was socked in ninhydrinereagent (prepare in2.1.5.1).The purplecolorin the site of colonies was noticed with brown backgroundof ninhydrine reagentindicated L-asparginase production and colonies were selected for quantitative enzyme assay.

2.2.5.2Quantitative screening of L-asparginase

Quantitative assay was carried according to the method described by Mokran (2003), by using direct Nesslerization method to determine the enzyme activity inbacteria.

2.2.5.2. A Preparation of cell free supernatant

P. aeruginosa was grown in 250 ml of brain heart broth at 37 °C over night; the culture was centrifuged for 30 minutes at 6,000 rpm.

2.2.5.2. B Standard curve of ammonia

Concentration gradient of ammonium sulfate (0, 1.6 , 3.2 , 4.8 , 6.4 , 8.0 , 9.6 , 11.2 , 12.8) mg / mL were prepared by adding suitable D.W volumes to certain volumes of ammonium sulfate stock solution and as shown in the following table:

Table(2-1): concentration and absorbance of ammonium sulfate for determination of L-asparginase activity.

Tube no.	Water Volume (ml)	Ammonium sulfate Vol. (0.64 mg / ml)	Final ammonium sulfate concentration (mg / ml)	Absorbance at 436 nm
1	8	0	0.00	0.00
2	7	1	1.60	0.054
3	6	2	3.20	0.11
4	5	3	4.80	0.16
5	4	4	6.40	0.21
6	3	5	8.00	0.26
7	2	6	9.60	0.32
8	1	7	11.2	0.39
9	0	8	12.8	0.45

Ammonia concentration was determined for each tubes in a duplicate by mixing 4.4ml of D.W, 0.1 ml sample and 0.5ml of Nessler reagent (2-1-4-5).The absorbance was measured at 436 nm (figure2-1).

Blank was prepared by mixing 4.5 ml of D.W and 0.5ml of Nessler reagent.

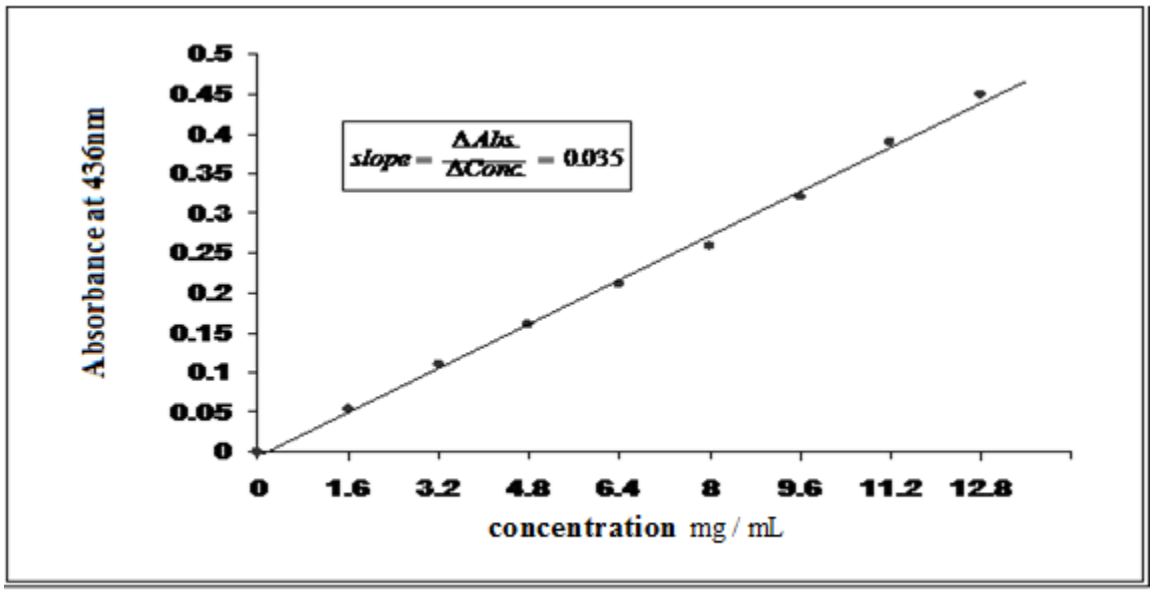

Figure (2-1) Standard curve for ammonia

2.2.5.2. C L-asparginase activity assay method:

L-asparginase activity was determined according to Cedar and Schwartz, (1968) as follows:

Enzymatic solution (0.1) ml was added to tris buffer (2.1.4.7) then incubated for 30 minutes at 37 ^{o}C.The reaction was stopped by adding 0.5 ml of the 5 % TCA (2.1.4.19) and the solution was centrifuged at 6000 rpm for 20 minutes. Then ammonium concentration was determined by mixing 4.4.ml of distilled water, 0.1 of L-asparginase and 0.5 ml of nissler reagent (2.1.4.5) ,the mixture was mixed by vortex and left it for one minute then measure the absorbance at 436 nm .

The blank sample was prepared by mixing 4.5 ml of distilled water and 0.5 ml of nissler reagent.

Ammonia concentration was determined in the sample as mentioned above and the enzymatic activity was calculated according to the following equation.

$$\text{Enzymatic activity (U / ml)} = \frac{\text{Concentration of ammonia liberated (mg / ml)}}{\text{Reaction time (30 minutes)} \times 14}$$

$$\text{Concentration of ammonia liberated} = \frac{\text{Absorbance at wave length at 436 nm}}{\text{Slop}}$$

International unit IU of L-asparginase is defined as the amount ofenzyme which liberates 1 micromole of ammonia in 1 min at37 ºC.

2.2.5.3 Determination of Protein Concentration

Protein concentration was determined according to the method of Bradford (1979) using bovine serum albumin standard curve as follow:

Different concentrations (0,20,40,60,80,100) were prepared from BSA stock solution (2-1-4-10) as shown in Table(2-2).Then 2.5 ml of Coomassi brilliant blue G-250 dye prepared in (2-1-4-11) was added, mixed and left to stand for 2 min at room temperature. Absorbance at 595 nm was measured; the blank was prepared from 0.45 ml of phosphate buffer prepared in (2-1-4-13) and 2.5 ml of the dye reagent.A standard curve was plotted between the BSA concentrations against the corresponding absorbance of bovine serum albumin at 595 nm.Protein concentration was estimated by mixing 0.05ml of the test sample,0.45ml of phosphate buffer and 2.5 ml of Comassie brilliant blue G- 250, and leftto stand for 2 min at room temperature before measuring the absorbance at 595 nm.

Table (2-2): Bovine serum albumin standard curve

BSA conc. (mg/ml)	Distilled water (ml)	BSA volume(ml)	Absorbance at 595 nm
0	10	0	0.00
2	8	2	0.27
4	6	4	0.50
6	4	6	0.68
8	2	8	0.85
10	0	10	0.90

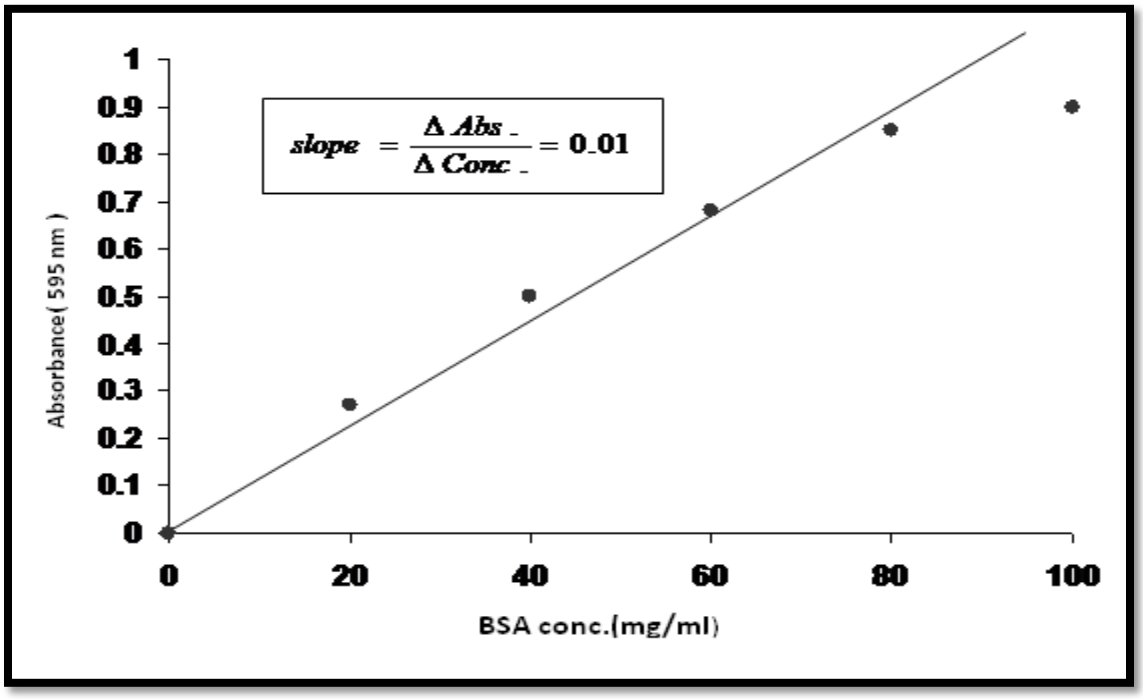

Figure (2-2): Standard curve of BSA.

2.2.6 Optimum Conditions for L-asparginase Production

Optimum conditions for L-asparginase produce by selectedisolate were determinedby inoculating 100 ml of the minimal salt medium (2-1-3-2-C) pH 7.5 with 1 ml of fresh culture (OD =0.6) of the bacterial isolate and incubated at 37 ºC for 24 hrs, in shaker incubator at 100 rpm, then L-asparginase activity and protein concentration in crude filtrate were estimated.Optimum factor when reached was used in the next experiment of optimization.

2.2.6.1 Optimum Carbon Source

Various carbon sources (glucose, mannitol, sucrose, glycerol, and maltose) wereadded, individually, to the production medium prepared in (2.1.3.2.C). All these sources were added to the minimal salt medium at a final concentration of 0.1 %. L-asparginase activity, protein concentration and specific activity were determined.

2.2.6.2 Optimum Nitrogen Source

Various nitrogen sources (peptone, casein, tryptone,and yeast extract)were added individually to the production medium (2.1.3.2.C).Supplemented with optimum Carbone source from previous step at final concentration of 0.1%.Also,L-asparginase activity, protein concentration and specific activity were determined.

2.2.6.3 Optimum Inoculum Size

Effect of different inoculums size of the selected *P. aeruginosa* isolate on L-asparginase production was studied. This was achieved by inoculating the production medium, individually with an inoculums size (10^3,10^4,10^5,10^6,10^7,$10^8$and 10^9CFU).

2.2.6.4 Optimum pH

Optimal pH for production of L-asparginase was determined by adjusting the pH of the production medium to different values (4.5, 5, 5.5,6, 6.5, 7, 7.5, 8, 8.5 and 9), then incubated at 37°C for 24 hrs.

2.2.6.5Optimum Temperature

The production medium was inoculated with the selected *P. aeruginosa*at different temperatures (20, 25, 30, 37and 42) ^{o}C .The optimum temperature for L-asparginase production was used in the next experiment of optimization.

2.2.6.6 Optimum Incubation Period

Effect of the incubation period on L-asparginase production by *P. aeruginosa* was studied by incubating the productionmedium at different periods of time (12, 24, 36, 48 and 60 hr.) to determine the optimum period for enzyme production.

2.2.7 L-asparginase Purification

Three steps were used in this study for the purification of L-asparginaseproduced under the optimum condition from *P. aeruginosa*.

2.2.7.1 Enzyme Production and Preparation of Cell Free Filtrate

The *P. aeruginosa* was grown in the production medium under the optimum conditions. Crude filtrate (crude L-asparginase) was collected by centrifugation at 6,000 rpm for 20 min. at 4°C in order to obtain a cell free filtrate, then L-asparginase activities and protein concentration were determined.

2.2.7.2Ammonium Sulfate Precipitation

Ammonium sulfate was added to the supernatant (crude enzyme) with saturation ratio 80%.The mixture was mixed gently on magnetic stirrer at 4°C for 20 minutes. Then centrifuged at 6,000rpm for 20 minutes at 4°C .The precipitated proteins were dissolved in a suitable volume of 0.05 M Tris- HCl buffer at pH8. Enzyme activity and protein concentration were estimated.

2.2.7.3 Purification By Ion Exchange Chromatography

A DEAE-Cellulose column was prepared according to Whitaker and Bernard, (1972) by dissolving 20 g of resin in 1L of distilled water. Then beads were left to settle down and washed several times with D.W until getting clear appearance. The suspension was filtered throughout Whattman No.1 using buchner funnel under discharging.The resin was re-suspended in 0.25 M sodium chloride and sodium hydroxide solution. The suspension was filtered again as mentioned above and washed several times with 0.25 M hydrochloric acidsolution and next by distilled water before it was equilibrated with 0.05 M Tris- HCl buffer pH 8.

