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Genetic Study on The Locally Isolated *Pseudomonas aeruginosa* and its Ability in Lectin Production

A Thesis

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Master of Science in Biotechnology

By

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Dedication

To the spring of sympathy

Mother and Father

To the most helpful peoples

Brother and Sister

I dedicate this work

Sinan

Acknowledgment

Praise to God, the first cause of all causes, the glorious creator of the universe.

I would like to express my thanks to my supervisor Dr. Hameed M. Al-Dulaimi for his great support and his big efforts during this study.

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Summary

A total of 34 samples from ear, blood, sputum, burns, CSF and cystic fibrosis infections were collected from Al-No'man hospital, Central Medicine City hospital and from sewage of Al-Dorra in Baghdad governorate. From these samples we got 18 isolates, 12 were identified to belong to *Pseudomonas* spp. according to their ability to grow on ceteremide agar.

These isolates were subjected to biochemical, morphological and physiological tests and as a result, 6 of them were *Pseudomonas aeruginosa* and it has been proved using API 20E system.

The ability of local isolates of *Pseudomonas aeruginosa* for lectin production and haemagglutination of RBC_s for human blood groups (A, B, O) and sheep RBC_s were studied, results showed that all these isolates has a high ability for haemagglutination of different RBC_s.

The ability of the locally isolated *Pseudomonas aeruginosa* S4 for lectin production was studied and it was producing for both types of lectins (PA-IL and PA-IIL), lectins were purified using precipitation by ammonium sulfate then dialyzed and eluted using gel filtration by sepharose-200 CL-6B. the same technique (gel filtration) has been used for molecular weight determination for both types of lectins by using standard proteins with known molecular weight (lysozyme, BSA and choline esterase). Results showed that the molecular weight of PA-IL approximately was 57500Da while for PA-IIL approximately was 42700Da.

Also the ability of antibiotic resistance for *Pseudomonas aeruginosa* S4 was studied for different antibiotics and it was resistant to vancomycin, chloramphenicol, carbenicillin, cefotaxime, cephalexin, tetracycline and bacitracin.

The effect of physical mutagen (using UV light) and chemical mutagen (using mitomycin C) on the ability of *Pseudomonas aeruginosa* S4 for lectin production were studied also. Results showed that physical mutagen was more effective than chemical mutagen on the ability of this isolate for lectin production in which haemagglutination activity of RBC_s for human blood groups (A, B, O) and sheep RBC_s has been reduced by the bacterial mutants, in the same time we have got a bacterial mutants which has a high haemagglutination activity for different blood groups compared to that of the wild type of *Pseudomonas aeruginosa* S4.

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Abbreviations

BDH	**Brutish Drug House**
BSA	**Bovine Serum Albumin**
CF	**Cystic Fibrosis**
CSF	**Cerebrospinal Fluid**
GE	**Grelet's Medium**
H. A.	**Haemagglutination**
HSA	**Human Serum Albumin**
LPS	**Lipopolysaccharides**
PA-IL	***Pseudomonas aeruginosa* 1^{st} lectin**
PA-IIL	***Pseudomonas aeruginosa* 2^{nd} lectin**
RBC	**Red Blood Cell**
RTF	**Resistance Transfer Factor**
V_e	**Elusion volume**
V_o	**Void volume**

Introduction

Pseudomonas aeruginosa is a rod shaped Gram-negative obligate aerobe belonging to the family Pseudomonadaceae. *P. aeruginosa* is capable of growing in a wide variety of differing niches and growth often occurs in moist environments which explains its associations with soil and water. It can also be isolated from plants and animals especially from moist sites such as the ear. As most human *P. aeruginosa* infections are nosocomial in nature, hospital reservoirs of growth are many and include respiratory equipment, solutions, medicines, disinfectants, sinks, mops, food mixers and vegetables (Palleroni, 1985). *P. aeruginosa* virulence is based on several properties, most particularly on the ability of this organism to adhere to surfaces, to form biofilms and to secrete hydrolytic enzymes and toxic compounds. The expression of virulence factor is controlled by numerous complex cascades that include quorum-sensing and two-component-system networks (Withers *et al*., 2001).

Since host carbohydrates have been known for many years to constitute specific attachments sites for microbial protein receptors, the lung mucins and above all those of cystic fibrosis patients, have been analysed thoroughly (Lamblin *et al*., 2001). On the other hand, some of the carbohydrate binding proteins of *P. aeruginosa* have been studied and their role in recognition and adhesion is far from being fully elucidated. Several types of receptors have been identified. Flagellin and flagellar cap protein FliD recognize mucin oligosaccharides, whereas type IV pilus adhesions have been shown to recognize glycosphingolipids asialo-GM1 and asialo-GM2 (Sheth *et al*., 1994). In addition, two soluble lectins PA-IL and PA-IIL specific for D-galactose and L-fucose, respectively, and containing divalent

cations have been characterized. These two lectins are produced at high levels by the bacteria in association with the cytotoxic virulence factors and under quorum sensing control (Winzer *et al*., 2000).

Aims of the study

Because of the importance of lectins in different immunological studies and applications, this study was aimed to:

1. Isolation and identification of *Pseudomonas aeruginosa*.
2. Studying the ability of local isolates of *Pseudomonas aeruginosa* in lectin production.
3. Purification of lectins produced by local isolates of *Pseudomonas aeruginosa* and determination its molecular weight.
4. Testing the effect of physical and chemical mutagens on locally isolated *P. aeruginosa* regarding lectin production.

1 Literature Review

1. 1 *Pseudomonadaceae*

The family *Pseudomonadaceae* comprise a large and important group of Gram-negative bacteria. Its members are found abundantly as free-living bacteria in soil, fresh water and marine environments. They also may be found in associations with plants and animals as agents of disease or normal flora. The family is informally known as "**Pseudomonads**"

Pseudomonads morphologically are Gram-negative rods, non spore formers, motile by means of one or more polar flagella (Todar, 2004).They are oxidase positive due to the presence of indophenol oxidase (cytochrome c oxidase) which means that it has an electron transport chain. Most of Pseudomonads are obligate aerobes (respire only), however, *Pseudomonas aeruginosa* which is the major pathogen of this group can survive in anaerobic environments by using nitrate as the terminal electron acceptor. Pseudomonads are nutritional versatile, to be survive, some of them can use trace amounts of 80 different carbon source, some require acetate and ammonia only (Microbiology syllabus, 2004).

Four genera are assigned to this family which are *Pseudomonas, Xanthomonas, Zoogloea* and *Frateuria. Pseudomonas* and its relatives includes many species which are pathogenic for humans, animals and plants. *Pseudomonas* species are among the most important bacteria that are pathogens of plants, they cause crop disease and crop loss world-wide. *Pseudomonas aeruginosa* infects both plants and animals and has evolved into one of the pathogens which are the most common and refractory nosocomial of the post-antibiotic era. Typical *Pseudomonas*

bacteria might be found in nature in a biofilm (attached to some surface or substrate) or in a planktonic form (as a unicellular organism, actively swimming by means of its flagellum). *Pseudomonas* species are able to degrade compounds that are very refractory to other organisms, these compounds includes aliphatic and aromatic hydrocarbones, insecticides, fatty acids and other environmental pollutants. Teflon, styrofoam and one-carbon organic compounds (methanol, formaldehyde, methane, etc) are the only organic compounds that these Pseudomonads can not attack.

The closest relative of *Pseudomonas* is *Xanthomonas,* which includes both phytopathogenic species and saprophytic strains. *Zoogloae* is ecologically more restricted, but it has an extremely active oxidase metabolism in its natural habitat, also it is an important participant in the carbon cycle as a component of the microflora of activated sludge. *Frateuria* strains are more restricted in habitat, but they transform sugars and other carbohydrates that are potentially important for the fermentation industries (Todar, 2004).

1.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic rod shaped bacteria, measuring 0.5-0.8μm width and 1.5-3.0μm long, oxidase and catalase positive. β-hemolysis is produced on blood agar in the presence of *Pseudomonas aeruginosa.* Their optimal temperature for growth is 37ºC (but can grow at 42ºC). It can denitrify and produces gelatinase (Ismar, 2003; Todar, 2004, Internet I). It has the ability to survive in numerous environmental niches, including soil and marine habitats, plants, animals and humans (de Kievit *et al.*, 2000; Ismar, 2003). Almost all *Pseudomonas aeruginosa* strains are motile using a single polar flagellum. *Pseudomonas aeruginosa* is also an opportunistic pathogen, responsible for numerous nosocomial infections in immunocompromised

patients. The bacteria colonise patients with a number of chronic lung diseases, particularly those on assisted ventilation and especially cystic fibrosis patients. Several properties can determine the virulence of this bacteria, most particularly on the ability of this organism to adhere to surfaces, to form biofilms and to secrete hydrolytic anzymes and toxic compounds (Bock *et al*., 1988; Hooper *et al*., 2001, Internet I).

Pseudomonas aeruginosa has a biofilm alginate layer surrounding its membrane and cell wall. This biofilm is formed by secretion of alginate (mucoid substance) to the cell surface which in turn connects with neighboring cells. As a sufficient amount of cells are joined together, the biofilm can be seen as a slimy substance. Biofilm contains numerous channels to enable transport of material and wastes through it. Biofilm protects the bacteria from antibiotics and antibodies (working as a physical barrier). Along with fimbriae on the cell surface, biofilm enable the bacterium to hold onto the host cells or other surfaces, therefore biofilm allows *Pseudomonas aeruginosa* to maintain a fairly constant environment for cells within it (much like a multicellular organism does) (Ismar, 2003).

Pseudomonas aeruginosa has very simple nutritional requirements that it can be seen "growing in distilled water" *in vitro*, the simplest medium for growing *Pseudomonas aeruginosa* consists of acetate for carbon and ammonium sulfate for nitrogen. (Todar, 2004).

Pseudomonas aeruginosa can form three types of colonies:

- Natural isolates (from soil or water) produce a small rough colony.
- Clinical samples produce one or another of two smooth colony types.

First type which has a fried-egg appearance which is large, smooth with flat edges and an elevated appearance. Second type (frequently

isolated from respiratory and urinary tract secretions, has a mucoid appearance (due to the production of alginate slime).

Pseudomonas aeruginosa considered to be dangerous and dreaded pathogen for its resistance to antibiotics. Its natural resistance to antibiotics is due to the permeability barrier afforded by its outer membrane LPS. Also its ability to form biofilm on surfaces makes the cells impervious to therapeutic concentrations antibiotics.

Because *Pseudomonas aeruginosa* natural habitat is the soil (which can live in associations with the bacilli, actinomycetes and molds) it has developed resistance to a variety of their naturally-occurring antibiotics. In addition *Pseudomonas aeruginosa* has antibiotic resistance plasmids, both R-factors and RTF_s, and it is able to transfer these genes by means of transduction and conjugation.

Few antibiotics are effective against *Pseudomonas aeruginosa* including fluoroquinolones, gentamicin and imipenem (these antibiotics are not effective against all strains) (Todar, 2004).

1.2 Virulence factors of *Pseudomonas aeruginosa*

The function of *Pseudomonas aeruginosa* in nature is saprophitic decomposition of organic materials for carbon and nitrogen cycling. But when it meets damaged patient tissues, unfortunately, it functions in the same efficient way (Gilboa-Garber, 1997).

Pseudomonas aeruginosa produces a variety of exoproduct virulence determinants and secondary metabolites (Winzer *et al.*, 2000; Todar, 2004; Microbiology syllabus, 2004) these includes:

1- Binding proteins and capsules

a-Fimbriae (N-methyl-phenylalanin pili): for attachment and colonization.

b-Pyochelin: siderophore in iron uptake system.

c-Alginate (polymers of D-mannuronic and L-gluluronic acid): exopolysaccharide capsule. Which is antiphagocytic and causes bacterial adherence to each other.

2- Enzymes

a-Phosphplipase C: is a hemolysin and its function is to lyse RBC_s.

b-Lecithenase: also a hemolysin.

c-Elastase: play an important role in damaging the host cells. It cleaves IgA, IgG, complement and collagen. Elastase lyses fibronectin, by this way it allows receptors for attachment of bacteria on the mucosa of the lung. It also destroys blood vessel linings.

d-Proteases: like Las A, Las B and alkaline protease which are used to split proteins and peptides or amino acids. Bacteria release proteases either to protect itself from dangerous proteins or to weaken host cells.

3- Pigments and toxins

a-Phenazine pigments:

- Pyocyanin (blue-green pigment): produced in large amount when *Pseudomonas aeruginosa* grown in media of low-iron content (diagnostic character of *Pseudomonas aeruginosa*).
- α-oxyphenazine (colorless): produced by breaking down of pyocyanin.
- Pyorubin (rust brown pigment): addition of glutamate to the medium enhance its production.

b-Fluorescein (yellow-green pigment): fluoresces when exposed to UV light (this character can be used to detect infection in burn patients).