The obtained ammonium sulfate precipitates was then applied to DEAE-Cellulose column (2x20cm) equilibrated previously with 0.05M Tris-HCl buffer pH8. Then column was washed with an equal volume of the same buffer, while attached proteins were stepwise eluted with gradual concentrations of sodium chloride (0.1–1 M). Flow rate throughout the column was 3ml/fraction and the absorbance of each fraction was measured at 280 nm using UV-VIS spectrophotometer. L-asparginase activity was determined in each fraction as described in (2.2.5.2.C).Fractions presents L-asparginase activity were pooled and kept for further steps of purification.

2.2.7.4 Purification By Gel Filtration Chromatography

Sephadex G-200was prepared as recommended by Pharmacia Fine Chemicals Company. Aquantity of Sephadex G-200 was suspended in 0.01 M Tris-HCl buffer pH8, degassed, and packed in a glass column (1.5×35 cm), then equilibrated with the same buffer.Concentrated L-asparginase obtained from the ion exchange step was applied onto the column. Elution was achieved at a flow rate of 30 ml/hrfraction and using the same buffer for equilibration. Absorbance of

each fraction was measured at 280 nm. L-asparginase activity was also determined in each fraction as described in (2.2.5.2.C).

2.2.8 Determination of Enzyme Purity and Its Molecular Weight

Purity of L-asparginase was determined by electrophoresis on SDS-polyacrylamide gel in the presence of standard proteins (Myosin, β-Galactosidase, phosphorylase, Bovine serum albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsine Inhibitor, lysozyme, and Aprotinin). Distances of protein migrated to anode were measured after electrophoresis on polyacrylamide gel to calculate the enzyme molecular weight.

2.2.9 L-asparginase Characterization

Some of the characteristics of purified L-asparginase were determined as follow :

2.2.9.1 Determination of pH Effects on L-asparginase Activity

Purified enzyme was incubated with 0.1 mM L-asparagine and 0.05M buffers ofPH ranging 4.5 to 9.Buffer used were sodium acetate buffer (2.1.4.3) pH(4.5,5,5.5 and 6), tris buffer (2.1.5.1.D) for pH values (6.5,7, 7.5, 8, 8.5 and 9).

Enzymatic activity was measured according to(2.2.5.2.C) and the relationship between different pH and enzyme activity was plotted.

2.2.9.2 Determination of pH effects on L-asparginase stability.

The enzyme was pre-incubated in buffer of various pH (4.5–9) for 30 min. at 37^{o}C. After that the tubes were cooled in ice bath.

Enzymatic activity then determined andthe remaining activity (%) for L-asparginase was plotted against the pH value.

2.2.9.3 Determination of Temperature Effects on Activity and Thermal Stability for L-asparginase.

The L-asparginase activity was measured at different temperatures (20, 25, 30, 35, 40, 45, 50, 55, and 60) °C. The enzyme activity was plotted against the temperature.

While for thermal stability, partially purified L-asparginase was pre-incubated in water bath at (20, 25, 30, 35, 40, 45, 50, 55and 60) °C for 30 min., and immediately transferred into an ice bath. Enzymatic activity was determined and the remaining activity (%) was plotted against the temperature.

2.2.9.4 Determination of Various Ions and Inhibitors Effect on L-asparginase Activity

The effect of different inorganic ions on enzyme activity was determined by pre-incubating with different salts (NaCl, $CaCl_2$, KCl, $HgCl_2$, $CuCl_2$ and $MnCl_2$) prepared at the concentrations of (1, 10 and 20 mM) for 1 hr at 37°C.Also, the effect of inhibitors and chelating agents(2-Mercaptoethanol,Cystien and EDTA)prepared at (0.5, 1,2 and 4) mM concentration was determined by incubating with the enzyme at 37°C for 1 hr.

Enzymatic activity was determined and the results werepresented as percentage remaining activity (%).

2.2.10 MTT (Cytotoxic Assay)

The cytotoxic effect of L-asparginase was performed by using MTT (Methylthiazolyldiphenyl-tetrazolium) ready to use kit:

2.2.10.1 Preparing and Adding Samples to The 96-Well Plate

Breast-cancer MCF-7 cell line supplied by Pharmacology Department/Medicine College/Malaya University. It was grown in 95% of RPMI-

1640 supplemented with 10% fetal bovine serum (FBS), the suspension of cells and medium incubated at 37^{o} C in 5% CO_2 incubator. In the Log- phase suspended cells. Then centrifuged at 250xg for 10 min. supernatant was removed, cells were re suspended in freezing medium, then vials were placed in -70^{o} C freezer, after 1-3 days at -70^{o} C, vials were transferred into standard freezer boxes, which they were transferred into a liquid N_2 container**.**

2.2.10.2 Procedure

One hundred micro litter of cell suspension was added onto the flat-bottomed micro culture plate wells. Solution was prepared by dissolving 5mg MTT crystals in 1 mL of PBS solution (Phosphate Buffer Saline).Aconcentrations of(400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5, 0.78, 0.39 and 0.19) µg/ml from L-asparginase was added to each well (three replicates for each concentration).MTT solution (10 ml) was added to each well of 96-well plate then incubated for 4 hours with testing sample in 37^{o} C (The solution became yellow).Then200 µlof DMSO (dimethylsulfoxid) was added to each well and shacked for 5 min (The DMSO solution became purple).After complete solubilization of the dye, the absorbance of the colored solution obtained from living cells was red at 575 nm with an ELISA reader.The mean absorbance for each group of replicates was calculated. Percentage of cell viability exposed to various treatments was obtained as follows:

Cell Viability %= [Absorbance of treated sample/Absorbance of non-treated sample] × 100

(Non-treated cultures in all experiments contained the medium only).

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Isolation of *Pseudomonas* spp

Samples collected from burns, ear infections and wounds were cultured on to Mackonkey agar. Out of 46 samples, 29 were able to show growth and only 6 isolates were able to produce β-hemolysis around their colonies on blood agar.

3.2 Identification of Bacterial Isolates

3.2.1 Cultural Characteristic

Suspected *P. aeruginosa isolates* were replicated on Mackonkey agar and incubated for 24 hrs at 37°C. Results showed that colonies were pale yellow in color, non-lactose fermenters. While pigmentwas more obvious when grew on selective media "Cetermide" .Colonies were surrounded by distinctive β hemolysis zones when grew in blood agar medium. These characteristics come in accordance with the corresponding cultural characteristics of *P. aeruginosa* that mentioned by El-Bessoumy*et al.*,(2004).Also, formation of such type of complete hemolysis zone is usually a characteristic of *P. aeruginosa* (Tenny*et al*., 2014). Also, they were able to grow on ceramides agar which is a selective medium for *Pseudomonas sp*.

3.2.2 Microscopical Examination

Bacterial isolates were also identified according to their Gram staining and other microscopically characteristic.The bacterial isolates were gram negative rods with one or more polar flagella (Fuchs *et al*., 2001).

3.2.3 Biochemical Characteristics

According to the results of morphological and microscopic characteristics, bacterial isolates suspected to bebelonging to *P. aeruginosa*were subjected to the related biochemical tests. Results illustrated in Table (3-1) showed that all the sixisolates were producing catalase and oxidase enzymes and positive in citrate test, as well as negative for Indole test. Adversely, they were unable to ferment

lactose that used as carbon sources. Macfadden, (2000) mentioned that such characteristics usually are coming in accordance with those belonging to *P. aeruginosa*.

Table (3-1): Biochemical tests for bacterial isolates.

Biochemical test	Isolates					
	P4	**P5**	**P9**	**P15**	**P25**	**P37**
Growth under aerobic condition	+	+	+	+	+	+
β-haemolysis	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Indole	_	-	-	-	-	-
Citrate	+	+	+	+	+	+
Lactose fermentation	_	_	_	_	_	_

3.2.4 Identification of Bacterial Isolates By VITEK System

Identification of bacterial isolates was also done by VITEK 2 system, installed at the Central Health Laboratory/Ministry of Health, by using the GN (Gram Negative) card.The six bacterial isolateswere identified by VITEK 2 system , Table (3-2) shows VITEK2system result for identification of *Pseudomonas aeruginosa* P4.

In general, the VITEK 2 system is an easy-to-handle system that provides a rapid (4 to 15 h) and reasonably accurate means for the identification of most commonly isolated species and accurately detects.

Joyanes *et al*.,(2001) tested 146 routinely isolated strainsof non-fermenting gram-negative rods using VITEK 2 system and ID-GNB cards with 91.6% similarity in identification .Also,Ines*et al*., (2009) founded that the correct identification rates of *P. aeruginosa*were 90.1%using the same VITEK 2 identification card.

Table (3-2): Biochemical tests for identification P. aeruginosa by VITEK 2 identification system

Test	***Result***	***Test***	***Result***	***Test***	***Result***	***Test***	***Result***	***Test***	***Result***
APPA	-	ADO	-	PyrA	-	IARL	-	Dcel	-
H_2S	-	BNAG	-	AGLTp	-	dGLU	+	GGT	+
BGLU	-	dMAL	-	dMAN	+	dMNE	+	BXYL	-
ProA	+	LIP	+	PLE	-	TyrA	-	URE	-
SAC	-	dTAG	-	dTRE	+	CIT	+	MNT	+
ILATK	+	AGLU	-	SUCT	+	NAGA	-	AGAL	-
GlyA	-	ODC	-	LDC	-	IHISa	-	CMT	+
O129R	+	GGAA	-	IMLTa	+	ELLM	-	ILATa	-
BGAL	-	OFF	-	BAIap	+	dSOR	-	5KG	-
PHOS	-	BGUR	-						

٣.٣Screening The Ability of Local Isolates *P. aeruginosa* Isolates In L-asparginase Production

Ability of the locally isolated *P. aeruginosa* in L-asparginase production was screened to select the most efficient one in enzyme production and as follow:

3.3.1 Qualitative Screening

Ninhydrin assay is one of the oldest methods used mainly for the qualitative determination of L-asparginase. This method depends on direct measurement of the area that contains lysis aspartic acid which produced due to the action of L-asparginase. Results showed thatcolonies producing an enzyme were detected which gave a purple spots color directly proportional to the quantity of enzyme produced and can be easily distinguished from the brown background of the filter paper which represents no bacterial growth . Among the six selected bacterial isolates, *P. aeruginosa* P4 represents the highest L-asparginase producer which gave more dark purple color when compared to the other isolates (Figure3-1).

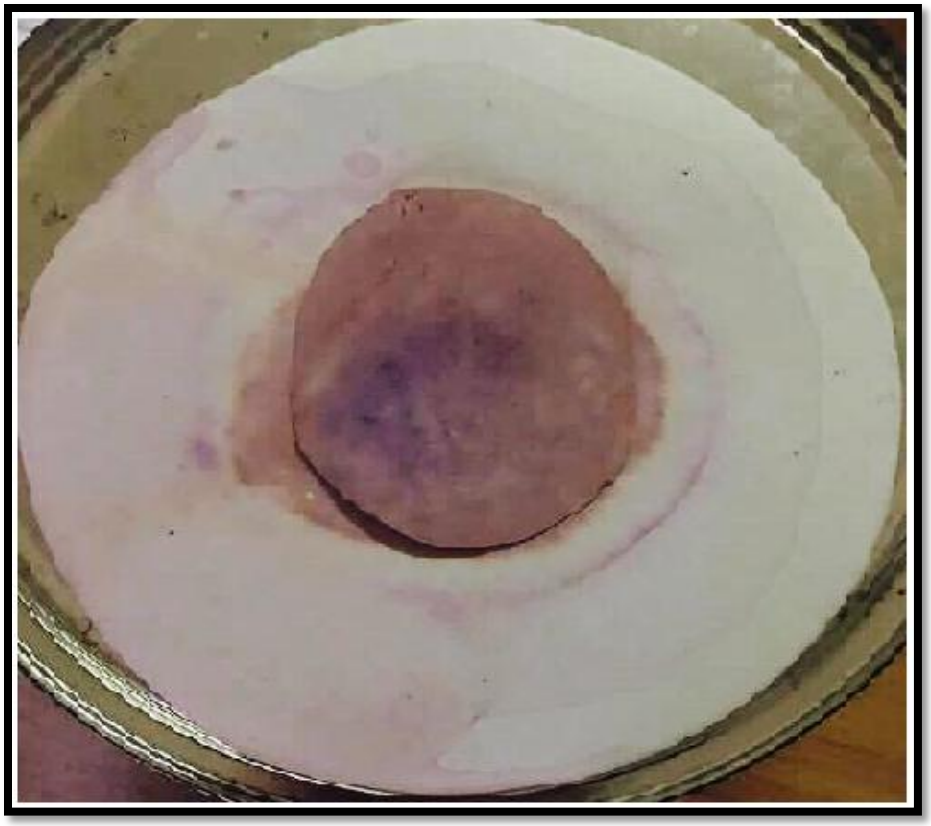

Figure (3-1): L-asparginasequalitative assay in*P. aeruginosa*P4 placed on ninhydrin socked filter paper.