All *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* strains can make this pigment.

c-Exotoxin S: also called ADP-ribosyl transferase, encoded by exo S gene. It has ADP-ribosylating activity (hence the name), it transfers ADP-ribosyl portion of NAD to proteins, this effect often kills eukaryotic cell or causes a tumor like growth (rare conditions).

d-Exotoxin A: Encoded by tox A gene. Exotoxin A binds to receptors, then enters the cytoplasm of the eukaryotic host cell and catalyzes the ADP-ribosylation of EF2 (exactly the same mechanism of diphtheria toxin) this process leads to inhibition of protein synthesis and kills the host cell. Exotoxin A is partially identical to diphtheria toxin, but antigenically it is distinct and use different receptors to bind on host cells. Induction of Exotoxin A production is done by iron limitation.

Toxigenic strains are more virulent than non toxigenic strains. *Pseudomonas aeruginosa* also produce pyocins which are bacteriocins which kills other Pseudomonads (Microbiology syllabus, 2004).

In addition to the above virulence factors, *Pseudomonas aeruginosa* also synthesizes two lectins termed PA-IL and PA-IIL (Gilboa-Garber, 1972a; Gilboa-Garber *et al.*, 1997). These lectins appears to function as adhesions (Wentworth *et al*., 1991) as well as cytotoxins for respiratory epithelial cells (Bajolet-Laudinat et al., 1994; Adam *et al*., 1997).

1.3 Quorum Sensing

Although bacteria are unicellular organisms, under certain conditions, these organisms need to communicate and interact with each other to perform certain activities. An example of this is the so-called "Quorum Sensing" behavior, where the bacterial population senses its density and alters its gene expression accordingly. Activities influenced by quorum sensing molecules include conjugation, luminescence, virulence, swarming and the production of antibiotics and enzymes. A variety of signalling molecules is used; Gram-positive bacteria use a range of extracellular molecules such as peptide pheromones eg. Amino acids and butyrolactone/butanolide metabolites (Kleerebezem *et al.*, 1997) whereas Gram-negative organisms use *N*-acylhomoserine lactone molecules (AHL_s) (Kaiser, 1996; Gray, 1997). Here, the concentration of a signal molecule reflects the number of bacterial cells, and the perception of a threshold level of that signal molecule indicates that the population is "quorate", that is, ready to make a behavioural decision. Quorum sensing thus constitutes a mechanism for multicellular behaviour in prokaryotes and is now known to regulate virulence, production of secondary metabolites, symbiosis and biofilm formation, as well as individual survival strategies such as the induction of stationary phase responses and motility for colony escape (Dunny and Winans, 1999; England *et al.*,1999; Shapero, 1998; Williams *et al.*, 2000). Cell-to-cell signalling does not, however, solely occur at high cell densities, and the term "Quorum Sensing" is now being applied to describe any bacterial intercellular communication that involves small diffusible signal molecules.

The key protein components of quorum sensing are the LuxI family of AHL synthases and the LuxR family of transcriptional activators. The LuxI proteins catalyze the formation of AHL molecules (More *et al.*, 1996; Jiang *et al.*, 1998). Small molecules termed autoinducers, are produced by the bacterial cell and accumulate in the environment at a high population density. Once an intracellular threshold level of an autoinducer is reached, the autoinducer binds to its cognate transcripional regulator protein to activate or repress target genes. This behavior was first identified in *Vibrio fischeri* as a mechanism of regulating the *lux* genes required for bioluminescence (Whitehead *et al.*, 2001). Since this discovery, Quorum sensing has been described in both Gram-positive and Gram-negative microorganisms (Whitehead *et al.*, 2001) and has been implicated in the regulation of cellular behavior and virulence (de Kievit *et al.*, 2000).

1.3.1 *Pseudomonas aeruginosa* Quorum Sensing

Pseudomonas aeruginosa possesses one of the best -studied models of quorum sensing, and two complete *lux*-like quorum sensing systems, *las* and *rhl*, have been identified (Pesci *et al.*, 1997).

The *las* system consists of the transcriptional regulatory protein LasR and its cognate signaling molecule, *N*-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL), whose production is directed by the autoinducer synthase encoded by *lasI*. The *rhl* system consists of the RhlR protein and an autoinducer synthase (RhlI), which is involved in production of the cognate autoinducer *N*-butyryl homoserine lactone (C4-HSL). These systems are intertwined in a hierarchical manner and the *las* system controls the *rhl* system at both the transcriptional and posttranslational levels (Figure 1-1). Quorum sensing has been shown to regulate the production of *Pseudomonas aeruginosa* virulence factors

(such as proteases, Exotoxin A, rhamnolipids and pyocyanin) and to be involved in biofilm formation and development, and it has been implicated in antibiotic resistance (Davis *et al.*, 1998; de Kievit *et al.*, 2000). Given these findings, it has been suggested that quorum sensing may contribute to the ability of *Pseudomonas aeruginosa* to initiate infection and to persist in a host. Data from many models of both acute infection and chronic infection have supported the hypothesis that quorum sensing is important in *Pseudomonas aeruginosa* pathogenesis (Tang *et al.*, 1996; Preston *et al.*, 1997; Rumbaugh *et al.*, 1999;de Kievit *et al.*2000).

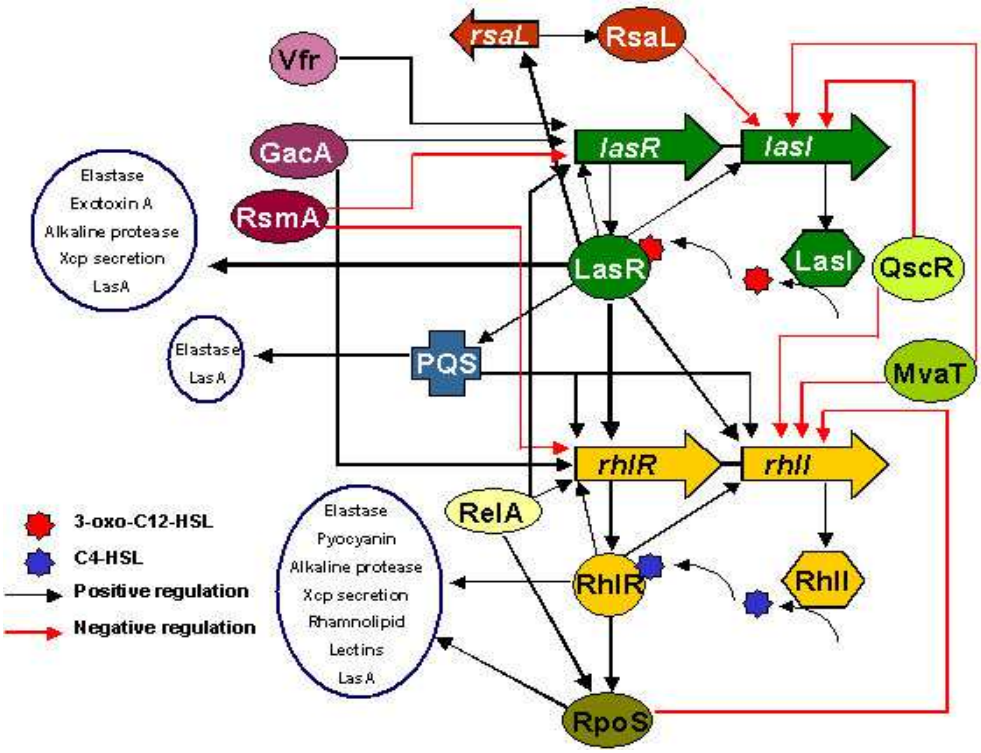


Fig (1-1) The *Pseudomonas aeruginosa* quorum sensing hierarchy (Cited from Steve Diggle *et al.*, 2004).

1.4 Lectins

Lectins are ubiquitous proteins, which, owing to their reversible carbohydrate/receptor-specific binding to macromolecules and cells, function like antibodies, hormones, and positioning sites of enzymes in controlling irreversible/key-enzyme-dependent reactions, in enabling macromolecule and cell protection, organization, transformation or lysis (in adaptation to environmental or life cycle drifts) and in supporting cell nutrition, special functions, contact and fusion with cells, proliferation or death (Gilboa-Garber, 1988; Gilboa-Garber and Garber, 1989,1992 and 1993).

Lectins exist in either soluble or cell-associated forms and possess carbohydrate-recognition domains with various specificities. Lectins are classified based on their interaction with specific carbohydrate structures. The specificity of lectins is determined by the exact shape of the binding site and the nature of amino acid residue to which the carbohydrate is linked (Sharon *et al.*, 1995).

1.4.1 Lectin classification

Lectins are classified into a small number of specificity groups-galactose, mannose, L-fucose, N-acetylgalactosamine, N-acetylglucosamine, N-acetylneuraminic acid -according to the monosaccharide which is the most effective inhibitor of the agglutination of RBC_s or precipitation of glycoprotein or polysaccharides by the lectin. Lectins whithin each group may differ markedly in their affinity for the specific monosaccharide or its derivatives, also some lectins combine more strongly with di-, tri-, and tetra-saccharides than with monosaccharides (Internet II).

Recognition of cell-surface carbohydrates by lectins has wide implications in various biological processes, including opsonization of microbes, phagocytosis, cell activation and differentiation, cell adhesion and migration, and apoptosis (Ni *et al.*,1996; Sharma *et al*., 1997).

Carbohydrate recognition by lectins often involves the side chains of tyrosine, tryptophan and histidine residues (Siebert *et al.*, 1997). A substitution of one or more amino acid residues can result in significant changes in lectin specificity.

1.4.2 Functions and importance of lectins

Lectins exhibit a wide range of functions. Animal lectins are included in many cellular processes, like tissue homing, enzyme trafficking and immune function. In plants, the biological function of lectins is not fully understood, but though to be involved in a number of intrinsic processes which include maintenance of seed dormancy, deposition of storage proteins, symbiosis, transport of carbohydrates, defense against pathogens and animal predators, mitogenic stimulation of embryonic plant cells, elongation of cell walls and recognition of pollen. Microbial lectins are largely function in host cell attachment, tissue colonization and invasion (Internet III).

Lectins play an important role in the adhesion of viruses, bacteria and protozoa to host cells, a prerequisite for infection to occur. For example, *Entamoeba histolytica* adhere to intestinal epithelial cells via galactose-inhibitable surface lectin interaction (Tannich *et al.*, 1998). Colonization of airways in respiratory tract infections is also facilitated by bacterial lectins that allow attachment to the glycoconjugates of the mucosa. Any measure to block this adhesion could be considered a significant part of antimicrobial therapy.

The role of lectins as tumor markers remains relatively unexplored, however, protein-carbohydrate interaction has gained some ground in cancer therapy where neoglycoconjugates have been designed to detect specific binding sites and evaluate potential therapeutic approaches to either block adhesion of lectins or direct drug-conjugates to cell surface (Mody *et al.*, 1995; Gabius *et al.*, 1995).

Lectins known as viscotoxins, extracted from mistletoe (*Viscum album*) are reported to exert strong immunomodulatory and apoptotic effect (Bussing *et al.*, 1999; Stein *et al.*, 1999). Galectin-3, a 31 kDa evolutionary conserved β-galactoside binding protein, has been associated with cellular transformation and metastasis in brain, thyroid and colonic mucosa (Schoeppner *et al.*, 1995; Xu *et al.*, 1995; Bresalier *et al.*, 1997).

1.5 *Pseudomonas aeruginosa* lectins

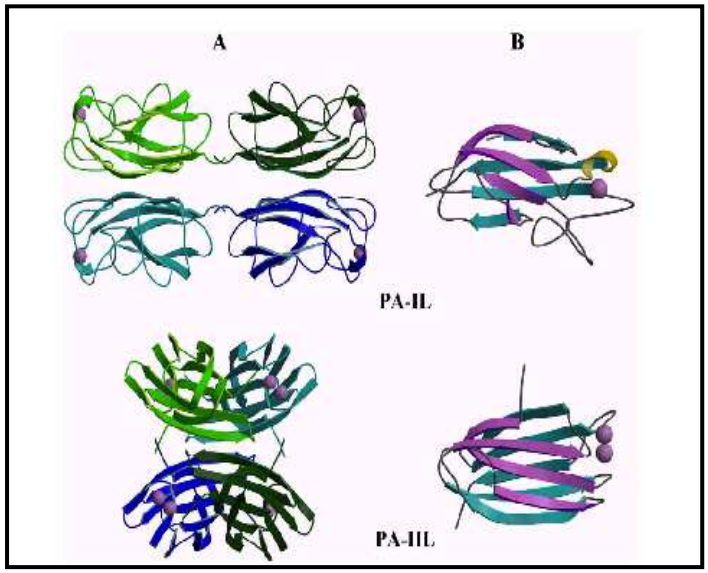
Pseudomonas aeruginosa, an important opportunistic pathogen associated with chronic airway infections, synthesizes two lectins, PA-IL and PA-IIL (Gilboa-Garber and N., 1982). These two lectins play an important role in human infections (Loris *et al.*, 2003).

These lectins appear to function as adhesins (Wentworth *et al.*,1991)as well as cytotoxins for respiratory epithelial cells (Adam *et al.*, 1997; Bajolet-Laudinat *et al.*, 1994) PA-IL and PA-IIL have been shown to interact with the ABO(H) and P blood group glycosphingolipid antigens which may contribute to the tissue infectivity and pathogenicity of *Pseudomonas aeruginosa* (Gilboa-Garber *et al.*,1994). However, in contrast to many *Pseudomonas* virulence determinants, there is little information concerning lectin expression at the molecular level. Cell density and age of the culture are known to affect lectin synthesis and the

production of PA-IL and PA-IIL lectins and that of several other virulence factors have been reported to be coregulated (Gilboa-Garber and N., 1983; Gilboa-Garber *et al.*, 1997), suggesting the existence of common regulatory mechanisms.

1.5.1 Biochemical properties of PA-IL and PA-IIL lectins

The lectin PA-IL (51 kDa), composed of four subunits of 121 amino acids, binds D-galactose plus its derivatives (Figure 2-1) and the lectin PA-IIL (47 kDa), composed of four subunits of 114 amino acids, binds D-mannose and L-fucose (N. Gilboa-Garber *et al.*, 1972 and 2000).



Fig(2-1) Stereo structures of the PA-IL/galactose and PA-IIL/fucose complexes. (A)Tetramers with stick representation of monosaccharides and space-filling representation of calcium ions. (B) Monomers with the two β -sheets represented by different colours (Cited from Anne Imberty *et al.*, 2004).

They are produced in *Pseudomonas aeruginosa* together with other secondary metabolism virulence factors under quorum-sensing control (Gilboa-Garber, 1997). Following their purification from the bacterial cell extracts, they behave as classical Ca^{++} dependent

tetrameric plant lectins, displaying haemagglutinating activity and relative resistance to heating, extreme pH and proteolysis. Therefore, the first step for their purification, preceding affinity chromotography, is heating to 65°C. The activities of both proteins are dependent upon the presence of Ca^{++} and Mg^{++} ions, with PA-IIL having the additional requirement of Zn^{++} cations. With these dependencies, EDTA, as would be expected, inactivates lectins activity (Gilboa-Garber, 1982).

1.5.2 *Psuedomonas aeruginosa* lectins genes

The PA-IL gene was isolated from *P. aeruginosa* ATCC 27853 genomic library (Avichezer *et al.,* 1992) whereas the PA-IIL gene was identified in the *P. aeruginosa* PAO1 genomic sequence from the sequence of 33 N-terminal amino acids. The genes, also referred to as *lecA* and *lecB,* are widely separated (about 867.5 kb) on the *P. aeruginosa* chromosome. Both mature proteins lack the initiator methionine and display acidic characters, but PA-IIL differs from PA-IL in lacking cysteine, methionine and histidine (Gilboa-Garber *et al.,* 2000).

1.5.3 *Pseudomonas aeruginosa* lectins and bacterial virulence

PA-IL and PA-IIL expression in the bacterium is co-regulated with certain other virulence factors (Gilboa-Garber and Garber, 1989) under the control of quorum-sensing and RpoS (Winzer *et al.*, 2000). These lectins were demonstrated to act directly in vitro and in vivo as cytotoxic compounds (Gilboa-Garber, 1997). When looking more particularly at respiratory epithelial cells, PA-IL was shown to be cytotoxic to these cells in primary cultures (Bajolet-Laudinat, 1994) whereas PA-IIL blocked their ciliary beating (Adam, 1997), which provides mechanical defence to the host airways. Another possible role that has been proposed for these lectins is a cooperative action with other virulence factors

(Gilboa-Garber, 1989). According to that proposal, the lectins would help in positioning the lytic enzymes of the bacteria into the right as well as the closest contact with the sensitive epitopes. Amongst the lectin receptors could be the glycoproteins transferrin (in serum and extracellular fluid) and lactoferrin (in mucosal secretions), which have high iron content and could be targets for the bacterial iron-scavenging proteins.

1.6 Physiological functions of *Pseudomonas aeruginosa* lectins

1. Protection

Protection of *Pseudomonas aeruginosa* cells by means of lectins may be passive or active. Passive protection includes clumping of the bacteria to masses, adherence to heterologous cells or to macromolecules, even entry to host cells for shelter. Active protection is the attacks on foreign organisms like bacteria, fungi and animals. (N. Gilboa-Garber, 1997).

2. Bacterial cell organization

The ability of multivalent lectins to bind several cells/molecules together make them very suitable for bringing together and repairing structures in the cells. Lectin-deficient strains or mutants of *Pseudomonas aeruginosa* are somewhat more sensitive than the lectin-producing wild type to freeze-drying and thawing (Gilboa-Garber *et al.*, 1994).

3. Bacterial cell nutrition

Presence of lectins in old cultures of *Pseudomonas aeruginosa* may help the bacteria to get nutrients by homing onto novel glycosylated organic materials (Gilboa-Garber, 1997).

4. Bacterial cell settlement

Bacterial lectin negative charges direct the bacterial cells to settle on damaged cells containing less sialic acid (Gilboa-Garber, 1972a,b) and bind to the glycolipids (Karlsson, 1989; Lanne *et al.*, 1994).

5. Bacterial functions in the nitrogen and carbon cycles in nature

Indirectly, *Pseudomonas aeruginosa* lectins support its saprophytic decomposition of organic materials (and their anaerobic denitrification) in both carbon and nitrogen cycles (Gilboa-Garber, 1997).

6. Adaptation to environmental and developmental drifts

Environmental conditions: composition of culture medium, temperature and aeration affect *Pseudomonas* lectin levels. When cell population of *Pseudomonas* becomes defect, lectin production is induced for the bacterium rescue (Gilboa-Garber, 1997).

7. Proliferation

Under normal conditions, lectin-less mutants or strains of *Pseudomonas aeruginosa* grow well, but their proliferation following freeze-drying damage is significantly lower (Gilboa-Garber *et al.*, 1994).

1.7 Applications of PA-IL and PA-IIL

PA-IL and PA-IIL resemble classical multivalent lectins in their application. So far, *Pseudomonas aeruginosa* lectins are the only bacterial lectins that are widely used for biotechnological, scientific and medical purposes (Gilboa-Garber, 1997). Some of these applications are:

1. Identification of free carbohydrates in solutions and body fluids

The most convenient way to exhibit the presence of sugar in solutions using lectins is by specific haemagglutination inhibition. Presence of galactose or its derivatives may be detected by PA-IL inhibition. Presence of mannose can be detected by PA-IIL and Con A. If PA-IIL is inhibited by a solution which does not inhibit Con A, the presence of L-fucose may be deduced and confirmed by *Ulex europaeus*-I lectin (UEA-I) whose affinity for free L-fucose is much lower (Garber *et al.*, 1987).

2. Examination of glycosylated macromolecules

Presence of glycosylated macromolecules in solutions may also be shown by inhibition of the lectin haemagglutinating activity. By means of the *Pseudomonas aeruginosa* lectins, it has shown that presence of high concentrations of mannosylated and fucosylated and lower level of galactosylated glycopeptides in human sera and seminal plasma (Lerrer *et al.*, 1996).

3. Separation and purification of glycosylated macromolecules

Various soluble glycosylated macromolecules including glycoproteins may be separated from others by means of *Pseudomonas aeruginosa* lectin-bearing sepharose (Gilboa-Garber, 1986).

4. Detection of specific carbohydrate presence on surfaces of various cells

Both PA-IL and PA-IIL bind to various bacteria, blood cells (including erythrocytes, leukocytes and thrombocytes) of different animals, spermatozoa (Gilboa-Garber, 1986), enterocytes (Grant *et al.*, 1995), respiratory (Bajolet-Laudinat *et al.*, 1994) and corneal epithelial cells (Wentworth *et al.*, 1991), cancer (Gilgoa-Garber *et al.*, 1986a; Avichezer and Gilboa-Garber, 1991), glial and brain capillary cells (Zambenedetti *et al.*, 1996). They may also be used for showing carbohydrates on surface of free-living unicellulars (Gilboa-Garber and Sharabi, 1980) and various algal cells.

5. Detection and activation of certain cell receptors

Both PA-IL and PA-IIL bind to lymphocyte receptors involved in mitogenic stimulation and stimulate enterocytes (Sharabi and Gilboa-Garber, 1979; Avichezer and Gilboa-Garber, 1987; Grant *et al.*, 1995).

6. Tracing and localization of cell surface carbohydrates

Labelled PA-IL and PA-IIL have been used for tracing and localization of glycosylated cell membrane components by using electron microscopy (Gilboa-Garber, 1997).

7. Typing and identification of microorganisms

Like plant lectins, *Pseudomonas aeruginosa* lectins can be used for typing microorganisms and differentiation between them according to the degree of agglutination of these cells (Garber *et al.*, 1981).

8. Human blood group typing

PA-IL may be used for differentiation between erythrocytes of type p and those bearing the P system antigens, especially P^k (Sudakevitz *et al.*, 1996), and between I positive and i erythrocytes (Sudakevitz *et al.*, 1995). PA-IIL may differentiate between the very rare H-negative (h), Bombay and H erythrocytes.

9. Tracing of cell aging and environmental or malignant cell alteration

The much stronger agglutination by PA-IL of adult human erythrocytes (treated by sialidase or papain), exhibiting I antigen, as compared to cord blood cells, is a pronounced example of this lectin efficacy in tracing cell surface developmental alterations. Similarly, both PA-IL and PA-IIL may be used (like peanut and soybean lectins) for differentiation between young and old erythrocytes. The agglutination of the latter is much stronger due to their desialylation. Examples of the ability of the *Pseudomonas* lectins to trace cell malignant transformation are numerous exhibited in stronger agglutination of various neoplastic cells as compared to their normal counterparts (Gilboa-Garber *et al.*, 1986a; Leibovici *et al.*, 1987; Avichezer and Gilboa-Garber, 1991).

10. Separation between cells

The selective cell agglutinating activity of *Pseudomonas* lectins enables their application for cell separation. Examples: younger from older RBC_s, leukocytes from erythrocytes (latter are agglutinated in both cases), viable spermatozoa from less viable ones, and transformed from normal cells (the first are agglutinated in both cases) (Lerrer *et al.*, 1995).

11. Activation or repression of specific metabolic pathways

Lectins of *Pseudomonas* activate specific pathways and function in various cells (Gilboa-Garber, 1997).

12. Mitogenic stimulation of human lymphocytes for genetic assays, stimulation of peripheral human mononuclear and various other cells for cytokine production and clinical analysis, and stimulation of peripheral human lymphocytes for cancer diagnosis (Gilboa-Garber, 1997).

13. Reduction of cancer cells tumorigenicity and viability (Avichezer and Gilboa-Garber, 1991; Avichezer and Gilboa-Garber, 1995).

14. Targeting of pharmacologically active molecules to specific cells

Targeting of drugs, enzymes, viruses or antibodies to specific organs may be done by *Pseudomonas aeruginosa* lectin selective affinities to them especially to lungs, kidneys, spleen and liver (Gilboa-Garber, 1997).

15. Vaccination against *Pseudomonas aeruginosa* infections by injection of a purified lectin preparations (Avichezer *et al.*, 1989; Sudakevitz and Gilboa-Garber, 1982).

16. Competitive direct inhibition of pathogen adhesion to host cells, of cancer cell adhesion and of cell-cell interactions (Gilboa-Garber, 1997).

2. Materials and Methods

2.1 Materials

2.1.1 Equipments and apparatus

The following equipments and apparatus were used in this study:

Apparatus	Company
Autoclave	Gallenkamp (England)
Balance	Memmert (Germany)
Centrifuge	Buchi (Switzerland)
Distillator	Mettler (Switzerland)
Hot plate/magnetic stirrer	Gallenkamp (England)
Incubator	Gallenkamp (England)
Microscope	Olympus (Japan)
Micropipettes	Gelson (France)
oven	Mettler (Switzerland)
pH meter	Mettler (Switzerland)
Sonicator	Branson (USA)
Spectrophotometer	Hitachi (Japan)
Shaker-incubator	Gallenkamp (England)
Sensitive balance	Gallenkamp (England)
UV lamp	Vilber Lourmat (France)
Vortex	Buchi (Germany)
Water bath	Memmert (Germany)

2.1.2 Chemicals

The following materials were used in this study:

Materials	Company
Agar	Difco (USA)
Bovine Serum Albumine	Sigma (USA)
Blue dextran	Sigma (USA)
Calcium chloride	BDH (England)
Crystal violet	Fluka (Germany)
Choline esterase	Sigma (USA)
Cholin Chloride	Miavit (Germany)
Di-Potassium hydrogen phosphate	Fluka (Germany)
Di-Potassium sulphate	BDH (England)
D-Glucose	BDH (England)
D-Galactose	BDH (England)
D-Mannose	BDH (England)
D-Fructose	BDH (England)
Ethanol	Reidel-De haeny (Germany)
Ferrous sulfate	BDH (England)
Gelatin	Biolife (Italy)
Glycerol	BDH (England)
Human Serum Albumine	Sigma (USA)
Hydrogen peroxide	Difco (USA)
Iodine	BDH (England)
Lysozyme	Sigma (USA)
Methylene blue	BDH (England)

Materials	Company
$MgSO_4.7H_2O$	Fluka (Germany)
$MnSO_4.4H_2O$	Fluka (Germany)
NNNN-Tetramethyl-P-phenylene diamine dihydrochloride	Difco (England)
Potassium-di-hydrogen phosphate	Sigma (USA)
Potassium nitrate	BDH (England)
Phosphate Buffer Saline (pH 7.3)	Kallestad (USA)
Peptone	BDH (England)
Phenol red	Difco (USA)
Sodium pyruvate	Merck (Germany)
Sucrose	BDH (England)
Tryptic peptone	BDH (England)
Trypsin	Fluka (Germany)
Urea	BDH (England)
Yeast extract	Biolife (Italy)

2.1.3 API 20E Kit (API Bio-Merieux)

API 20E Kit consists of:

A- The gallery:
It is a plastic strip with 20 microtubes containing dehydrated reactive Ingredients.