Many investigators reported the validity of this method for the primary screening of L-asparginase from *Aspergillus* and *Penicillium*sp. (Sarquis *et al*., 2004; Theantana *et al*., 2009).

The results that have been reached are also consistent with what indicated by several studies that the majority of microorganisms belonging to the family of intestinal bacteria (Enterobacteriaceae), in particular, the bacterium*P.aeruginosa* and the bacteria *E. coli*have high capacity to produce L-asparginase, when compared to the genus *Bacillus* ,which are less efficient in the production of the enzyme (Imada *et al*., 1973).

3.3.2 Quantitative Screening of Isolates Producing L-asparginase

The six local isolates of *P. aeruginosa* were screened quantitatively to examine their ability in L-asparginase production. Specific activity of L-

asparginase in the crude filtrates was determined. All of the isolates were L-asparginase producers with variable degreeof production (Table 3-3). P4 isolate showed the highest specific activity (0.144 U\mg) protein indicating the existence of a correlation between spot color and specific activity of the enzyme in the broth.

Table (3-3) Specific activity of L-asparginase produced by local isolates of *P. aeruginosa*after 24hrs of incubation at 37°C.

Isolate number	Specific activity (U\mg)
P4	**0.144**
P5	**0.092**
P9	**0.104**
P15	**0.084**
P25	**0.119**
P37	**0.128**

The differences in the ability of the isolates to produce L-asparginasecould be due to genetic variations of the genes responsible for the production of L-asparginase (Mokrane, 2003)

According to these results, the isolate P4 was selected to be used for improving its ability in L-asparginase production by optimization.

3.4 Optimum Conditions For L-asparginase Production

Optimization studies were carried out by one factor at a time method; varying only single factors and keeping the remaining factor constant.

3.4.1 Optimal Carbon Source

Five carbon sources (glucose, mannitol, sucrose, maltose and glycerol) were used as a sole source for carbon and energy to determine the optimum for

production of L-asparginase by *P. aeruginosa*P4. These carbon sources were added separately to basal medium in a concentration of 0.1 % w/v. Results indicated in Figure (3-2) shows that the maximum production of L-asparginase by *P. aeruginosa*P4 was achieved when Glycerol was used as a sole source for carbon and energy.

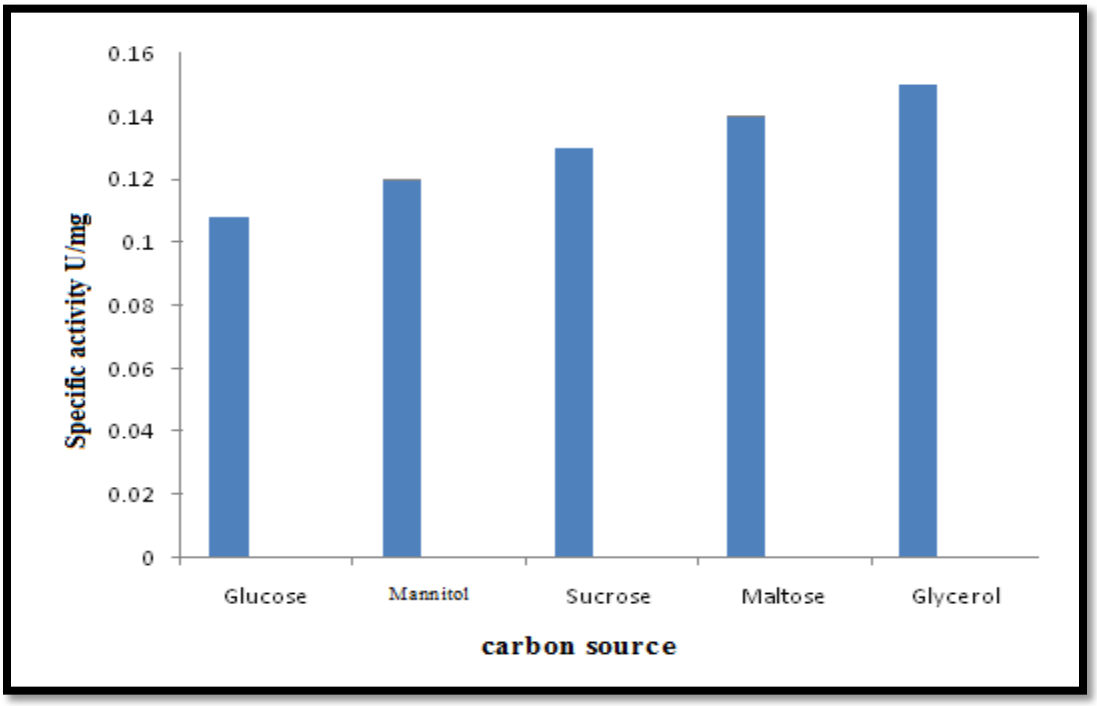

Figure (3-2): Optimum carbon source on L-asparginase produce by *P.aeruginosa*p4 after incubation at 37°C for 24 hrs in shaker incubator at 150 rpm.

Among the different sugars supplemented in the liquid media,glycerol showed the highest L-asparginase specific activity of (0.15 U/mg) followed by maltose (0.14U/mg), sucrose (0.135 U/mg), mannitol (0.13 U/mg)and glucose(0.12 U/mg).

Repression of L-asparginase synthesis by glucose has been shown in some bacteria like *Escherichia coli* and *Enterobacter aerogenes* (Geckil and Gencer, 2004). In line with these findings, glucose showed an inhibitory effect on L-

asparginase synthesis by *P. aeruginosa*. Morkan, (2003) mentioned that best carbonsource of culture medium for L-asparginase production from *P. aeruginosa* was glycerol in a concentration of 0.1%.

3.4.2 Optimum Nitrogen Source

Nitrogen sources includes peptone, yeast extract, casein hydrolysate, and tryptone were added to the production medium separately in a concentration of 0.1%.Maximum production of L-asparginase was achieved when the production medium was supplemented with tryptone because enzyme specific activity reached 0.210 U/mg protien when this nitrogen source was used(Figure 3-3).

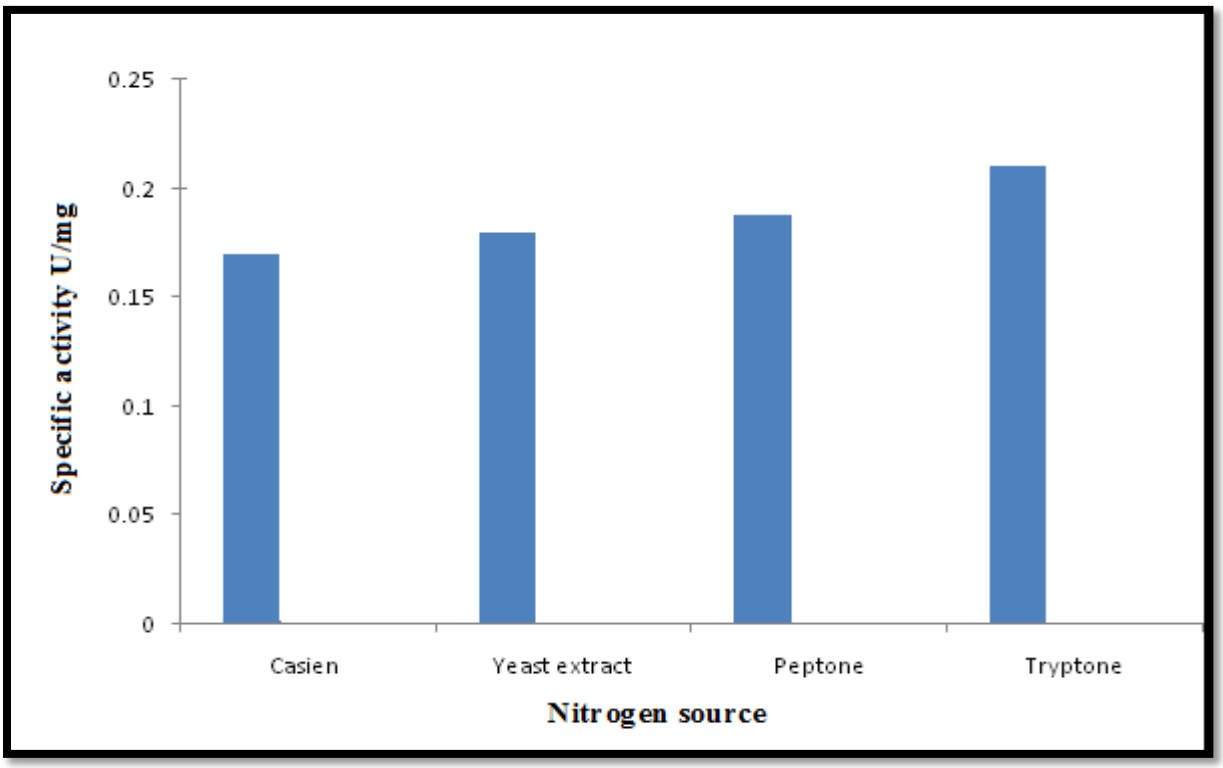

Figure (3-3): Optimum nitrogen source for L-asparginase production by *P.aeruginosa*P4 after incubation at 37°C for 24 hrs.

This result may be related to the fact that trypton is easier to be utilized by microorganism than the other sources to achieve requirements for growth, cell division and production of different metabolites (Basha *et al*.,2009). Also,Usha *et*

al., (2010) noticed that the best nitrogen source of culture medium for L-asparginase production from *P. aeruginosa* was tryptone, while Verma *et al*., (2007) reported that yeast extract is an important for cell growth and L-asparginase synthesis, but in high concentrations L-asparginase production was inhibited.

3.4.3 Optimum Inoculums Size

Inoculums sizes ranged between (10^3–10^9) cells/ml were used to inoculate the production medium. Results illustrated (Fig.3-4) shows that production of L-asparginase by *P. aeruginosa* was affected by the inoculums size of microorganism, since, it was increased slightly with the increase of the inoculum size to 10^8 cells/ ml, and then it was decreased. However, maximum L-asparginase production was achieved with 10^8cell/mlwithenzyme specific activity of 0.28 U/mg proteins.

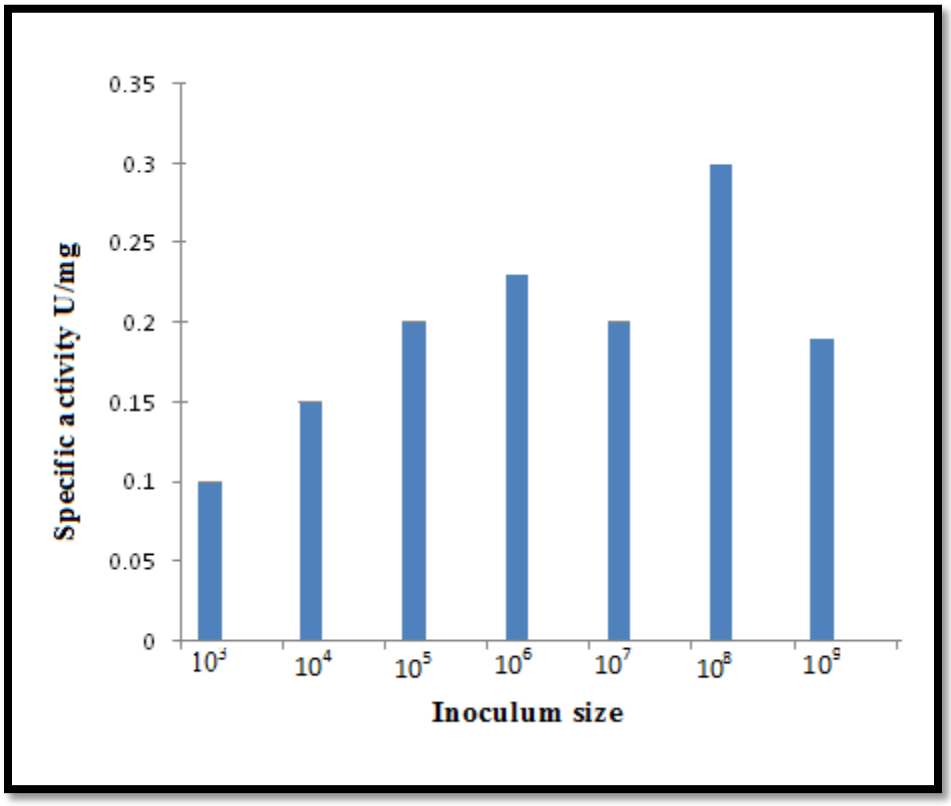

Figure (3-4): Effect ofinoculums size on L-asparginase production by*P.aeruginosa*P4after incubation at 37°C for 24 h.

In another report, both *Bacillus subtilis* and *Bacillus cereus* exhibited highest enzyme activity at 10^8 cell\ ml inoculums size (Hegazy and Moharam, 2010).Also, an inoculums size of $1x10^8$spores/ml showed the highest yield (11.0 IU) of L-asparginase by *Streptomyces albidoflavus*(Amena *et al*, 2010).

3.4.4 Optimum pH

The initial medium pH was adjusted in ranged between pH 4.5 to pH 9 , the maximum L-asparginase production was obtained when the pH value of the production medium was adjusted to 7, at this value the enzyme specific activity recorded was 0.41 U/mg (Figure 3-5).

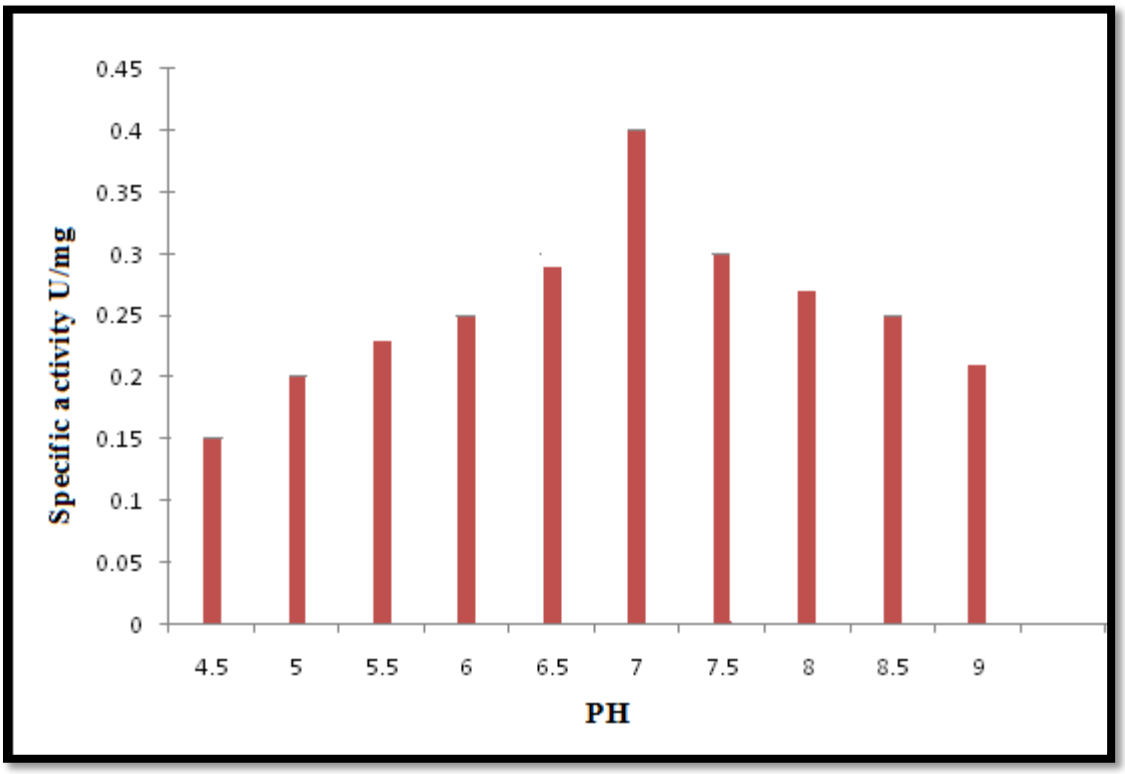

Figure (3-5): Effect of medium pH on L-asparginase production by *P. aeruginosa* P4after incubation at 37°C for 24 hrs.

A decrease or increase in hydrogen ions(H^+) concentration causes pH changes in the culture medium which may lead to drastic changes in the three-dimensional structure of proteins because H^+ and/or OH^-compete with hydrogen

bonds and ionic bonds in an enzyme, resulting in enzymes denaturation (Tortora*et al*., 2004).

On the other hand, the effect of pH on enzyme production resulted from its role in the solubility of the nutritional substances and its effect on the ionization of the substrate and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme (Bull and Bushnel, 1976).

Khamna *et al*. (2009) reported that the optimum pH for L-asparginase production from actinomycetes was observed at 7.0.

Also Narayana *et al*., (2007) have reported the optimum pH for L-asparginase production by *Streptomyces albidoflavus* to be 7.5.

3.4.5Optimum Incubation Temperature

In this study different incubation temperatures (20, 25, 30, 37 and 42^{o}C) were used to determine the optimum for L-asparginase production by *P. aeruginosa*P4. Figure (3-6) shows that maximum production of L-asparginase was occurred when the microorganism was grown in the production medium and incubated at 37^{o}C, with specific activity of 0.4 U/mg.

For any enzymatic reaction, temperature below or above the optimal will drastically reduce the rate of reaction. This may be due to the enzyme denaturation, or to losing its characteristics of three-dimensional structure. Denaturation of a protein involves the breakage of hydrogen bonds and other non-covalent bonds (Tortora *et al*., 2004).

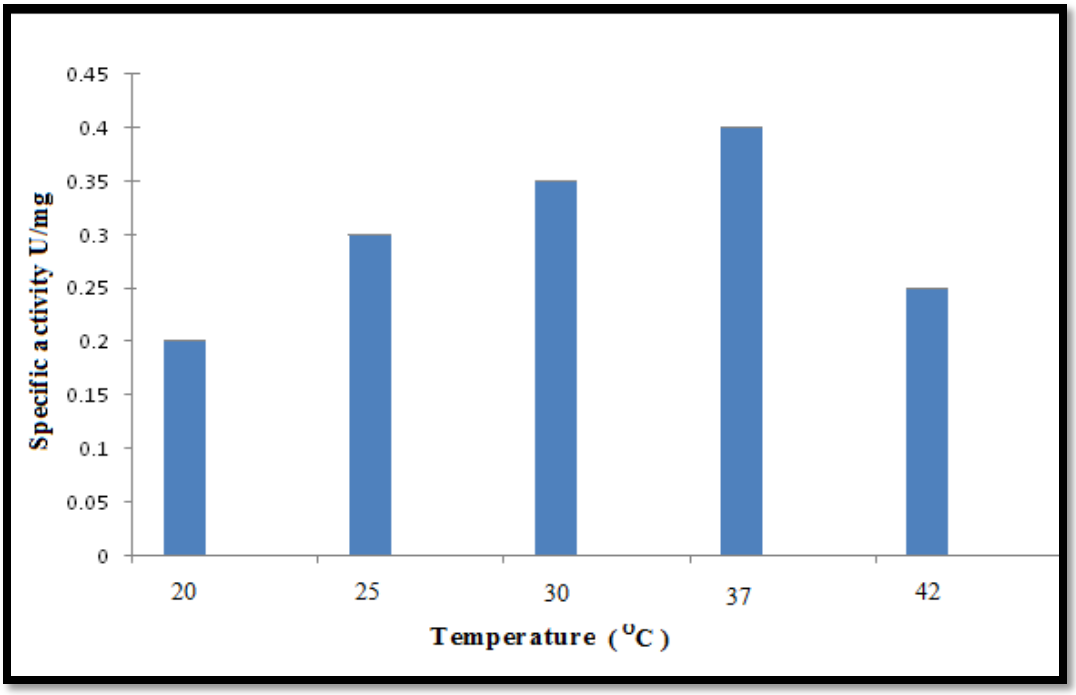

Figure (3-6): Effect of incubation temperature on L-asparginase production by *P. aeruginosa*P4

According to Kushwaha *et al*., (2012) the bacterial L-asparginase showed maximum activity at 37°C under optimized conditions.Also, the maximum enzyme activity from endophytic bacteria appeared as 0.65 IU/ml was at 37°C; (Joshi *et al*.,2015).

3.4.6 Optimum Incubation Period

L-asparginase production by locally isolated *P. aeruginosa*P4 was determined after different incubation periods (12, 24, 36, 48 and 60hrs). Results in Figure (3-7) showed a gradual increase in enzyme productivity with an increase in the incubation period until reach its maximum at 48 hr, with specific enzyme activity of 0.6 U/mg. After that, the activity of enzyme was decreased to 0.3U/mg after 60 hours of incubation.

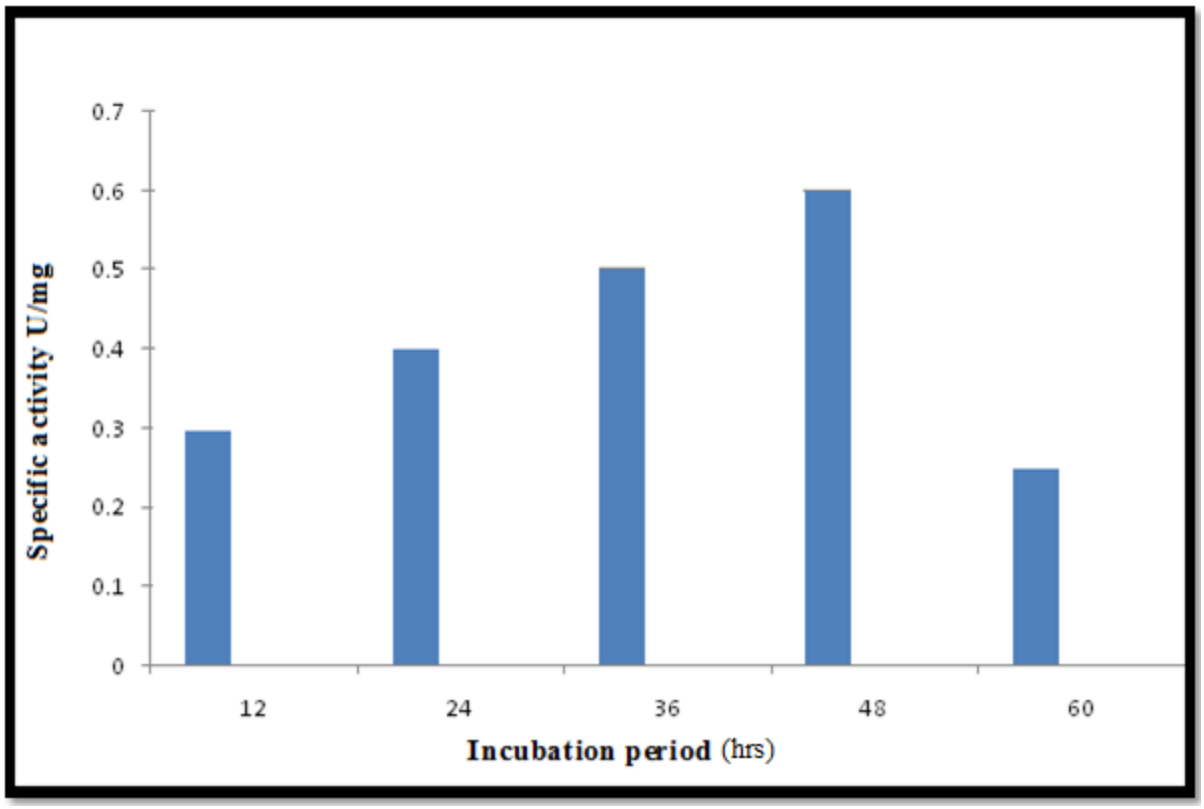

Figure (3-7): Optimum incubation period for L-asparginase production by locally *P. aeruginosa*p4 after incubation at 37°C.

The probable reason for decrease in L-asparginase production beyond 48 hr may be due to rapid depletion of nutrients in the medium, accumulation of excess acid in the media as a result of sugar utilization and developed oxygen tension or it could be attributed to the accumulation of acetate which is inhibitory to cell growth and generation of toxic byproducts that often limit protein yields (Ferreira *et al.*,2004).Also, production of L-asparginase from *P. aeruginosa* decreased while increasing the incubation time from 2 to 7 days (Patel *et al.*, 2011).

Hosamani *et al*., (2011) founded that L-asparginase production by *Fusarium equiseti* using soya bean meal under solid state fermentation with maximum enzyme yield (8.51U/ml) at 48 hrs. incubation period.

In another research, Makky *et al*., (2013) reported that L-asparginase production by *Bacillus sp*.was successfully enhanced and exhibited higher productivity at 37°C for 48 h incubation period.

3.5 Purification of L-asparginase

3.5.1 Ammonium Sulfate Precipitation

Concentration of enzymes is one of the most widely used techniques in enzyme purification: this process depends on precipitation by ammonium sulfate salts (salting out); it's rather used as inexpensive way for concentrating a protein extract (Clive, 2002).

In this study, ammonium sulfate precipitation was achieved using saturation ratios 80% (Table 3-4)and the enzyme specific activity was increased to (1.25U/mgprotein) compared to that of the crude extract (٠.6U/mg proteins).