B- API 20E Reagents:
1. Oxidase reagent (1% tetra-methyl-P-phenylene diamine).
2. Kovac's reagent.
3. Voges-Proskauer reagent.
4. Ferric chloride 3.4%

2.1.4 Antibiotic disks

Antibiotic agents	Symbol	Concentration	Company (origion)
Gentamycin	CN	10 μg	Bioanalyse (Turkey)
Amikacin	AK	30 μg	Bioanalyse (Turkey)
Streptomycin	S	10 μg	Bioanalyse (Turkey)
Vancomycin	VA	30 μg	Bioanalyse (Turkey)
Chloramphenicol	C	30 μg	Bioanalyse (Turkey)
Carbenicillin	PY	100 μg	Bioanalyse (Turkey)
Cefotaxime	CTX	30 μg	Bioanalyse (Turkey)
Cephalexin	CL	30 μg	Bioanalyse (Turkey)
Tetracycline	TE	30 μg	Bioanalyse (Turkey)
Bacitracin	B	10 U	Bioanalyse (Turkey)

2.1.5 Culture media

2.1.5.1 Ready prepared media

The ready prepared media used in this study were:

Medium	Company
Nutrient agar	Oxoid (England)
Nutrient broth	Oxoid (England)
MacConkey agar	Oxoid (England)
Ceteremide agar base	Difco (England)

These media were prepared according to the manufacturer instructions.

2.1.5.2 Synthetic media

1. R2A-broth (Fiorina *et al*., 2000).

This medium consists of the following:

Components	Weight (gm)
Yeast extract	0.5
Tryptic peptone	0.5
Sodium pyruvate	0.3
Di potassium hydrogen phosphate	0.1
$MgSO_4.7H_2O$	0.05
Calcium chloride	0.01

These components were dissolved in 950 ml of distilled water, adjusted pH to 7.2, then the volume was completed to 1000 ml.

2. Grelet's medium (GE medium) (Gilboa-Garber, 1982)

This medium consists of the following:

Components	Weight (gm)
Potassium nitrate	10
Potassium sulphate	0.144
Potassium dihydrogen phosphate	6.8
$MgSO_4.7H_2O$	0.123
$CaCl_2.4H_2O$	0.183
$MnSO_4.4H_2O$	2.23
Ferrose sulphate	0.02
$ZnSO_4.7H_2O$	0.144
Potassium hydroxide	0.3
Yeast extract	4

The first 3 components were dissolved together with yeast extract in 950 ml of distilled water, adjusted pH to 7, then the volume was completed to 1000 ml and sterilized by autoclaving, while other components were autoclaved separately then added to the medium according to their concentration mentioned above.

3. Gelatin medium (Atlas *et al.*, 1995))

Prepared by adding 12% w/v gelatin to nutrient broth , then sterilized by autoclaving.

4. Urea agar (Christensen) for urease test (Atlas *et al.*, 1995)

Components	Weight (gm)
Phenol red	0.012
NaCl	5
KH_2PO_4	2
Peptone	1
Glucose	1
Agar	15

These components were dissolved in 850 ml of distilled water, adjusted pH to 7, sterilized by autoclaving and cooled to 50°C. Aseptically added 100 ml of 20% solution of urea (sterilized by filtration) to give a final concentration of 2% urea, then the volume was completed to 1000 ml. After that, 3 ml of this media were aseptically added to sterile small tubes and allowed to solidify in a slant position.

2.1.6 Solutions

1. Choline chloride solution (0.2%)
   This solution was prepared by dissolving0.02 g of choline chloride in 10 ml of distilled water and sterilized by autoclaving.

2. Saline solution (0.15M)
   This solution was prepared by dissolving 0.85 g of sodium chloride (NaCl) in 10 ml of distilled water and sterilized by autoclaving.

3. Phosphate buffer saline (pH 7.3)
   This solution was prepared by dissolving 11.34 g of the phosphate salt in 950 ml of distilled water, pH was adjusted to 7.3, then the volume was completed to 1000 ml and sterilized by autoclaving.

4. Bovin Serum Albumin
   This solution was freshly prepared as stock solution at a concentration of 60 μg/ml in phosphate buffer saline solution.

5. Trypsin solution
   This solution was prepared by dissolving 1gm of trypsin powder in 100ml of distilled water containing 0.1gm of L-cystine.

6. Magnesium chloride solution (0.001M)
   This solution was prepared by dissolving 10mg of $MgCl_2$ in 100ml of phosphate buffer saline solution.

2.1.7 Reagents preparation

A- Catalase test reagent

This solution was prepared by adding one volume of 30% H_2O_2 to 9 volume of sterile distilled water (Atlas *et al*., 1995).

B- Oxidase test reagent

This solution was prepared by dissolving 0.1g of N,N,N,N-Tetramethyl-p-phenylene diamine dihydrochloride in 10 ml of sterile distilled water (Baron *et al.*, 1994).

2.2 Methods

2.2.1 Sterilization

1. Autoclaving

Media and solutions were sterilized by autoclaving at 121°C (15Ib/in^2) for 15 min.

2. Oven sterilization

Glasswares were sterilized using electric oven at 180°C for 3 hr.

3. Filter sterilization

Millipore filter paper (0.45μm) used to sterilize urea that was sensitive to be sterilized by autoclaving.

2.2.2 Isolation of *Pseudomonas aeruginosa*

2.2.2.1 Sample collection

Two types of samples were collected in order to isolate *P. aeruginosa* which they were:

- **Clinical samples**

  4 blood samples from patients suffering from septicemia, 9 samples from sputum, 12 samples from burns and injuries, 2 from CSF, in addition to 2 samples from ears and 1 from cystic fibrosis infections were collected from Al-No'man hospital and Central Medicine City hospital in Baghdad governorate.

- **Environmental samples**

  Water and soil samples (2 samples from each) were collected from Al-Dorra city in Baghdad governorate also.

2.2.2.2 Sample preparation

Serological samples were cultured directly in nutrient broth containing tubes and immediately transferred to the department of biotechnology labs, then they were platted on MacConkey agar plates and incubated overnight at 37°C, while environmental samples from water and soil were diluted using sterilized distilled water and as following:

1. **Sewage water samples:**

One ml of each sample was diluted in 9ml of sterilized distilled water, mixed thoroughly, serial dilutions for each sample were done separately, then 100µl aliquots from the appropriate dilution were taken out and spreaded on MacConkey agar plates and incubated over night at 37°C .

2. **Soil samples:**

One gram of each soil sample was added to 9ml of sterilized distilled water, mixed thoroughly, serial dilution for each sample were done separately, then 100µl aliquets from the appropriate dilution were taken and spreaded on MacConkey agar plates and incubated at 37°C for 16 hours.

After incubation, non fermentive colonies which appear as pale color on MacConkey agar plates were selected and streaked on ceteremide agar plates (as a selective medium for *Pseudomonas* spp.) at 37°C for 16 hour.

2.2.3 Identification of *P. aeruginosa* isolates

Different isolates from clinical and environmental samples were identified according to their morphological, physiological and biochemical properties and as follows:

2.2.3.1 Morphological characteristics

As an early step of identification, different local isolates were examined according to their ability to staining, shape, color, size, production of pigments, transperancy and mucoidal properties after plating on nutrient agar and it was explained by Palleroni (1985).

2.2.3.2 Physiological and Biochemical tests

In order to identify the locally isolated bacteria, all the isolates were subjected to further identification using some biochemical tests and as it was mentioned by Bradbury (1986):

1. Catalase test

This test was performed by adding few drops of 3% hydrogen peroxide solution to colonies grown previously on nutrient agar plates separately. The production of gas bubbles indicates a positive result (Atlas *et al.*, 1995).

2. Oxidase test

Whattman filter paper was moistened with a few drops of tetramethyl-p-phenylenediamine dihydrochloride solution, then a loopful of each isolate grown previously on nutrient agar plate was smeared separately on the moistened filter paper using a sterile wooden stick. The development of a violet or purple color within 10 seconds indicates a positive result (Atlas *et al.*, 1995).

3. Fluorescein pigment production test

Bacterial isolates grown on ceteremide agar plates were examined under the source of UV light. Yellow-green fluorescent pigment indicates the presence of pyoverdin and then a positive result (Todar, 2004;Internet 6).

4. Gelatinase test

A gelatin medium-containing tube was inoculated with the bacterial isolate and incubated at 37°C for 24 hour, after that the tubes were placed in a refrigerator for 30 minutes. Liquefaction of the media indicates a positive result (Atlas *et al*., 1995).

5. Urease test

The surface of the Christensin urea agar slants were inoculated with the bacterial isolate and incubates at 37°C for 24 hour. After incubation, the appearance of red-violet color indicates a positive test, while the appearance of a yellow-orange color indicates a negative test (Atlas *et al.*, 1995).

6. Growth at 4°C

Bacterial isolates were grown at 4°C, by inoculating 50 ml of nutrient broth with 50 µl of freshly prepared cultures of these isolates separately. Appearance of growth (O.D=600nm) indicates a positive result (Palleroni , 1985).

7. Growth at 41°C

Bacterial isolates were grown at 41°C, by inoculating 50 ml of nutrient broth with 50 µl of freshly prepared cultures of these isolates

separately. Appearance of growth (O.D=600nm) indicates a positive result (Palleroni , 1985).

2.2.4 Characterization of bacterial isolates using API 20 E system

Local isolates that have the same features and characteristics of *P. aeruginosa* on nutrient agar plates, subsequently identified using biochemical tests were further characterized using API 20 E system as a standardized characterization system for Enterobacteriaceae and other non-fastidious Gram-negative rods. The system consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents.

Biochemical tests included in this system are:

1. ONPG: Beta-galactosidase test.
2. ADH: Arginine dihydrolase test.
3. LDE: Lysine decarboxylase test.
4. ODC: Ornithine decarboxylase test.
5. CIT: Citrate utilization test.
6. H_2S: Hydrogen sulphide test.
7. URE: Urease test.
8. TDA: Tryptophane deaminase test.
9. IND: Indole test.
10. VP: Voges proskauer test.
11. GEL: Gelatin liquifaction test.

12. GLU: Glucose fermentation test.
13. MAN: Manitol fermentation test.
14. INO: Inositol fermentation test.
15. SOR: Sorbitol fermentation test.
16. RHA: Rhamnose fermentation test.
17. SAC: Sucrose fermentation test.
18. MEL: Melibiose fermentation test.
19. AMY: Amygdalin fermentation test.
20. ARA: Arabinose fermentation test.

2.2.4.1 Preparation of the strip

Five ml of distilled water dispensed into wells of the strip in order to provide a humid atmosphere during incubation.

2.2.4.2 Preparation of the inoculum

A single, pure isolated colony was picked up from the bacterial growth. This colony was suspended in a test tube containing 5 ml of distilled water and mixed thoroughly.

2.2.4.3 Inoculation of the strip

According to the manufacture instructions, both the tube and cupule section of CIT, VP and GEL tests were filled with the bacterial suspension. Other tests, only the tubes were filled. The tests ADH, LCD, ODC, URE and H_2S were overlaid with mineral oil to create anaerobic conditions. After inoculation, the plastic lid was placed on the tray and incubated at 37°C for 24 hr.

2.2.4.4 Reading the strip

After incubation, the following reagents were added to the corresponding microtubes:

1. One drop of VP reagent to VP microtube and wait for 10 min then the result was recorded immediately.
2. One drop of 3.4% ferric chloride to TDA microtube then the result was recorded immediately.
3. One drop of Kovac's reagent to the IND microtube.

The results were recorded and compared to that of identification table, identification of isolates was performed using analytical profile index.

2.2.5 Maintenance of isolates

Maintenance of *Pseudomonas aeruginosa* isolates were performed according to Maniatis *et al.* (1982):

1. Short -Term Storage

Bacterial isolates were maintained for periods of 2-3 weeks on the nutrient agar plates, wrapped in parafilm and stored in refrigerator at 4°C.

2. Medium-Term Storage

Bacterial isolates were maintained for periods of 2-3 months on nutrient agar slants in a small screw-capped tubes and stored in refrigerator at 4°C.

3. Long-Term Storage

Bacterial isolates were stored for long periods (12-18 months) in test tubes containing nutrient broth and glycerol (15%) and stored in freezer at -20°C.