It was noted that ammonium sulfate is the most widely used in the concentration of enzymes due to its high solubility and low cost, compared with the other organic solvents. In addition, it has no effect on pH or the stability of the enzyme (Whitaker and Bernard, 1972).

Purification of *Pseudomonas fluorescens* L-asparginase was achieved using 80% ammonium sulphate saturation enhanced the specific activity of the enzyme with respect to the crude enzyme from 39.9 IU/mg to 91.6 IU/mg (Sinhar *et al* .,2015) .Also, Kumar and Selvam, (2011) reported that 80% ammonium sulfate saturation were contained high L-asparginase activity and the specific activity was 938.88, IU/mg protein as an initial step for the purification of L-asparginase from *Streptomyces radiopugnans*.

3.5.2Ion exchange chromatography

The ion exchange chromatography technique was used to purify negatively charged L-asparginase produced by *P. aeruginosa*p4 after ammonium sulfate precipitation.

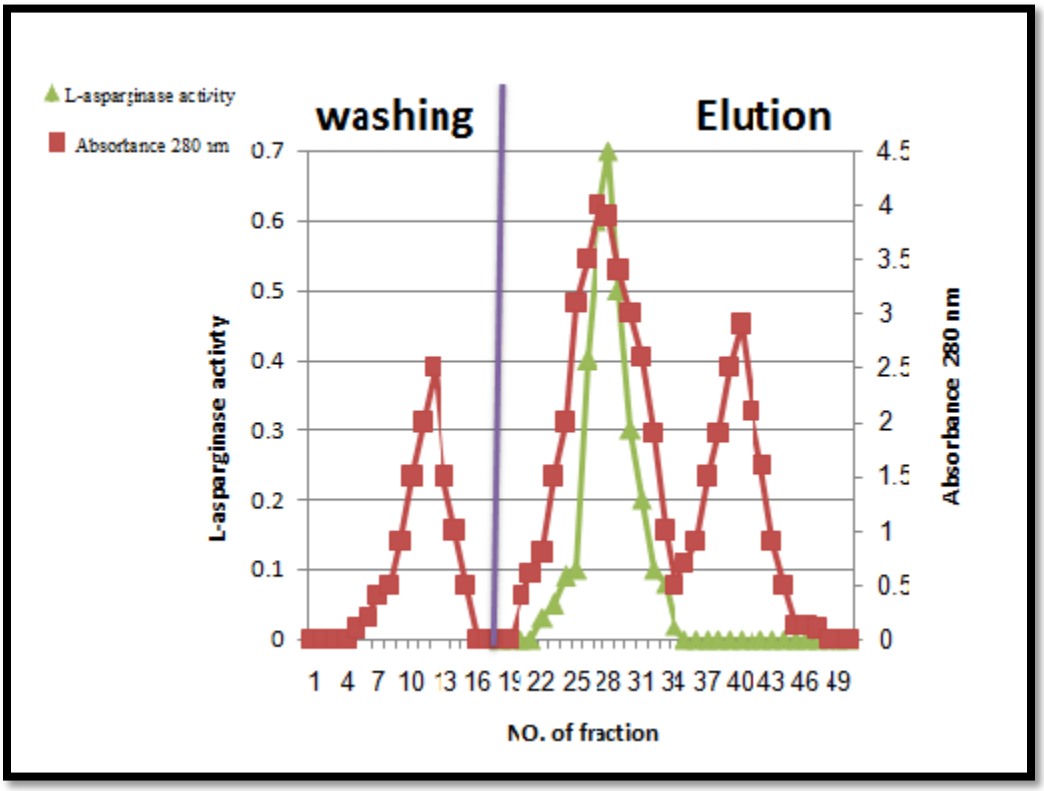

Figure (3-8): Ion exchange chromatography of L-asparginase produced by locally isolated *P. aeruginosa*P4 using DEAE-Cellulose column (2x20cm) with a flow rate of 30ml/hr.

Results showed in (Fig. 3-8) that there was one protein peak which appeared in the washing step, and two protein peaks were appeared after elution by the gradient concentrations of sodium chloride. All these protein peaks were assayed to detect L-asparginase activity. Results showed that eluted proteins (Fractions ٢٥ to ٣٢) contained most of the L-asparginase activity. Enzyme specific activity was measured to be (7U\mg protein), also its purification fold 11.6with 70 % overall yield Table (3-4).

DEAE-cellulose exchanger was used to purify L-asparginase from *Bacillus lichniformis* (Richi *et al*., 2014). In another research, L-asparginase purified by the ion exchange chromatography technique was further purified using the gel filtration chromatography technique (El-Bessoumy *et al*., 2003)

3.5.3 Gel Filtration Chromatography

After purification by the ion exchange, fractions representing L-asparginase activity were collected and pooled for applying to sephadex G-200 column. This column has separation limits ranging between (5000-600,000) Dalton which allowed more efficient ability of separation with high degree of purification. Furthermore, it is characterized by simple preparation, fast runing, high recovery and stability for a long time which permits the re-use of the gel for several times in protein separations (Pharmacia, 1985; Stellwagen, 1990)

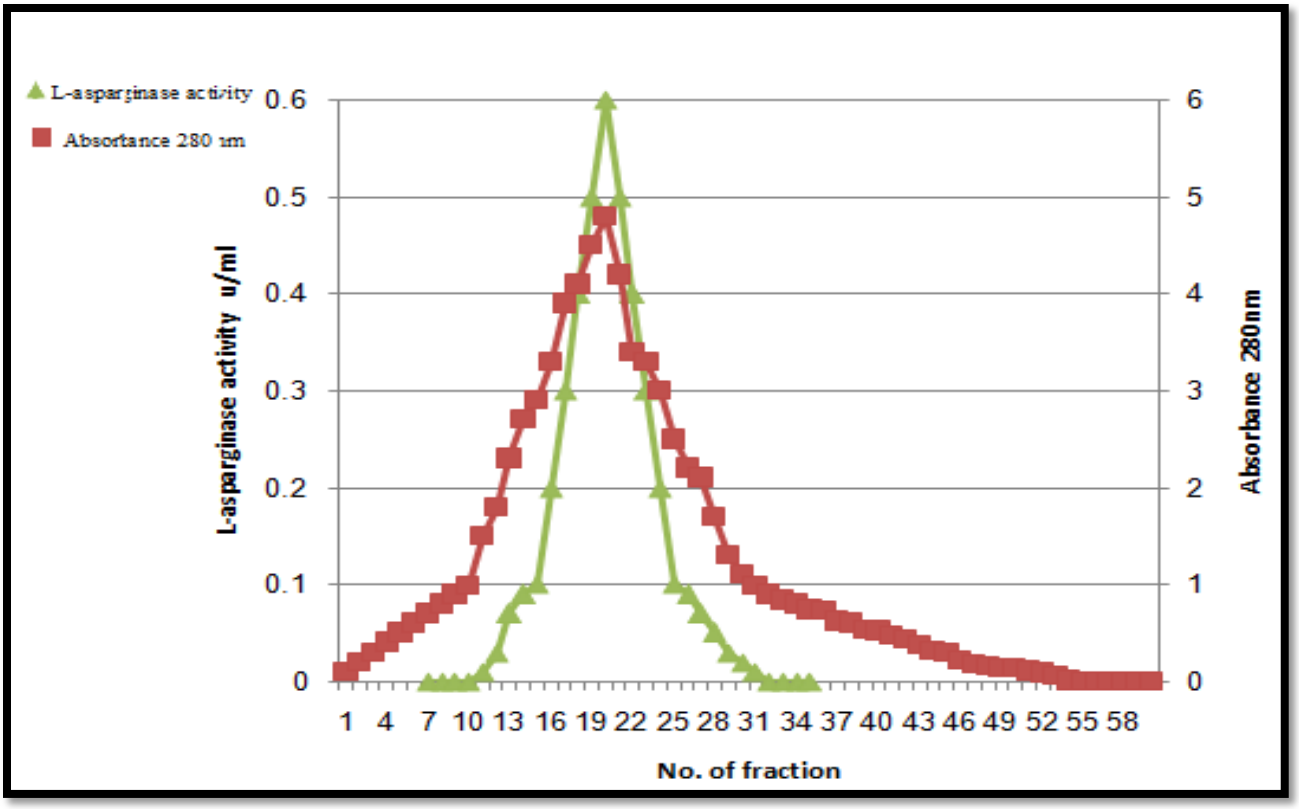

Figure (3-9): Gel filtration chromatography of L-asparginase produced by locally isolated *P. aeruginosa P4* using SephadexG-200 column (1.5cmx35cm) equilibrated with 0.1 M Tris-HCL buffer pH8.

Results in (Fig. 3-9) showed that only one peak representing L-asparginase activity appeared after elution with Tris- HCl buffer. These fractions were pooled and concentrated by lyophilization for further study.

Also, protein concentration, activity and specific activity of L-asparginase were measured, and results in (Table 3-4) shows that there is an increase in the specific activity of the purified enzyme (15 U/mg) with purification fold of (25) and the L-asparginase yield (60%).

In other study, L-asparginase produced by *Aspergillus*sp. ALAA-2000 strain was purified by gel filtration using sephadex G-200 as third step of purification

(after ammonium sulfate precipitation and DEAE-cellulose) to get purified enzyme with specific activity of 0.4 U/mg and 43.6% yield (Abbas Ahmed *et al*.,2015).

Table (3-4): Purification steps for L-asparginase produced by *P. aeruginosa*P4

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	100	0.15	0.25	0.6	15	1	100
Ammonium sulfate precipitation (80%)	25	0.5	0.4	1.25	12.5	2	83.3
DEAE-cellulose	15	0.7	0.1	7	10.5	11.6	70
Sephadex G-200	15	0.6	0.04	15	9	25	60

3.6 Determination of L-asparginase Molecular Weight

The degree of L-asparginase purity was detected by electrophoresis using polyacrylamide gel. Analysis of protein profile of purified L-asparginase from *P. aeruginosa* gave one band after ion exchange step with a molecular weight of 120 kDa which indicates the purity of enzyme (Fig. 3-10).Electrophoresis on polyacrylamide gel was used as another step of purification, and considered as an

improvement technique for determining the efficiency of purification steps (Las, 1998).

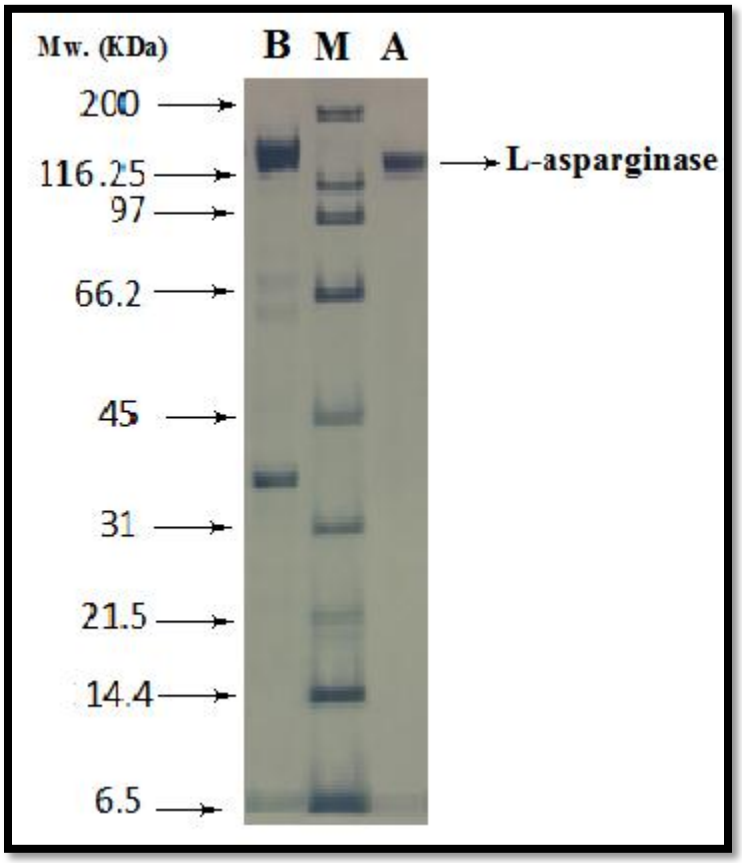

Figure (3-10): Polyacrylamide gel electrophoresis of purified L-asparginase produced by *P. aeruginosa*.

M: Proteins marker

A: Proteins bands after gel filtration step

B: Proteins band after ion exchange step

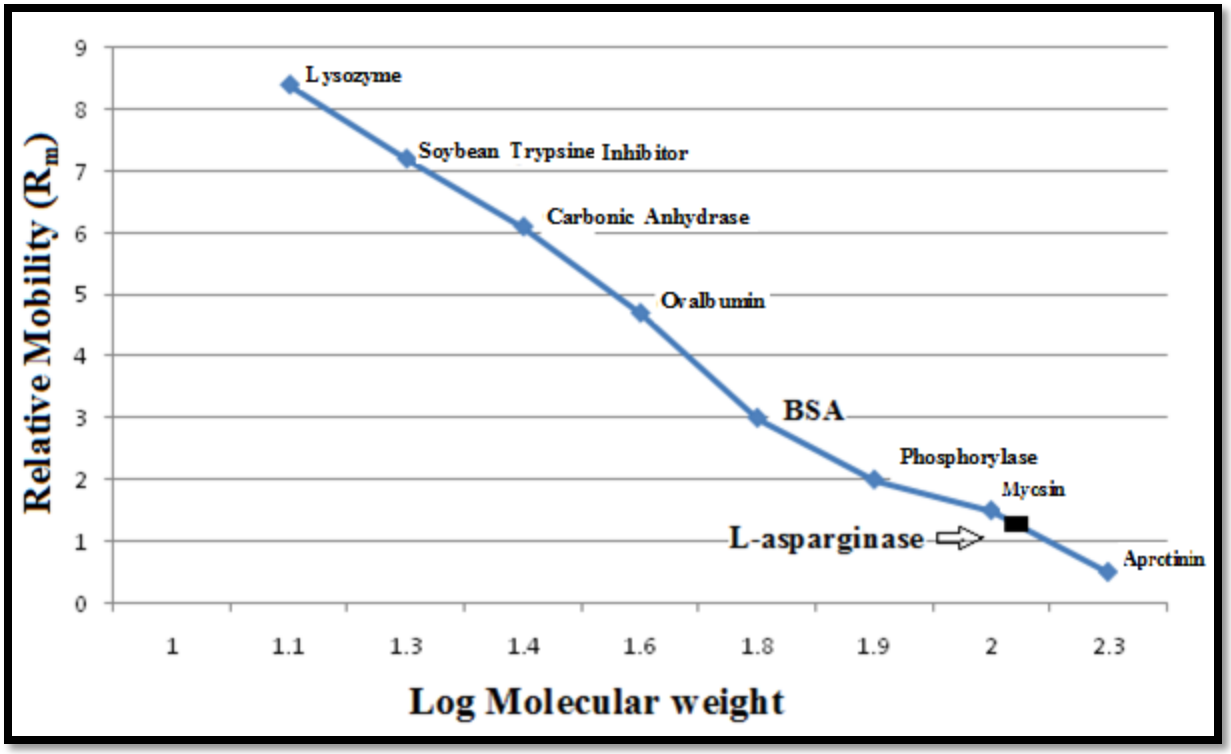

Figure (3-11): Log molecular weight of L-asparginase produced by *P. aeruginosa* P4 after electrophoresis

The molecular weight was determined by interpolation from linear molecular mass versus the R_m value (relative mobility).Depending on the relative mobility,the molecular weight of the protein band was calculated to be 120kDa, which coincided with the band of Myosin (Figure 3-11)

Patro and Gupta, (2012) stated that L-asparginase of *Penicillium* sp. had anapparent molecular weight of 66 kDa.The molecular weight of L-asparaginase produced by *Aspergillusniger*is48kDa(Akilandeswari *et al.*,2012). Also, Amena*et al.*,(2009) founded that L-asparginase produced from *S. gulbargensis* had an apparent molecular weight of 85 kDa. While Narayana *et al.*,(2007) reported that purified L-asparginase from *Streptomyces* sp.PDK2 and *Streptomyces albidoflavus* exhibited a molecular weight of 140 kDa and112 kDa respectively.

3-7 Characterization of Purified L-asparginase

3-7-1 Effect of pH on Enzyme Activity

The effect of pH on L-asparginase activity was determined in a range between 4.5and 9. A gradual increase in L-asparginase activity was observed from pH 4.5 to 7 followed by gradual decrease in its activity beyond this pH(Figure3-12).

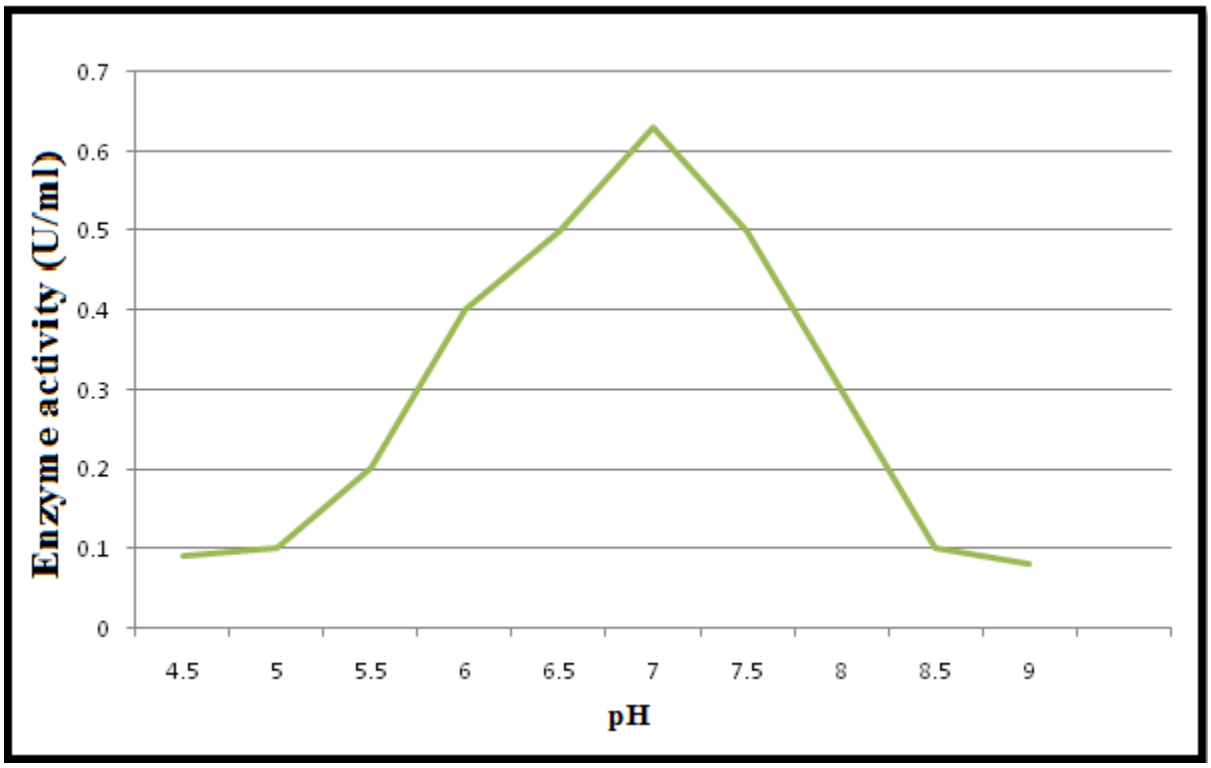

Figure (3-12): Effect of pH on activity of purified L-asparginase produced by *P. aeruginosa*p4

In comparison to previous research, Prema *et al*., (2013) had reported that pH 8 gave the optimum activity of L-asparginase from *Pseudomonas fluorescens*.Also, optimum activity of L-asparginase produced from *Grimontiahollisae (Vibrio)* was at pH range of 6 to 8(Khobragade *et at*., 2015). Saritaand Azmi, (2012) reported that the optimum pH for L-asparginase production by *E. carotovora*MTCC 1428 was found to be 7.0.

While Akilandeswari *et al.*, (2012) founded that pH 8 is best value for maximum activity of L-asparginase produced by *A. niger*.

3.7.2 pH Stability for L-asparginase

Thermal stability studies revealed that L-asparginasewas more stable at pH 7.5, because at this pH, the enzyme gains maximum remaining activity (100 %).While the remaining activity was decreased when the enzyme was incubated at pH less or more than this value (Figure 3-12).

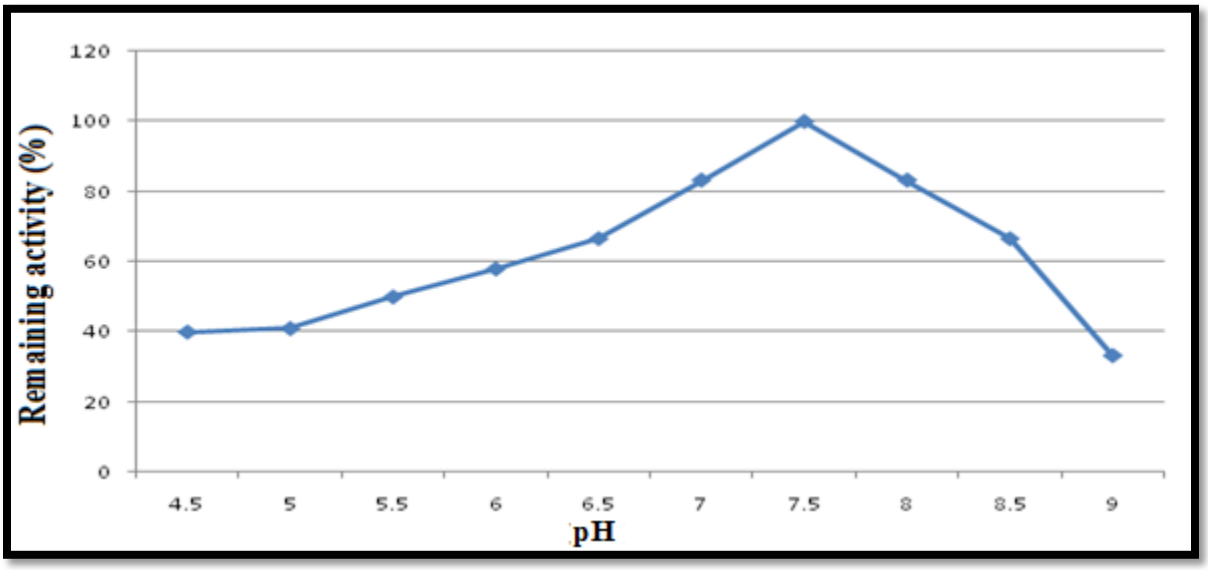

Figure (3-13): Effect of pH on stability of purified L-asparginase produced by *P. aeruginosa*P4

L -asparginase obtained by *Pseudomonas stutzeri* MB 405 was more stable at alkaline pH than at the acidic one. The enzyme was maximally stable at pH 7.5 (Siddalingeshwara and Lingappa, 2011).Another observation regarding the pH stability showed that L-asparginase purified from *Streptomyces gulbargensis* retained 80% of its activity in the pH range 7-10 (Amena *et al*.,2009)

3.7.3 Effect of Temperature on L-asparginase Activity

Temperature is an important factor which affects enzyme activity. The favorable temperature for L-asparginase activity may differ with different L-asparginase sources. Enzyme activity was assayed at temperatures which ranged between 20 and 60^{o}C.

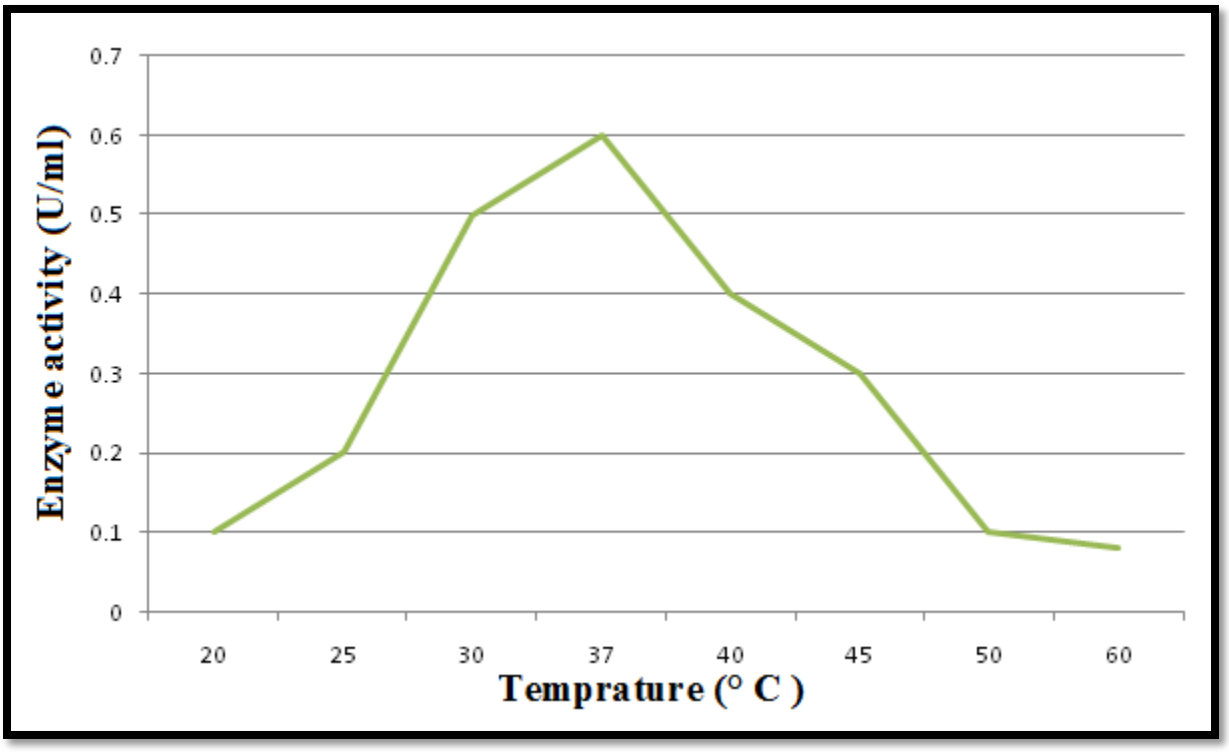

Figure (3-14): Effect of temperature on activity of purified L-asparginase produced by *P. aeruginosa P4*

The enzyme had maximum activity at 37^{o}C with observed decrease in activity at less or a higher temperature of incubation (Figure 3-14)

Similar results were recorded for L-asparginase from *Pseudomonas stutzeri* MI-405, *Erwinia carotovora*, and *Staphylococcus* (Sobis and Mikucki ,1991;Rajesh *et al*.,2011). Kushwaha *et al*., (2012) noticed that the bacterial L-asparaginase showed maximum L-asparginase activity at 37°C.