2.2.6 Detection of the ability of locally isolated *P. aeruginosa* in lectin production

In order to identify the ability of locally isolated *P. aeruginosa* for lectin production as a virulence factor, haemagglutination test was achieved for each isolate and as follows:

2.2.6.1 Haemagglutination ability and lectin profiles

This test was done according to the procedure mentioned by Fiorina *et al*., (2000). Single colony of each locally isolated *P. aeruginosa* was used to inoculate R2A broth-containing flasks separately, then flasks were incubated in shaker incubator at 150 rpm for 24 hour at 30°C. After incubation, cells were collected by centrifugation at 6000 rpm under aseptic conditions, washed twice by phosphate buffer saline (pH 7.3), then they were resuspended in the same buffer solution containing 1mM magnesium chloride to obtain an absorbance at 600 nm of 2, corresponding to 2×10^{10} bacteria/ml.

The haemagglutination capacity of each isolate was tested with human blood group A, B, O and sheep RBC_s according to Glick *et al*., (1987). All tests were performed in duplicate in U microtiter plates. After centrifugation of blood samples, RBC_s suspension was prepared by washing all blood groups with 0.15M NaCl solution and suspended to a concentration 5% in PBS (pH 7.3). In order to obtain RBC_s suspension treated with trypsin, 9 volumes of 5% RBC_s suspension were incubated with 1 volume of 1%

trypsin solution at 37°C for 30 minutes, then RBC_s were washed 3 times in 0.15M NaCl solution and suspended in PBS (pH 7.3) at a concentration of 5%.

Haemagglutination test was performed as a two fold serial dilutions in 0.2ml of PBS solution containing 0.001M $MgCl_2$ up to 1/128, then a 50µl of RBC suspension treated with trypsin was added and incubated at 25°C for 60 minutes. Results were recorded as a titration which represents the greatest dilutions causing haemagglutination.

2.2.6.2 Haemagglutination inhibition test

Bacterial suspension (0.2 ml) was incubated with 0.2 ml of 50mM of sterilized sugar solutions (D-galactose, D-mannose, D-glucose, D-fructose and sucrose) for 30min at room temperature. Haemagglutination inhibition test was performed with human blood group A, B, O and sheep blood RBC_s and as described above.

2.2.7 Extraction and purification of *Pseudomonas aeruginosa* lectins

Extraction and purification of the two types of lectins (PA-IL and PA-IIL) produced from the wild type of *Pseudomonas aeruginosa* was performed according to the procedure mentioned by Gilboa-Garber (1982) and as follows:

2.2.7.1 Extraction of *Pseudomonas aeruginosa* lectins

Grelet's medium (GE medium) was used for high and dominant PA-IL production, while nutrient broth was used for high and dominant PA-IIL production

Both GE medium and nutrient broth (200 ml for each) were inoculated separately with 2ml of the freshly prepared inoculum of *P.aeruginosa* and incubated at 30°C with shaking at 200 rpm for 72 hr. Choline chloride (0.2%) was added daily to the growth medium, then cells were harvested by centrifugation at 6000 rpm, washed 3 times in saline solution (0.15M), then 10 ml of cell suspension was added to 40 ml of saline (0.15M). Cells were disrupted by sonication for 10 minutes at 0°C using 20 KH_z (Soniprep 150). Sonication was performed for 30 second sonication with stop intervals of 1 minute. A lectin-containing crude preparation (supernatant) obtained after centrifugation at 18000 rpm for 20 min was kept at -20°C.

2.2.7.2 ammonium sulfate precipitation

Heat-labile foreign proteins were removed by heating at 70°C for 15 min. Both lectins (PA-IL and PA-IIL) were precipitated separately by 60% saturation of ammonium sulfate at 4°C then stirring for 30 min. Lectins were precipitated by centrifugation at 1800 rpm for 15 min. The precipitate were dissolved in PBS (pH 7.3) to 1/5 of the original volume. Dialized against PBS (pH 7.3) with three increments of substitutions at 4°C had been performed until sulfates were removed. Lectins solutions then filtered using filter paper and kept at -20°C.

2.2.7.3 Purification by gel filtration technique

Lectin solution obtained after ammonium sulfate precipitation step was purified by gel filtration chromatography technique using sepharose-200 CL-6B which was prepared according to the instructions of manufacturer (sigma). Gel matrix was poured into the column to give a dimension of (2.8, 15 cm) of the column size, the column was equilibrated and washed for 24 hours using phosphate buffer saline solution (pH 7.3), the flow rate was 1 ml/min, then 1.8 ml of lectin solution (PA-IL) was added to the column accurately and eluted with phosphate buffer saline (pH 7.3) in a flow rate of 1 ml/min, proteins in eluted fractions were detected using UV-visible spectrophotometer at a wave length of 280 nm. After the complete elution, the column was rewashed by phosphate saline solution for 24 hours and then the PA-IIL lectin solution was added to the column in the same manner. results were used for plotting the relationship between optical density and fraction number.

2.2.8 Haemagglutination test for purified lectins (Gilboa-Garber, 1982)

2.2.8.1 Preparation of RBC suspension

RBC suspension was prepared by washing all human blood groups A, B, O and sheep RBC_s with 0.15M NaCl solution and suspended to a concentration of 5% in PBS (pH 7.3).

2.2.8.2 Haemagglutination test

Twofold dilutions were used for purified lectins in PBS solution containing BSA (at a concentration of 60µg/ml). 50µl of RBC suspension

aliquets were added to 200µl of purified lectin solution and incubated at 25°C for 1 hour. The haemagglutination titer was the greatest dilution causing haemagglutination.

2.2.8.3 Haemagglutination inhibition test

Haemagglutination inhibition test was performed using two types of sugars (galactose and mannose) with a final concentration of 50mmol/l to make sure that weather the resulted purified protein (lectin) was the desired one by determining its specificity to a particular type(s) of sugar(s).

The purified lectin (0.2 ml) was incubated with 0.2ml of both types of sugar solutions for 30 minutes at room temperature and tested for haemagglutination as described above.

2.2.9 Growth curve measurement

The locally isolated *P.aeruginosa* S4 which gives the greatest dilution causing haemagglutination for each human blood group (A, B, and O) and sheep RBC_s, was selected and used in the next experiments of antibiotic sensitivity and mutagenesis by the physical and chemical mutagens. For this purpose, growth curve for *P.aeruginosa* S4 was performed to determine the mid exponential phase.

The growth curve for *P.aeruginosa* S4 was performed by growing this isolate in nutrient broth at 37°C for 24 hour with shaking (180 rpm). O.D was measured with intervals of 1 hr during the period of incubation. Then the relationship between optical density and time was plotted.

2.2.10 Antibiotic sensitivity (Baron and Finegold, 1994)

To determine the antibiotic sensitivity for the selected isolate, 10 ml of nutrient broth was inoculated with 100µl of *P. aeruginosa* S4 and incubated at 37°C to mid log phase. 100 µl of the inoculum was streaked on nutrient agar plates, then placed for 10 minutes at room temperature in order to allow the absorption of excess moisture. The selected antibiotic disks were placed on the inoculated plates using sterile forceps and incubated over night at 37°C in an inverted position.

After incubation, the zone of inhibition of growth were measured using mm units according to the national committee for laboratory standards (NCCLS, 1986).

2.2.11 Mutagenesis of *P. aeruginosa* S4

In order to improve the ability of the locally isolated *P. aeruginosa* S4 in lectin production, two types of mutagenesis were used for this purpose, physical mutagenesis by UV light and chemical mutagenesis by mitomycin C and as follows:

2.2.11.1 Physical mutagenesis

Physical mutagenesis of locally isolated *P. aeruginosa* S4 was performed by the direct subjection of cell suspension to the effect of UV light at 254 nm according to Carrasco and Soto (1987).

Single colony of *P. aeruginosa* S4 was firstly used to inoculate 25 ml of nutrient broth containing flasks and incubate in shaker incubator (150 rpm) at 37°C till mid-exponential phase, 5 ml of cell suspension was poured aseptically onto a 100 mm petridish to covers all over the surface of its area,

then it was subjected to the effect of UV ray emitted from electric UV lamp (Vilber Lourmat) for 100 seconds. A 100 µl aliquets of irradiated cell suspension were plated on nutrient agar within 10 seconds intervals, incubated in dark place at 37°C for 24 hours, then cells viable count was calculated to determine the bacterial survival curve.

2.2.11.2 Chemical mutagenesis

Chemical mutagenesis of locally isolated *P.aeruginosa* S4 was performed by incubation of growth culture of this isolate with the chemical mutagen (Mitomycin C) according to the procedure described by Ramirez *et al*. (1988).

Single colony of *P. aeruginosa* S4 was first used to inoculate 25 ml of nutrient broth containing flasks and incubated in shaker incubator (150 rpm) at 37°C till mid-exponential phase. 1 ml of Mitomycin C was added to 5 ml cell suspension (the final concentration was 200µg/ml) then incubated at 37°C for four hours with shaking.100µl aliquets was taken every 20 minutes of incubation with the mutagen and plated on nutrient agar. Cells viable count was calculated to determine the bacterial survival curve.

2.2.12 Selection of lectin over producer mutants

After subjection of *P.aeruginosa* S4 to both physical and chemical mutagens, colonies that found within 0-10% survivals were picked-up and used to inoculate 25 ml nutrient broth containing flasks, and incubated in shaker incubator (150 rpm) for 18 hour at 37°C, then 100µl aliquets were taken separately to achieve haemagglutination test for screening lectin over producer mutants.

3. Results and Discussion

3.1 Isolation of *Pseudomonas aeruginosa*

In order to isolate *P. aeruginosa*, thirty four samples were collected from different clinical cases and environments, from different hospitals and environments in Baghdad governorate during the period from October 2004 to December 2004.

Table (3-1): Local isolates from different clinical and environmental samples

Source	No. of samples	No. of isolates	Growth on Ceteremide	*P. aeruginosa*
Sputum	9	1	1	1
Cystic Fibrosis	1	1	1	1
Ear	2	1	1	0
Blood	4	1	1	1
Cerebrospinal Fluid	2	2	2	0
Burns	12	10	6	3
Sewage water	2	1	0	0
Soil	2	1	0	0
Total	**34**	**18**	**12**	**6**

Results in table (3-1) showed that there were 16 isolates were obtained from cystic fibrosis patients, sputum, blood, burns and ears, while there were only 2 isolates obtained from different water samples.

Among the total isolates, only 12 isolates were able to grow on Ceteremide agar plates , which might gives an indicator that these isolates were belonged to *Pseudomonas* spp and all of them were pathogenic. These 12 isolates were further characterized and identified according to the cultural, morphological and biochemical tests.

From the other results mentioned in table (3-1) we can see that there were other 6 isolates from clinical and environmental samples may belong to other pathogenic or nonpathogenic bacteria from different genera.

3.2 Identification of bacterial isolates

Local isolates that were able to grow on Ceteremide agar plates, which may be suspected to be *P. aeruginosa* were further identified according to morphological characteristics and biochemical tests. For the former, colonies of each isolate that were plated on nutrient agar show different morphological characteristics of *P. aeruginosa* such as mucoidal growth, smooth in shape with flat edges and elevated center, whitish or creamy in color, has fruity odor, all of them were pyoverdin producers.

Microscopical examination of each isolate showed that they were all having single cells, non-spore forming, Gram negative and rod shape.

3.2.1 Biochemical and physiological characteristics

Some biochemical tests were done to ensure that these 12 isolates were *P. aeruginosa*. Results in table (3-2) showed that all of these isolates

gives a positive results for catalase, oxidase, pyoverdin production, gelatinase and urease. On the other hand, results in table (3-2) showed that these isolates differ in growth at 41 and 4°C, six of them (S3, S5, S6, S8, S9 and S11) were unable to grow at 41°C but they were able to grow at 4°C, while the other six isolates (S1, S2, S4, S7, S10 and S12) were able to grow at 41°C but they cannot grow at 4°C. From these results we can conclude that the last six isolates were *P. aeruginosa*, so they were selected and further characterized using API 20 E system.

Table (3-2) Biochemical and physiological characteristics of the locally isolated *P. aeruginosa*

Isolate / Test	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Pyoverdin	+	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 4°C	-	-	+	-	+	+	-	+	+	-	+	-
Growth at 41°C	+	+	-	+	-	-	+	-	-	+	-	+

From these results we can observe that the local isolates S1, S2, S4, S7, S10 and S12 are *P. aeruginosa.* Further more, these results were agreed with those observed by Palleroni, (1985); Hawkey and Lewis, (1989).

Further identification of the isolates was performed using API 20 E system and as shown in figure (3-1):

Fig (3-1) API 20 E system for *P. aeruginosa*

These isolates were able to utilize arginine, citrate, urea, gelatin and glucose. While they gave negative results for β-galactosidase, lysine decarboxylase, ornithine decarboxylase, H_2S, tryptophane deaminase, indole, VP, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. These results were also in agreement with those mentioned by Mandelle *et al*., (1995) and Collee *et al*., (1996).

3.3 Screening of the locally isolated *P.aeruginosa* for their ability in lectin production

Lectins are protein adhesions that bind specifically and reversibly to carbohydrates (Mirelman and Ofek,1986). The affinity of bacterial lectins for cell surfaces or in exocellular polymers is of biological significance in *P. aeruginosa* infections (Ramphal *et al.* 1991; Gilboa-Garber *et al.* 1994; Plotkowski *et al*. 1996). Lectins also play a role in the colonization of prosthetic devices by *P. aeruginosa* since blocking the lectin with carbohydrates significantly decreases the adherence of the bacteria to the coated surfaces (Jansen *et al*. 1990). Because of their wide range of surface

carbohydrates the haemagglutination of human or animal erythrocytes is widely used to screen for lectins on bacteria, whereas inhibition of haemagglutination by carbohydrates indicates the specificity of lectins (Goldhar 1995).

3.3.1 Haemagglutination ability test

All the locally isolated *P. aeruginosa* has shown a haemagglutination activity but in different degrees as it was shown in table (3-3). The highest haemagglutination titer was scored for the isolate S4 which gave the highest haemagglutination titer against different blood groups. These isolates also had a higher affinity for human blood groups than for sheep RBC_s. From the other results indicated in table (3-3) we can conclude that all these clinical isolates of *P. aeruginosa* have greater affinity for human RBC_s than for sheep RBC_s, this may be resulted from their adaptation to human ecosystem. These results were in accordance to that obtained by Fiorina *et al.*, (2000).

Table (3-3) Haemagglutination activity for the locally isolated *P. aeruginosa* against human blood groups A, B, O and sheep RBC_s

Strains	Maximum H.A titer			
	A	B	O	Sheep
S1	32	32	32	16
S2	32	32	16	16
S4	128	64	64	32
S7	16	16	8	8
S10	8	8	4	2
S12	8	4	8	2

3.3.2 Haemagglutination inhibition test

Haemagglutination inhibition test was performed for the S4 isolate that gave the highest haemagglutination titer using each of mono and disaccharides (glucose, fructose, galactose, mannose and sucrose). Results of haemagglutination inhibition test mentioned in table (3-4) showed that the greatest inhibition of haemagglutination was scored by D-mannose, while other sugars showed no detectable inhibition except fructose which shows little inhibition of haemagglutination at the titers 32, 64 and 128. The ability of mannose in inhibition of haemagglutination of RBC_s was due to its ability to bind the active sites on the surface of RBC_s and prevent the attachment of lectins to those active sites and then inhibits haemagglutination of these RBC_s (Beely, 1985).

Table (3-4) Haemagglutination inhibition activity of locally isolated *P. aeruginosa* S4 against human blood groups A, B, O and sheep RBC_s using mono and disaccharides (glucose, fructose, galactose, sucrose and mannose).

Blood group	Sugar	Haemagglutination titer							
		1	2	4	8	16	32	64	128
A	Glucose	+++	+++	+++	+++	+++	+++	+++	++
	Fructose	+++	+++	+++	+++	+++	++	++	+
	Galactose	+++	+++	+++	+++	+++	+++	+++	++
	Sucrose	+++	+++	+++	+++	+++	+++	+++	++
	Mannose	+	+	-	-	-	-	-	-
B	Glucose	+++	+++	+++	+++	+++	+++	++	++
	Fructose	+++	+++	+++	+++	++	++	++	+
	Galactose	+++	+++	+++	+++	+++	+++	++	++
	Sucrose	+++	+++	+++	+++	+++	+++	++	++
	Mannose	+	-	-	-	-	-	-	-
O	Glucose	+++	+++	+++	+++	+++	++	++	++
	Fructose	+++	+++	+++	+++	+++	++	++	+
	Galactose	+++	+++	+++	+++	+++	+++	+++	++
	Sucrose	+++	+++	+++	+++	+++	+++	+++	++
	Mannose	+	-	-	-	-	-	-	-
Sheep	Glucose	+++	+++	+++	+++	+++	+++	+++	++
	Fructose	+++	+++	+++	+++	++	++	++	+
	Galactose	+++	+++	+++	+++	+++	++	++	++
	Sucrose	+++	+++	+++	+++	+++	++	++	++
	Mannose	+	-	-	-	-	-	-	-

3.4 Extraction and Purification of *P. aeruginosa* S4 lectins

It was well known that *P. aeruginosa* can produce two types of lectins which they are PA-IL and PA-IIL (Gilboa-Garber, 1982), so the ability of the locally isolated *P. aeruginosa* S4 in production of PA-IL and PA-IIL was detected by the extraction and purification of both types of lectins and as the followings:

3.4.1 Extraction of *P. aeruginosa* S4 lectins

For the extraction of PA-IL and PA-IIL from the locally isolated *P. aeruginosa* S4, this isolate was grown in GE medium for 72 hours of incubation with shaking (180 rpm) at 30°C, this medium was selected as a production medium for PA-IL because of the high and dominant activity of PA-IL that was attained in the extracts of *P. aeruginosa* grown in salts and yeast extract-containing medium (Gilboa-Garber, 1982). Whereas nutrient broth was used for culturing *P. aeruginosa* S4 as a production medium for PA-IIL. If any change occurs in composition of these culture media, it will lead to a profound decrease in the lectin activities. Addition of choline to the medium increases the levels of both lectins (Gilboa-Garber, 1982). Cell density and age of culture are important factors in lectin production. Highest levels of PA-IL and PA-IIL were obtained at the stationary phase, linked with some virulence secondary metabolites, including proteolytic activities (e.g. those of elastase and alkaline protease), hemolysin, pyocyanin (Gilboa-Garber, 1983), cyanide and chitinase activity (Camera *et al*., 1995).

3.4.2 Purification of *P. aeruginosa* S4 lectins

Crude lectins Produced in the previous step were purified by gel filtration chromatography technique using Sepharose-200 Cl-6B, by applying the crude extracts of both PA-IL and PA-IIL to the column separately, and eluted using 0.1M phosphate buffer. Eluted fractions was detected spectrophotometrically at 280 nm. Results in figure (3-2 A) showed that there were four peaks appeared throughout the elution process of PA-IL crude extract, which may represent four types of different proteins. Haemagglutination inhibition test was performed for these 4 sample proteins to determine its specificity to make sure of the type of the selected protein (PA-IL) which gives an inhibition of haemagglutination with galactose. The second peak (tube no. 18) gives an inhibition of haemagglutination with galactose.

In figure (3-2 B), 3 peaks appears which gave the highest absorbancy among the 50 fractions collected. Haemagglutination inhibition also was performed for these 3 sample proteins, PA-IIL gave an inhibition of haemagglutination with mannose. The second peak (tube no. 19) gave an inhibition of haemagglutination with mannose. Molecular weight for both lectins was determined according to the plot obtained by elution of HSA, lysozyme and choline esterase and as shown in figure (3-3) . M.W for PA-IL has been determined which was ≈ 57500 Da, while for PA-IIL it was ≈ 42700 Da. Figure (3-4). These results were very close to that recorded by Gilboa-Garber, (1982, 1986 and 1997); Cioci, (2003); Loris, (2003); Imberty, (2004).

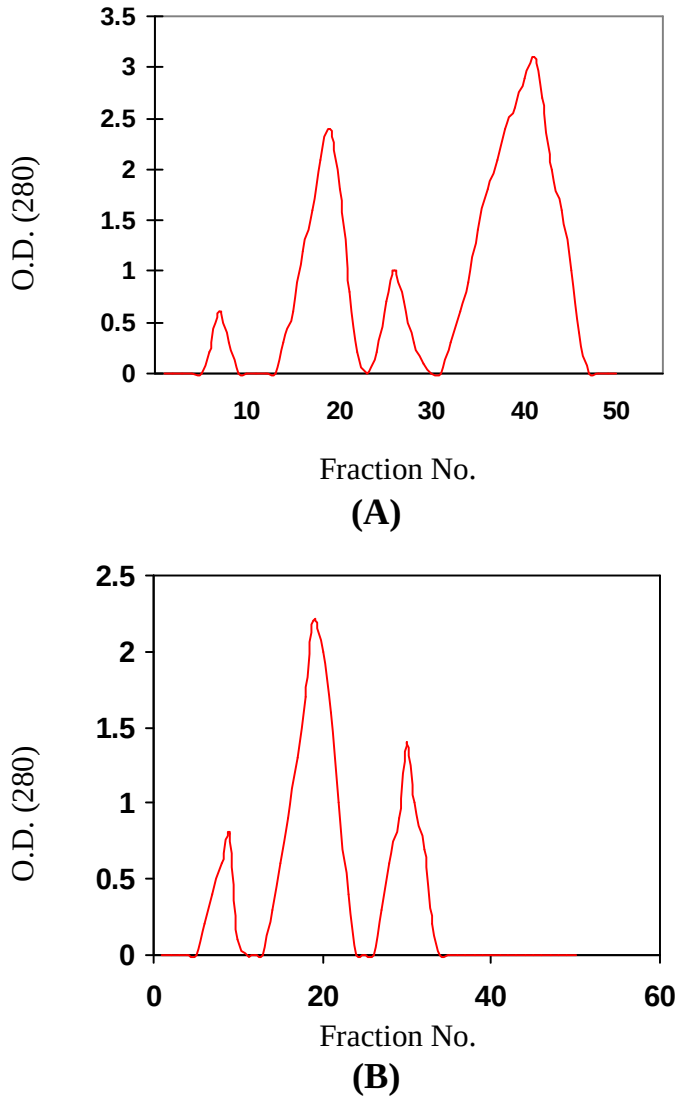
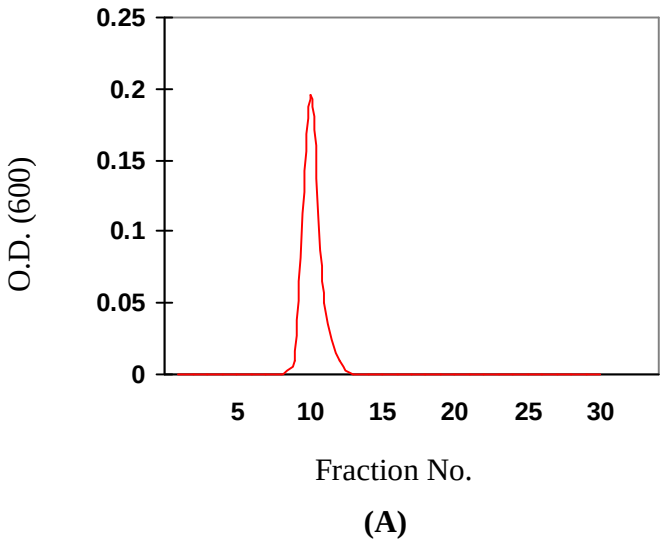
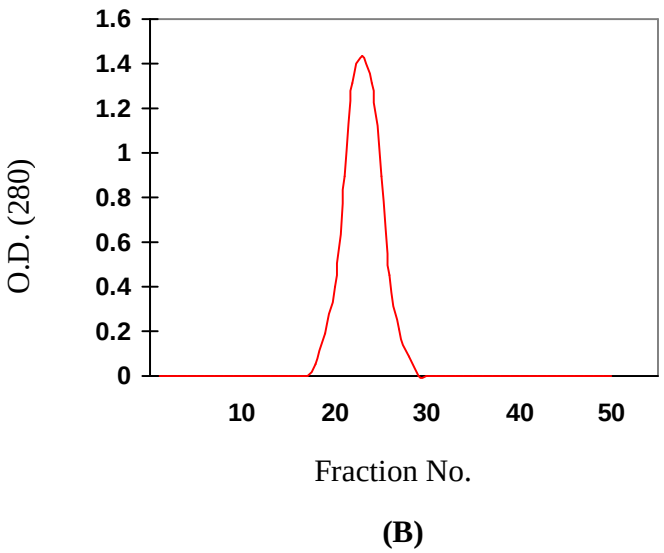


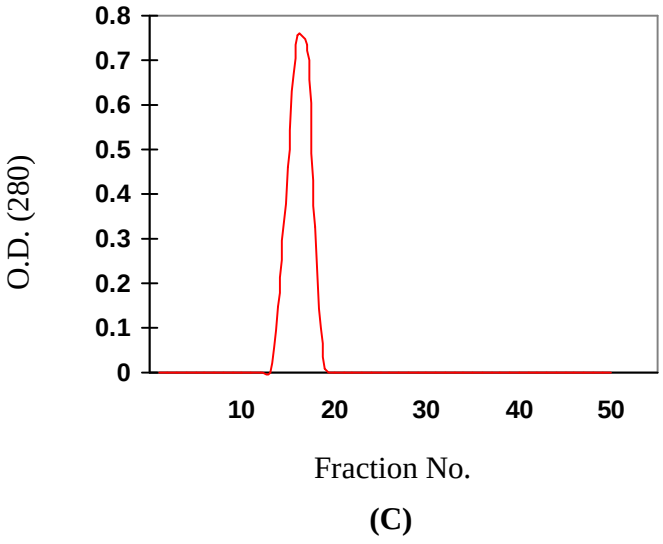
Fig (3-2) Gel filtration of (A) PA-IL and (B) PA-IIL, through sepharose-200 CL-6B. flow rate (1 ml/min.) , fraction volume (3 ml).