3.7.4 Temperature Stability of L-asparginase

The temperature stability for L-asparginase (Figure 3-15) showed that the enzyme retained all its activity (100%) when incubated in temperatures ranges between 20 and 37 ^{o}C. However, the enzyme began to lose its activity beyond this temperature and remaining activity reached 0% at 60 ^{o}C.

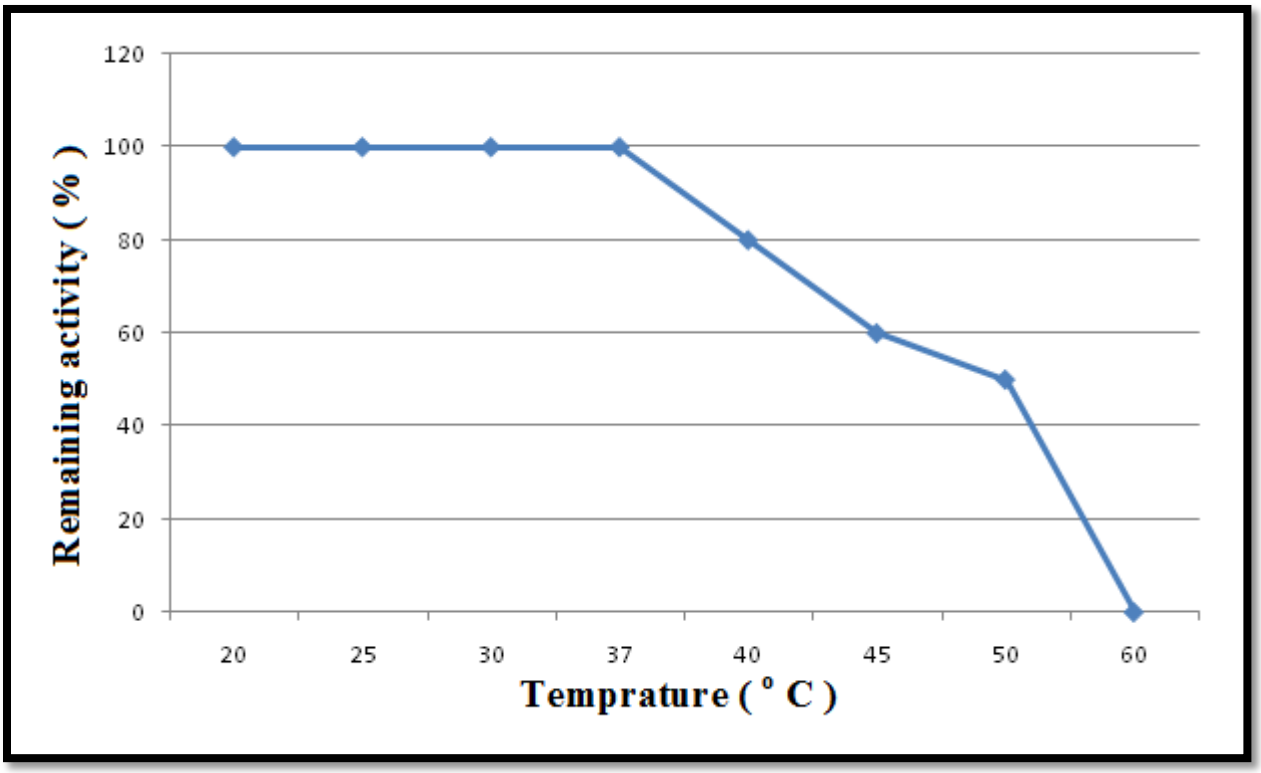

Figure (3-15): Temperature stability of L-asparginase produced by *P. aeruginosa*P4

It has been established for some time that the tertiary structure of proteins is stable. The conformational stability of a protein is the sum of a large number of weak, non-covalent, interactions, including hydrogen bonds, van der Waal interactions and others. All of these forces are affected by environmental conditions, including, for example, solvent and temperature (Roy *et al*.,1996).

Segal, (1976) stated that crude and partially purified enzymes are more stable than purified enzymes due to the existence of carbohydrates and other proteins

protecting them. Similar results were obtained by Hazim*et al*.,(2010) who founded that L-asparginase produced from pathogenic *E.coli* gave full activity (100%) after 15 min incubation at 20-37 ºC, while 70% of its activity was lost when incubated at 60 ºC.

3.7.5 Effect of Ions And Inhibitors on L-asparginase Activity

The effect of some inorganic ions on L-asparaginase activity was studied. Table (3-5) showed that none of NaCl, $CuCl_2$andKClat 20mM concentration significantly affects enzyme activity. Also, $HgCl_2$ and $CaCl_2$(20 mM) were found to inhibit enzyme activityby71% and 44% lost respectively. On the other hand, the enzyme showed an increase in its activity when $MnCl_2$was applied.

Regarding the reducing and chelating agents , the enzyme showed a constant decrease of about 87% at all tested concentration of 2-Mercaptoethanol. Cysteine showed 44% and 20% loses of original activity at 2 and 4mM respectively. While the enzyme still resistant to EDTA at 4mM, where the remaining activity was 98% of original, suggesting that L-asparginase isn't a mettalo enzyme (Table 3-6).

Pekhov and Zanin (1987) stated that the presence of manganese chloride lead to reduce only 15% of L-asparginase activity that produced by *Pseudomonas boreopolis* cells.

Abbas Ahmed *et al*.,(2015) reported the effect of Ni^{+2},Ba^{+2}, Ca^{+2}, Na^{+}, Co^{+2}, Mn^{+2}, and $Mg^{+2}$with final concentration of 0.5mM on partially purified L-asparginase activities only Ni^{+2} achieved an increase in the L-asparginase activity about 16%, while others inhibited enzyme activity. The highest inhibition was 22% by Na^{+}.

Table (3-5): Metal ions and inhibitors effect on L-asparginase activity produce by *P.aeruginosa*P4

Metal ions	Concentration (mM)	Remaining activity (%)
NaCl	1	98.35
	10	97.53
	20	94.25
$CuCl_2$	1	91.79
	10	91.79
	20	88.51
KCl	1	94.25
	10	94.25
	20	94.25
$HgCl_2$	1	52.45
	10	41.79
	20	28.68
$CaCl_2$	1	97.53
	10	63.92
	20	55.73
$MnCl_2$	1	278.66
	10	131.95
	20	**102**

Table (3-6): Reducing and chelating agent's effect on L-asparginase Activity produce by *P.aeruginosa*P4.

Agent	Concentration (mM)	Remaining activity
2-mercaptoethanol	0.5	13.93
	1.0	13.93
	2.0	13.93
	4.0	13.93
Cystein	0.5	100
	1.0	97.53
	2.0	80.32
	4.0	55.73
EDTA	0.5	100
	1.0	100
	2.0	100
	4.0	97.53

3.8 Cytotoxic Activity of L-asparginase Using MTT Assay

The assay of (MTT) was used to determine the cytotoxic effect of crude and purified L-asparginase on breast cancer Adeno-carcinoma (MCF-7) cell line .MTT assay is a non-radioactive colorimetric assay which is used to measure the cell viability in response to a variety of cytotoxic stimuli. The assay is based on the reduction of the yellow, water soluble tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) within metabolically active cells. The reduction of the tetrazolium salt occurs by the action of mitochondrial dehydrogenases present only in viable cells, yields a purple formazan product which can be quantified spectrophotometrically. The percentage viability of the

treated cells was calculated by compare it with normal cell line WRL (Manish and Thankamani, 2015).

Purified L-asparginase enzyme showed a cytotoxic effect on MCF-7 cell line significantly higher than crude enzyme andthis effect was increased with the increasing in concentration (Table 3-7).

Table (3-7): Inhibitor ratio of crude and purified enzyme at different Concentrations

Concentration (**µg/ml**)	IR (%)of crude enzyme	IR (%) of purified enzyme
400	33.0 ± 6.508	47.15± 7.580
200	25.0 ± 0.536	34.42 ± 9.869
100	13.2 ± 7.206	21.84 ± 8.541
50	06.1 ± 2.400	20.25 ± 8.735
25	05.1 ± 3.130	16.21 ± 7.450
12.5	03.6 ± 2.052	13.71 ± 6.175
6.25	02.2 ± 2.680	10.66 ± 3.444
3.125	01.6 ± 3.035	07.84 ± 2.339
1.5	00.3 ± 0.755	06.18 ± 2.580
0.78	0.00	04.46 ± 2.580
0.39	0.00	03.32 ± 3.949
0.19	0.00	01.97 ± 3.930

The IC_{50}which is the half maximal inhibitory concentration for cell growth constructing adose-response curve was measured. Crude L-asparginase showed IC_{50} value of355.6 µg/ml, whereas an IC_{50} of 185.7 µg/ml was obtained from the purified L-asparginase against MCF-7 cell line.

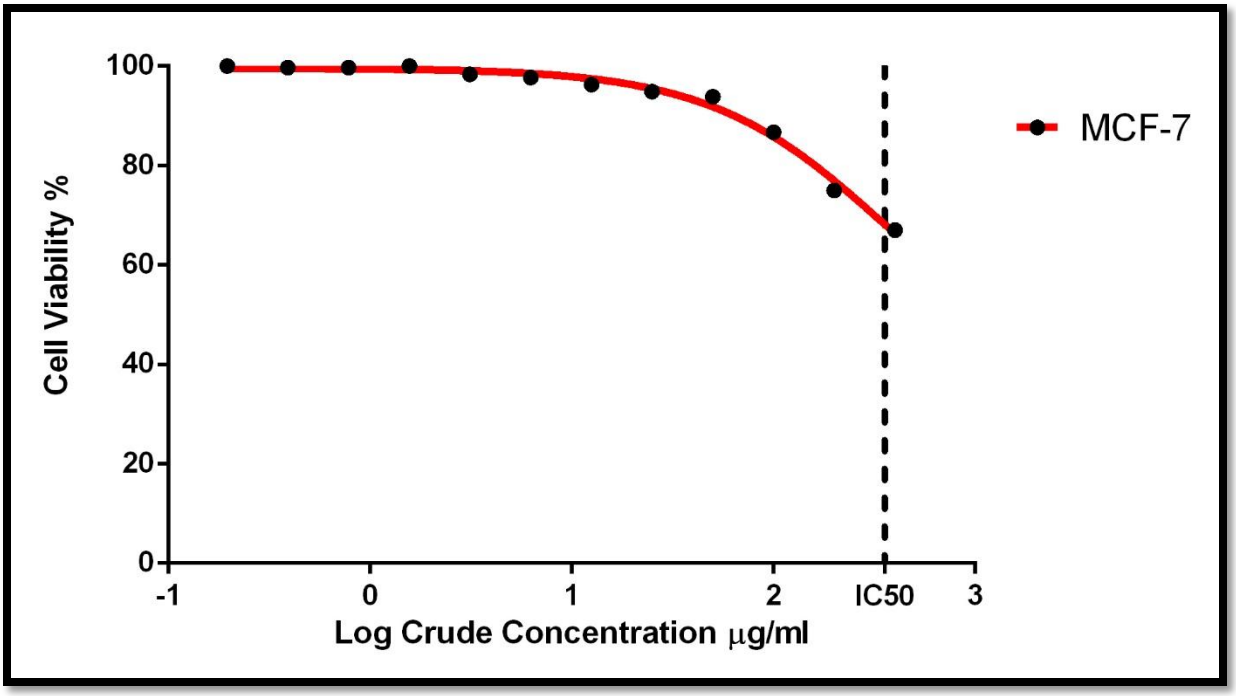

Figure (3-16): Cytotoxic activity of Crude L-asparginase produced from *P. aeruginosa* on MCF-7 breast cancer cell line by using MTT test after 24 h. and 37^{o} C.