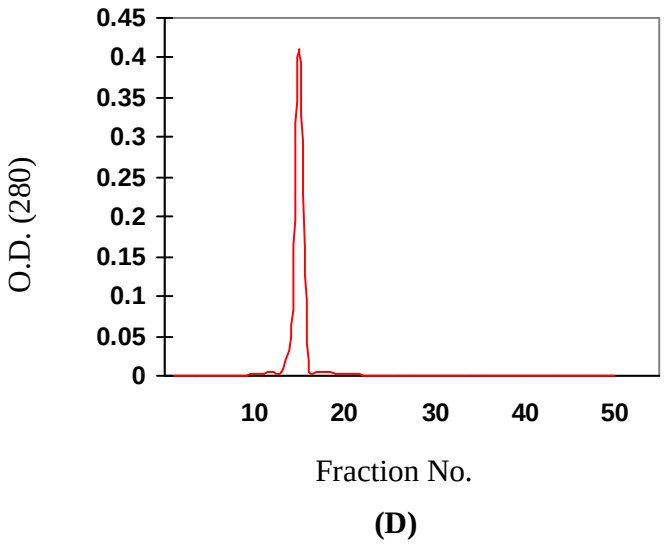
(A)



(B)



(C)



(D)

Fig.(3-3) Gel filtration of (A) blue dextran , (B) lysozyme , (C) HSA and (D) choline esterase through sepharose-200 CL-6B. Flow rate (1ml/min.), fraction volume (3 ml)

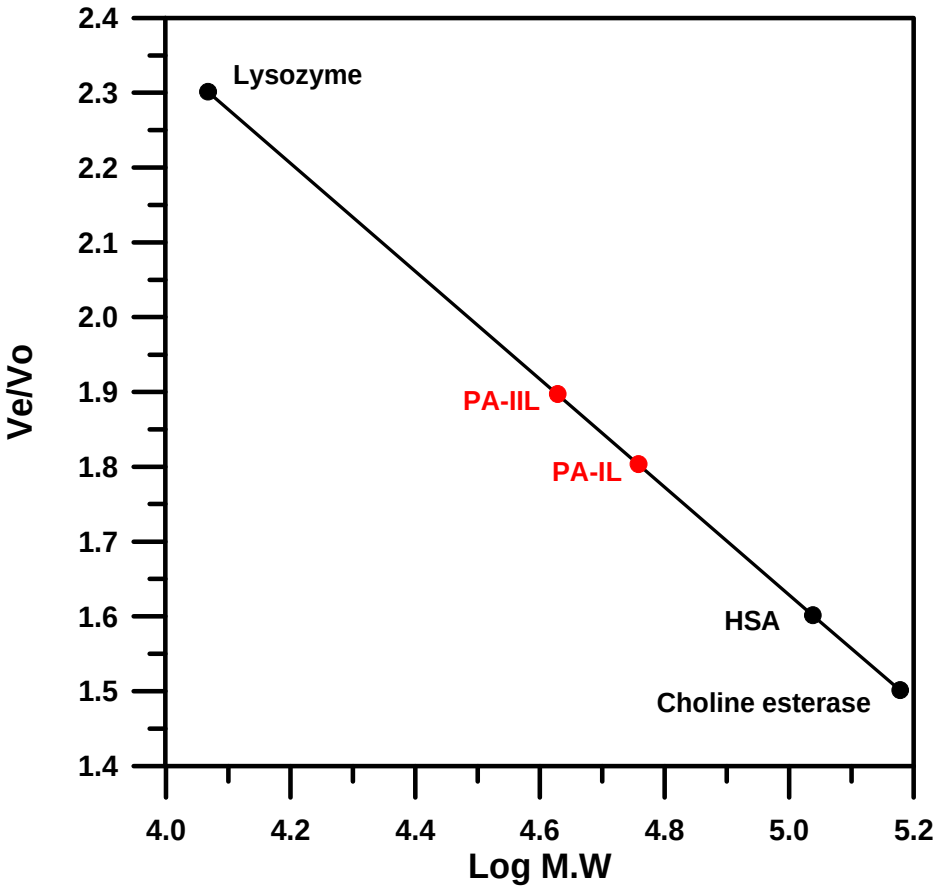


Fig. (3-4) selectivity curve for PA-IL , PA-IIL and standard proteins

3.5 Growth curve of *P. aeruginosa* S4

Growth curve of *P. aeruginosa* S4 was plotted to determine the mid-log phase. From the figure (3-5) we can see that the mid-log phase for *P. aeruginosa* S4 is at 10 hour period of growth. This result was exploited in mutagenesis experiment

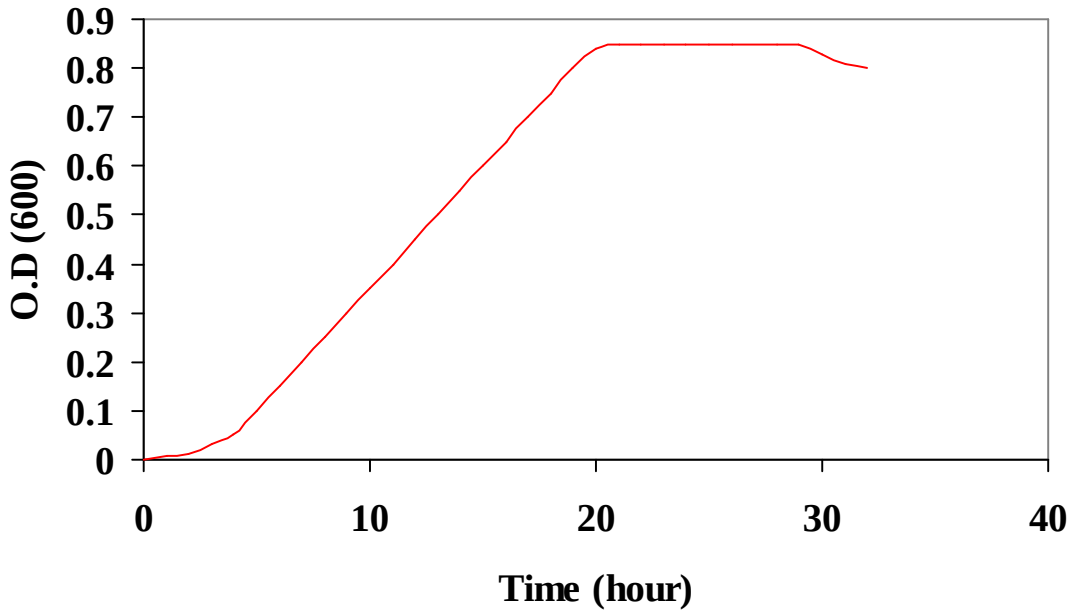


Fig (3-5) Growth curve for *P. aeruginosa* S4

3.6 Antibiotic sensitivity test

The development of antibiotic resistance is considered a major therapeutic problem that can be explained by some hypothesis such as, the influence of excessive and/or inappropriate antibiotic use (Sotto *et al.*, 2001).

Standard disk diffusion test has been performed for detection of susceptibility of pathogenic bacteria for antibiotics.

P. aeruginosa S4 has been tested using 10 different antibiotic disks. Decision for considering an isolate resistant or sensitive was taken in

comparison of the diameter of inhibition zone with that of standard value of $NCCL_s$ (1986).

As shown in table (3-5), *P. aeruginosa* S4 was resistant to vancomycin, chloramphenicol, carbenicillin, cefotaxime, cephalexin, tetracycline and bacitracin, but was sensitive to gentamicin, amikacin and streptomycin. The greater sensitivity observed with amikacin which gives the largest zone of inhibition compared with other antibiotics. From these results we can conclude that *P. aeruginosa* S4 is probably has the ability to produce more than one enzyme among them β-lactamases.

Table (3-5) Antibiotic sensitivity test for *P. aeruginosa* S4

Antibiotic	Symbol	Sensitivity
Gentamycin	**CN**	**S**
Amikacin	**AK**	**S**
Streptomycin	**S**	**S**
Vancomycin	**VA**	**R**
Chloramphenicol	**C**	**R**
Carbenicillin	**PY**	**R**
Cefotaxime	**CTX**	**R**
Cephalexin	**CL**	**R**
Tetracycline	**TE**	**R**
Bacitracin	**B**	**R**

3.7 Mutagenesis of *P. aeruginosa* S4

In order to study the effect of mutagenesis on *P. aeruginosa* S4 ability in lectins production, two types of mutagens were used for this purpose, which they were the physical mutagen (by the direct subjection of the isolate to the effect of UV light at 254nm), and the chemical mutagen (by Mitomycin C). Haemagglutination activity was measured after the exposure to both mutagens separately.

For physical mutagenesis, *P. aeruginosa* S4 was exposed to UV light (254nm) within 100 seconds Through this period, 100µl aliquets were taken every 10 seconds and spreaded (after serial dilutions) on nutrient agar plates and incubated at 37°C for 24 hours in dark place, then the viable count for each period was taken to determine the survival curve. Results indicated in figure(3-6) showed that over 70% and 95% of the viable cells were killed after 10 and 20 seconds respectively. After that the killing percentage was ranged between ≈ 96% and ≈ 99% after 40 to 60 seconds respectively. The survival colonies were selected randomly to determine its ability in lectin production.

While for chemical mutagenesis, *P. aeruginosa* was exposed to Mitomycin C with a final concentration of 200 µg/ml and incubated at 37°C for four hours with shaking. 100µl aliquets was taken every 20 minutes of incubation with mutagen, then plated on nutrient agar. Results were scored which represents the total viable count and used to determine the bacterial survival curve and as shown by figure (3-7).

From table (3-6) we can see that that haemagglutination ability were decreased for each of the isolates (S42, S45, S47, S411, S413, S415, S422, S423, S428, S430, S431, S432, S439, S441, S445, S447, S449, S450 and S451) and this was resulted from decreasing in lectin production.

Also from table (3-7) we can see that haemagglutination ability were decreased for each of the isolates (S458, S461, S462, S464, S465, S466, S471, S474, S475, S480, S485) and this was also resulted from decreasing in lectin production.

The RhlR protein drives expression of *rhlI* to generate C4-HSL. (via an unknown mechanism) and LasR/ 3- oxo- C12- HSL also positively influence *rhlI* expression. The RhlR/ C4- HSL quorum sensing circuit is responsible for controlling multiple target genes, including the stationary phase sigma factor, RpoS. For example, expression of the *lecA* gene, which codes for the PA-IL lectin, depends on both *rhlRI* and *rpoS*. Mutation of either of these results in complete loss of lectin synthesis (Withers et al., 2001)

While for each of the isolates (S48, S418, S419, S429 and S433) that were subjected to UV light, we can see that haemagglutination ability were increased and this belongs to the increasing in lectin production. Also in the chemical mutagenesis for the isolates (S459, S460, S463, S476, S477 and S484), we can see that haemagglutination ability were increased and this also belongs to the increasing in lectin production.

Other isolates has no detectable change in haemagglutination ability and this refers to the absence of any mutation at least to the regulatory genes responsible for lectin production.

The genetic effect of UV irradiation is markedly dependent on the wavelength since DNA has a characteristic absorption peak around 260nm in the UV region. The correlation between UV absorption of the DNA and the genetic effectiveness of the radiation is remarkably good. UV irradiation may cause direct breakage in the genetic material by various forms of photolysis. One of the most common effects, however, is the formation of pyrimidine (mainly thymine) dimmers (Dale, 1998).

Mitomycin C blocks DNA synthesis by cross-linking the guanine bases in DNA to each other. Sometimes the cross-linked bases are in opposing strands. If the two strands are attached to each other, they cannot be separated during replication. Even one cross-link in DNA that is not repaired will prevent replication of the chromosome (Synder and Champness; 1997)

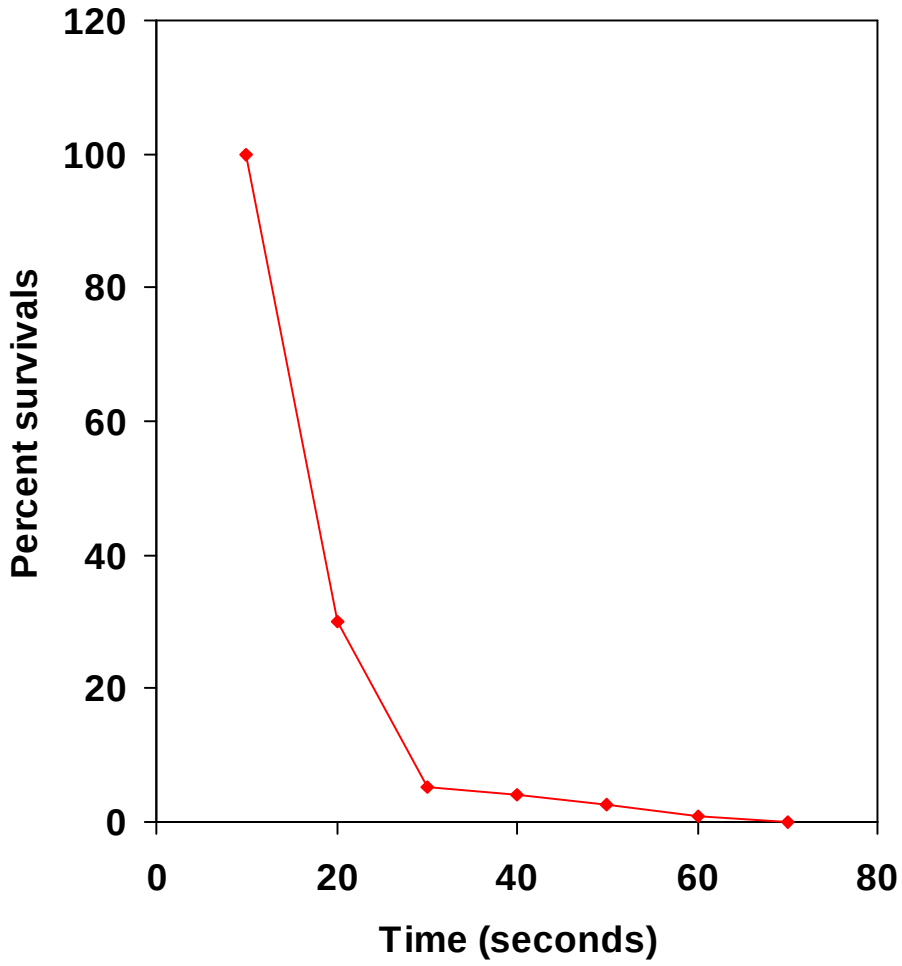


Fig (3-6) Survival curve represent the effect of UV irradiation on a suspension of *P .aeruginosa*

Table (3-6) Haemagglutination activity for the mutant *P. aeruginosa* S4 after subjection to UV light

Blood group / Mutant	A	B	O	Sheep
S41	128	64	64	32
S42	32	32	32	16
S43	128	64	64	32
S44	128	64	64	32
S45	32	32	32	16
S46	128	64	64	32
S47	64	64	64	32
S48	128	128	128	128
S49	128	64	64	32
S410	128	64	64	32
S411	64	64	64	32
S412	128	64	64	32
S413	64	64	64	32
S414	128	64	64	32

S415	**64**	**64**	**32**	**16**
S416	**128**	**64**	**64**	**32**
S417	**128**	**64**	**64**	**32**
S418	**128**	**128**	**128**	**128**
S419	**128**	**128**	**128**	**128**
S420	**128**	**64**	**64**	**32**
S421	**128**	**64**	**64**	**32**
S422	**64**	**64**	**64**	**32**
S423	**32**	**32**	**32**	**16**
S424	**128**	**64**	**64**	**32**
S425	**128**	**64**	**64**	**32**
S426	**128**	**64**	**64**	**32**
S427	**128**	**64**	**64**	**32**
S428	**64**	**32**	**32**	**16**
S429	**128**	**128**	**128**	**128**
S430	**32**	**32**	**32**	**16**
S431	**64**	**64**	**64**	**32**
S432	**16**	**16**	**8**	**8**
S433	**128**	**128**	**128**	**128**
S434	**128**	**64**	**64**	**32**

S435	**128**	**128**	**128**	**64**
S436	**128**	**64**	**64**	**32**
S437	**128**	**64**	**64**	**32**
S438	**128**	**128**	**128**	**64**
S439	**32**	**32**	**32**	**8**
S440	**128**	**128**	**128**	**128**
S441	**64**	**64**	**32**	**16**
S442	**128**	**64**	**64**	**32**
S443	**128**	**64**	**64**	**32**
S444	**128**	**64**	**64**	**32**
S445	**32**	**32**	**32**	**8**
S446	**128**	**64**	**64**	**32**
S447	**32**	**32**	**32**	**8**
S448	**128**	**64**	**64**	**32**
S449	**64**	**64**	**32**	**16**
S450	**32**	**32**	**32**	**8**
S451	**64**	**64**	**32**	**16**

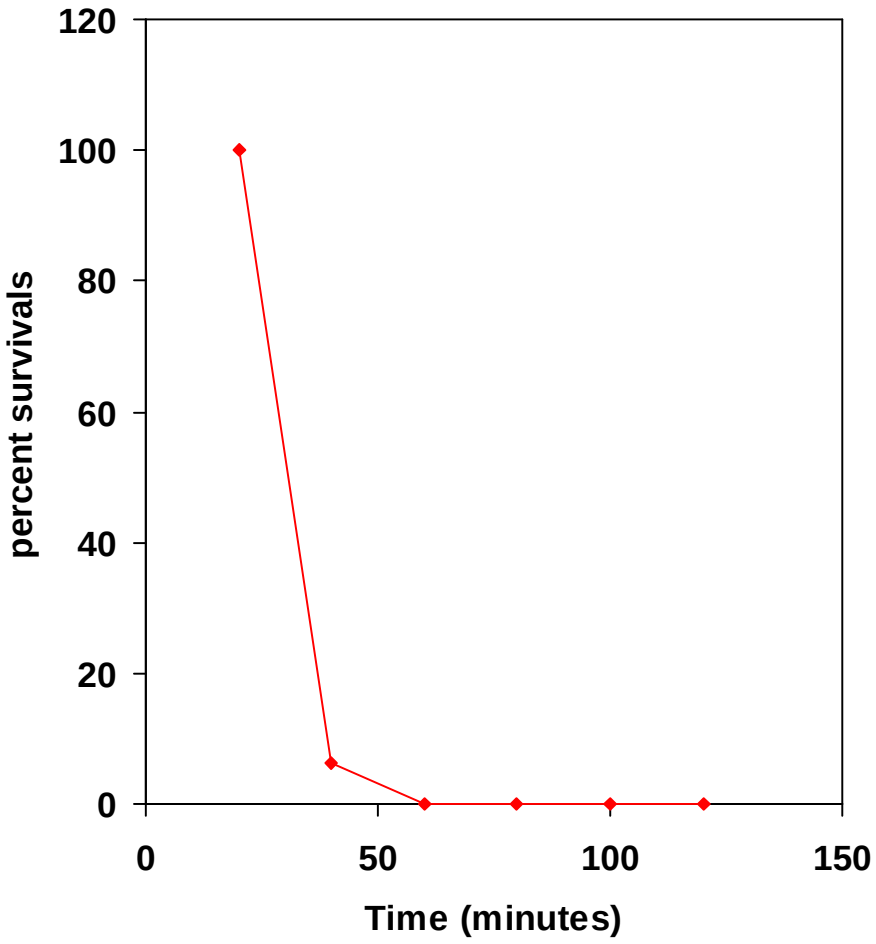


Fig (3-7) Different durations of exposure of *P .aeruginosa* to Mitimycin C

Table (3-7) Haemagglutination activity for the mutant *P. aeruginosa* S4 after subjection to Mitomycin C

Blood group / Mutant	A	B	O	Sheep
S452	128	64	64	32
S453	128	64	64	32
S454	128	64	64	32
S455	128	64	64	32
S456	128	64	64	32
S457	128	64	64	32
S458	64	64	64	32
S459	128	128	128	128
S460	128	128	128	128
S461	64	64	32	16
S462	64	64	32	16
S463	128	128	128	128
S464	64	64	32	16

S465	**64**	**64**	**64**	**32**
S466	**64**	**64**	**64**	**32**
S467	**128**	**64**	**64**	**32**
S468	**128**	**64**	**64**	**32**
S469	**128**	**64**	**64**	**32**
S470	**128**	**64**	**64**	**32**
S471	**64**	**64**	**32**	**16**
S472	**128**	**64**	**64**	**32**
S473	**128**	**64**	**64**	**32**
S474	**32**	**32**	**32**	**16**
S475	**64**	**32**	**64**	**16**
S476	**128**	**128**	**128**	**128**
S477	**128**	**128**	**128**	**64**
S478	**128**	**64**	**64**	**32**
S479	**128**	**64**	**64**	**32**
S480	**32**	**32**	**32**	**16**
S481	**128**	**64**	**64**	**32**
S482	**128**	**64**	**64**	**32**
S483	**128**	**64**	**64**	**32**

S484	**128**	**128**	**128**	**128**
S485	**32**	**32**	**16**	**16**
S486	**128**	**64**	**64**	**32**
S487	**128**	**64**	**64**	**32**
S488	**128**	**64**	**64**	**32**
S489	**128**	**64**	**64**	**32**
S490	**128**	**64**	**64**	**32**

Conclusions

1. All of the *Pseudomonas aeruginosa* isolates have a good activity in haemagglutination of human blood groups and sheep RBC_s.
2. *Pseudomonas aeruginosa* isolated from blood infections is the most virulent among the other isolates from clinical cases according to its ability in lectin production and haemagglutination of human blood groups and sheep RBC_s.
3. Physical and chemical mutagens affect the ability of lectin production by *Pseudomonas aeruginosa*.
4. Physical mutagenesis by UV light is more effective than chemical mutagenesis by Mitomycin C in altering the ability of *Pseudomonas aeruginosa* in lectin production.

Recommendations

1. Determination of the optimum conditions for lectin production by *Pseudomonas aeruginosa*.
2. Studying the role of quorum sensing in production of virulence factors from *Pseudomonas aeruginosa* especially lectin.
3. Determination the molecular structure of *Pseudomonas aeruginosa* lectins (amino acid sequence, subunits and sugar binding sites).
4. Large scale production of *Pseudomonas aeruginosa* lectins for immunological studies and applications.

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(A)

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الخلاصة

جمعت ٣٤ عينة من اصابات الأذن، الدم، البلغم، الحروق، سائل النخاع الشوكي و التكيس الرئوي من مستشفى النعمان و مدينة الطب و من مياه المجاري لمدينة الدورة في محافظة بغداد و قد تم الحصول على على ١٨ عزلة شخصت ١٢ منها على انها .*Pseudomonas* spp على اساس اختبار النمو على وسط السترمايد. اخضعت هذه العزلات الى الاختبارات الكيموحيوية و الدراسات المظهرية و الفسلجية و قد تبين ان ست منها كانت *Pseudomonas aeruginosa* وقد تم التأكد من ذلك باستخدام نظام التشخيص API 20E.

درست قابلية العزلات المحلية لبكتريا *Pseudomonas aeruginosa* على انتاج اللكتين و فعالية تلازن كريات الدم الحمراء لمجاميع الدم البشرية(A, B,O) و كريات دم الاغنام، وقد اظهرت النتائج ان جميع العزلات كانت ذات فعالية عالية في تلازن كريات الدم الحمراء المختلفة. و قد اختيرت العزلة المحلية *Pseudomonas aeruginosa* S4 على اساس فعاليتها العالية في تلازن كريات الدم الحمراء المختلفة مقارنة بالعزلات الاخرى.

درست قابلية العزلة المحلية *Pseudomonas aeruginosa* S4 على انتاج اللكتينات و كانت منتجة لكلا النوعين من اللكتين PA-IL و PA-IIL و قد تم تنقية اللكتينات المنتجة بطريقة الترسيب بمحلول كبريتات الامونيوم ثم الديلزة ثم الترشيح الهلامي خلال عمود sepharose-200 CL-6B . واستخدمت نفس التقنية (الترشيح الهلامي) في تقدير الوزن الجزيئي لكلا النوعين من اللكتينات بوجود بروتينات قياسية معلومة الأوزان الجزيئية (اللايسوزايم، البومين المصل البقري و انزيم choline esterase) وقد اشارت النتائج الى أن الوزن الجزيئي التقريبي للكتين PA-IL هو ٥٧٥٠٠ دالتون في حين كان الوزن الجزيئي التقريبي للكتين PA-IIL هو ٤٢٧٠٠ دالتون.

كما درست قابلية *Pseudomonas aeruginosa* S4 على مقاومة مضادات الحياة المختلفة و كانت مقاومة لكل من الفانكومايسين، الكلورامفينيكول، الكاربنسلين، السيفوتاكسيم، السيفالكسين، التتراسايكلين و الباستراسين.

درست ايضا تأثير المطفرات الفيزيائية (بوساطة الأشعة فوق البنفسجية) و الكيميائية (بمادة المايتومايسين سي) على قابلية العزلة المحلية *Pseudomonas aeruginosa* S4 في انتاج اللكتينات و قد اشارت النتائج الى أن التطفير الفيزيائي كان اكثر كفاءة في التأثير على قابلية هذه العزلة في انتاج اللكتينات أذ انخفضت فعالية تلازن كريات الدم الحمراء لمختلف اصناف الدم البشرية (A, B, O) و كريات الدم الحمراء للأغنام بوساطة معظم الطافرات البكتيرية و في نفس الوقت تم الحصول على طافرات بكتيرية ذات فعالية تلازن عالية لمختلف اصناف الدم أذا ما قورنت بالنوع البري لبكتريا *Pseudomonas aeruginosa* S4.

وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم

دراسة وراثية على بكتريا *Pseudomonas aeruginosa* المعزولة محليا و قابليتها على انتاج اللكتين

رسالة

مقدمة الى كلية العلوم في جامعة النهرين

و هي جزء من متطلبات نيل درجة الماجستير علوم

في علوم التقانة الاحيائية

من قبل

سنان عزت سعيد بحو

بكالوريوس تقانة احيائية-كلية العلوم-٢٠٠١

جامعة النهرين

أيلول **2006**

شعبان
١٤٢٧

Dedication

To the spring of sympathy

Mother and Father

To the most helpful peoples

Brother and Sister

I dedicate this work

Sinan

Name: Sinan Izzat Saeed Baho
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