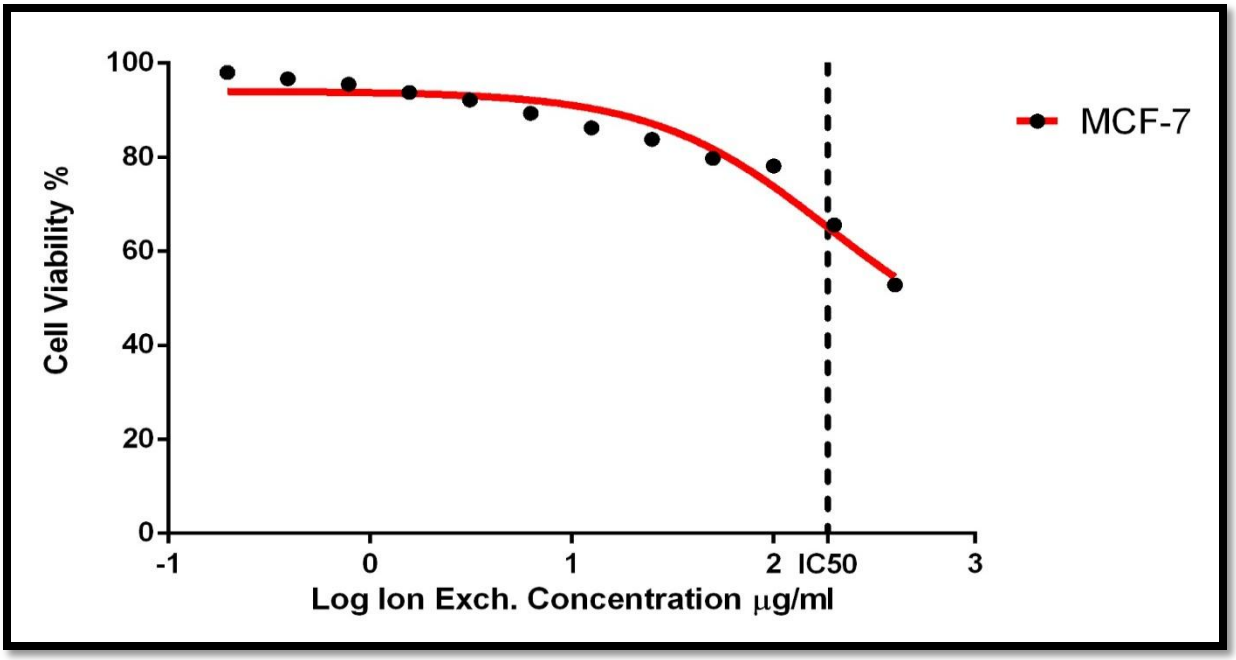

Figure (3-17): Cytotoxic activity of purified L-asparginase produced from *P. aeruginosa* on MCF-7 breast cancer cell line by using MTT test after 24 h. and 37° C.

The sensitivity of MCF-7cell line to crude and purified L-asparginase appeared to be dose dependent, resulting in a significant decrease in viable cells. At a concentration of 25 µg/ml, 85% and 80% cell viability for crude and purified L-asparginase were observed, respectively. However, cell viability reached to 50% using 400 Mg/ml concentration of purified L-asparginase (Figure3-16) and (Figure 3-17).

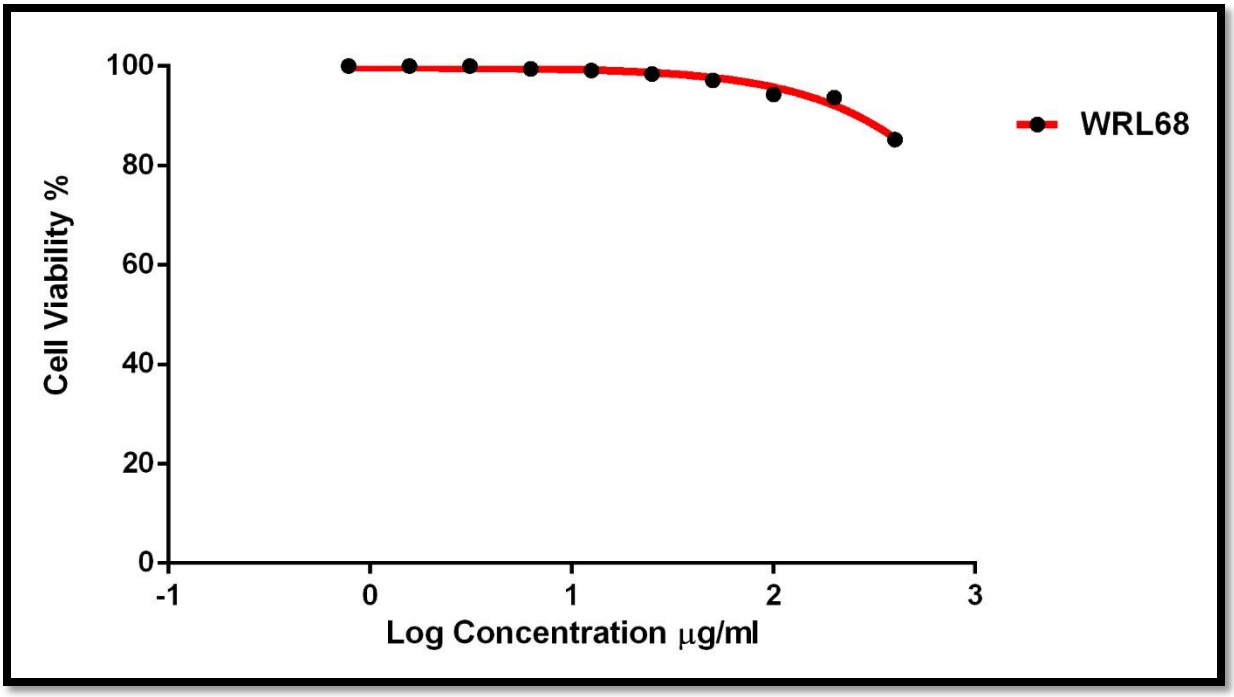

Figure (3-18): Anticancer activity ofL-asparginase produced from *P. aeruginosa* on WRL normal cell line by using MTT test after 24 h. and 37^{o} C.

However, *P, aeruginosa* P4 L-asparginase didn't show significant toxicity on normal cell tested WRL cell line (Figure 3-18).

Ando *et al*., (2005) stated that the cytotoxic potential of purified L-asparginase is comparable to commercial L-asparginase. Mechanistically, purified enzyme induced apoptosis by arresting cell cycle in G0/ G1 phase and also dysfunctioning of mitochondrial integrity. It is noteworthy that *P. aeruginosa*is nontoxic for human noncancerous (WRL) cells and had no hemolytic effect on erythrocytes.

In similar research, L-asparginase purified from *Helicobacter pylori* and tested for its cytotoxicity against tumorcells, these cells were affected in different levels (Cappelleti *et al*., 2008).

EL-sayed *et al.,* (2011) demonstrated that the IC50 for L-asparginase was greater than 10μg/ well using MCF-7 (breast carcinoma) cell line. The purified L-

asparginase from *Bacillus licheniformis* showed cytotoxic effect against MCF-7 andK-562 (Mahajan *et al.*, 2014).

Conclusions and Recommendatios

Conclusions:

1- *Pseudomonas aeruginosa* can be used as a good source for the production of L-asparginase, an enzyme that has gained industrial and pharmaceutical significant.

2- Under the optimized condition, the enzyme can be produced in a medium supplemented with glycerol as a best Carbone source, 0.1 % Tryptone as a best nitrogen source, adjusted at pH 7 inoculated with 10^8 cells /ml and incubated at 37 ^oC under aerobic conditions.

3- Applying successive purification procedures including :

A- Ammonium sulfate precipitation at 80 % saturation ratio.

B- Ion exchange chromatography on DEAE-cellulose and

C- Gel filtration chromatography on sephadex G-200. Resulted a purified enzyme with a single protein band considered as an indication of enzyme purity.

4- Optimum pH (7) and temperature (37 ^{o} C) for L-asparginase in a values similar to that of human body making the enzyme preparation suitable for therapeutic use.

5- The purified L-asparginase showed cytotoxic activity against MCF-7 cell line with less toxicity against normal cell favoring its use for further pharmaceutical application as antitumor candidate.

6- Metal chelators ion as EDTA didn’t affect enzyme activity suggesting that the enzyme was not a metalloenzyme.

Recommendations:

1- Improving the production of L-asparginase enzyme using genetic engineering techniques.

2- Immobilization of the enzyme onto a suitable matrix to increase its half-life and its therapeutic potential.

3- Investigating the antitumor abilities of L-asparginase and its chemical treatment for tumors transplanted into mice *in vivo*.

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الخلاصه

جمعت في هذه الدراسه ٤٦ عينة لعزل*P. aeruginosa* .تشمل ١٤ عينة مأخوذة من الجروح، ٢٠ عينة من الحروق و ١٢ عينة من التهابات الأذن والتي تم جمعها من مستشفى اليرموك. من بين هذه العينات، تم الحصول على مجموعه ٢٩ عزلة بكتيرية اخضعت ستةعزلات منها الى التشخيص على اساس صفاتها المظهريه و المزرعيه و خصائصها الكيموحيويه وفحص الفايتك. وقد اشارت نتائج التشخيص الى ان هذهالعزلات شخصت على انها *P. aeruginosa*.

تمت غربلة العزلات البكترية على اساس قابليتها على انتاج انزيم الاسبارجينيز وقد اظهرت النتائج ان جميع هذه منتجة للانزيم وبدرجات متفاوتة. وقد كانت العزلة التي سميت P4هي الاكفأ. اذ بلغت الفعالية النوعيه للانزيم في رائق مزرعتها١٥.٠وحدة/ملغم بروتين .لذا فقد تم انتقاء هذه العزلة لتحسين قابليتها في انتاج الاسبارجينيز .

تم دراسة الظروف المثلى لانتاج انزيم الاسبارجينيزوقد اشارت النتائج ان اعلى *انتاج للانزيم* كان باستخدام الوسط ذو الرقم الهيدروجيني ٧ المدعم بكليسيرول ١.٠% و١.٠% تريبتون قبل تلقيحه ب ^{٨}١٠خلية/مل وحضنه بدرجة ٣٧^{o}م بحاضنة هزازة بسرعة ١٥٠ دورة /دقيقة لمدة ٢٤ ساعة.

نقي انزيم الاسبارجينيز المنتج تحت الظروف المثلى بثلاث خطوات تضمنت الاولى الترسيب بكبريتات الامونيم بنسبة اشباع ٨٠ % ، ثم التبادل الايوني باستخدام المبادل الايوني الموجب - DEAE cellulose , ثم خطوة التنقيه باستخدام هلام الترشيح السيفادكس G-200 وقد بلغت الفعالية النوعيه للاسبارجينيز المنقى ١٥ وحده/ ملغم بروتين بعدد مرات تنقيه ٢٥ و حصيلة انزيمية مقدارها ٦٠ % .

درست خلال البحث بعض الصفات الكيموحيوية للانزيم المنقى ووجد ان الوزن الجزيئي للاسبارجينيز هو ١٢٠٠٠٠ دالتون ، وقد اعطى الانزيم اعلى فاعلية برقم هيدروجيني ٧و لثبات الانزيم ٨ ، كما كانت اعلى فعالية انزيمية بدرجة حرارة ٣٧ ^{o}م وكان الانزيم ثابتا بدرجات حراره تراوحت مابين(٢٠-٣٠)^{o} م .

خلال هذه الدراسة، تم اختبار التأثير السمي الخارجي لانزيم الاسبارجينيز الخام والنقي ضد خلايا MCF7 السرطانية وباستخدام فحص MTT. وقد ثبط الانزيم النقي الخلايا السرطانية وبتركيز مثبط وسطي بلغ ١٨٥.٧ مايكروغرام/مل مقارنة مع ٣٣٥.٦ مايكروغرام/مل للانزيم الخام. على كل حال فان الانزيم لم يظهر اي تاثيرسمي على الخلايا الطبيعية مما يشير الى التأثير الفعال ضد الخلايا السرطانية.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

انتاج، تنقية ، توصيف و تأثير سمية انزيم الاسبارجينيز من بكتريا الزائفة الز نجاريه المعزولة محليا

رساله

مقدمه الى كلية العلوم / جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير علوم في التقانه الاحيائيه

من قبل

ايلاف اسماعيل الخاصكي

بكلوريوس تقانه احيائيه / كلية العلوم/ جامعة النهرين (٢٠١٤)

باشراف

د. اسماء علي حسين

(استاذ مساعد)

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