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Hasan

وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

إنتاج وتوصيف الالجينيت المنتج من عزلة محلية لبكتريا *Pseudomonas aeruginosa*

رسالة مقدمة إلى
كلية العلوم في جامعة النهرين
كاستكمال جزئي لمتطلبات نيل درجة ماجستير علوم
في التقانة الاحيائية

من قبل

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ذو القعدة ١٤٢٧ **كانون الأول ٢٠٠٦**

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1. Introduction and Literature Review

1.1 Introduction

Many species of bacteria produce extracellular polymers that may facilitate nonspecific adhesion to surfaces and provide the framework for biofilms (Costerton *et al*., 1995).

Alginate is one of those extracellular polysaccharide that produce by a variety of gram-negative bacteria including *Pseudomonas aeruginosa* ,*Pseudomonas fluorescens* , and *Azotobacter vinelandii* (Evan and Linker, 1973).

Alginate plays an important role in the pathogenecity of *P. aeruginosa* in that it is provides the bacteria with selective advantages such as resistance to antibiotics and opsonization (Learn *et al*., 1987). Alginate also has several applications in life such as in food industry , water treatment and pharmaceutical preparations (Whistler and Beniller, 1973; Rosevear, 1988).

All alginates used for commercial purposes are currently being produced by the harvesting of brown seaweeds. However, considering the quality of bacterial alginate and the environmental impact associated with seaweed harvesting and processing, it is more probable that bacterial alginate may become commercial products. Furthermore, alginate with unique qualitative properties has the advantage that it may potentially be sold at higher prices and this may open new markets for this polymer (Yalpani and Sandford, 1987).

The microbial production of alginate would also have the considerable advantage of assured yield of known composition, being unaffected by marine pollution and tides, and the location of production can also be arranged to utilize convenient or cheap substrates. It is worth noting that the

global consumption of alginates in 1985 was 23,000 tons with a marketing value of 115 million $/year. Thus market volume of this seaweed polymer should stimulate the development of an alternative microbial sources to reduce the cost of this polysaccharide (Sutherland, 1996).

According to the points mentioned above, this study was aimed to:-

1. Isolation and identification of *P. aeruginosa* from different clinical and environmental samples.
2. Screening the isolates for their ability to produce alginate and determine the efficient one in alginate production.
3. Determine the optimum conditions for alginate production.
4. Purification and characterization of alginate produced by the locally isolated *P. aeruginosa.*

1.2 Literature Review

1.2.1 The General Characteristics of *P. aeruginosa*

P. aeruginosa is a Gram-negative, aerobic rod, motile by means of a single polar flagellum,bacilli measuring about (1.5-3.0x0.5µm), non spore forming, non capsulated and it is occur as a single bacteria, in pairs and occasionally in short chains, belonging to the bacterial family *Pseudomonadaceae* (Jawetz *et al*.,1998).

The organism can be isolated from soil and water and it is may be pathogenic for human, as well as for certain animals, insect and plants (Gilardi *et al*., 1985). *P. aeruginosa* isolates may produce three colony types. Natural isolates from soil or water typically produce a small rough colony. Clinical samples in general, yield one or two smooth colony types.

One type has a fried-egg appearance, which is large, smooth, with flat edges and elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has a mucoid appearance, which is attributed to the production of alginated slime. The smooth and mucoid colonies are presumed to play a role in colonization and virulence.

P.aeruginosa produces two types of soluble pigments pyocyanin and fluorescent pyoverdin. The latter was produced abundantly in media of low iron content , and could function in iron metabolism in the bacterium.

Pyocyanin (from "Pyocyaneus") refer to "blue pus" which is a characteristic of supportive infections caused by *P. aeruginosa* (Todar, 1997; Jawetz *et al*., 1998).

1.2.1.1 Pathogenecity of *P. aeruginosa*

P. aeruginosa is ubiquitous organism that can be readily isolated from the natural environment and human (Lopy, 1990; Vasquez *et al*., 1992).

P.aeruginosa is now widely recognized as an important opportunistic pathogen that produces sever infection since it's pathogenic only when introduced in to area devoid of normal defense. This may lead to the death of the compromised host (Sakata *et al*., 1996). Diseases caused by *P. aeruginosa* are as following:

A. Respiratory infections: Respiratory infections caused by *P. aeruginosa* occur almost exclusively in individuals with a compromised lower respiratory tract or a compromised systemic defense mechanism. Primary pneumonia occurs in patients with chronic lung disease and congestive heart failure (Hoiby and Rosendal,1980). Lower respiratory tract colonization of cystic fibrosis patients by mucoid strains of *P.aeruginosa* is common and difficult, if not impossible, to treat (Giwercman *et al*., 1990; Marty *et al*., 1998).

B. Endocarditis: *P.aeruginosa* infects heart valves of intravascular drug users and prosthetic heart valves (Mandelle *et al*., 1995). The organism establishes itself on the endocardium by direct invasion from the blood stream (Pollack, 1998).

C. Bacteremia: *P.aeruginosa* causes bacteremia primarily in immunocompromised patients. Predisposing conditions include hematologic malignancies, immunodeficiency relating to AIDS, neutropenia, diabetes mellitus, and severe burns. Most *Pseudomonas* bacteremia is acquired in

hospitals and nursing homes. *Pseudomonas* accounts for about 25 percent of all hospital acquired Gram-negative bacteremias (Todar, 1997).

D. Ear infections including external otitis: *P.aeruginosa* is the predominant bacterial pathogen in some cases of external otitis including "swimmer's ear". The bacterium is infrequently found in the normal ear, but often inhabits the external auditory canal in association with injury, maceration, inflammation, or simply wet and humid conditions (Strauss *et al*., 1982; Mims *et al*., 1993).

E. Eye infections: *P.aeruginosa* can cause devastating infections in the human eye. It is one of the most common causes of bacterial keratitis (Jawetz *et al*., 1998).

F. Wounds and burns infection: Almost any opportunistic pathogen can infect burns and wounds, but one of the most common and hardest to treat is the gram negative rod *P. aeruginosa* , which can actually color the burns or damaged tissue with it's blue-green fluorescent pigments and may lead later to septicemia (Nester, 1982).

G. Urinary tract infection: The bacterium enters the urinary tract by catheters and instrument or in irrigation solutions (Worth, 1982; Martinez *et al*., 1999). *P. aeruginosa* is more frequently found in hospital-acquired urinary tract infection due to antibiotics which fervor it's selection in hospital patients (Glauser, 1986).

1.2.1.2 Virulence Factors of Pathogenic *P. aeruginosa*

As in other pathogenic bacteria, *P.aeruginosa* don't produce the virulence factors until it response to environmental signal, which it's production variant according to stage of inflammation, the virulence factors which produced by *P.aeruginosa* was summarized in table (1-1) (Cryze, 1984; Todar, 1997).

Table (1-1) Virulence factors produced by *P. aeruginosa.*

Function	Virulence determinant
Adhesion	Fimbriae, alginate slime(biofilm)
Invasion	Elastase,alkaline protease, pyocyanin diffusible pigment
Toxins	Exoenzyme S, Exotoxin A, Lipopolysaccharide(LPS)
Defense against immune response	Capsules, slime layers, protease enzymes
Defense against serum bactericidal reaction	Protease enzymes, capsules, slime layers, LPS
Antiphagocytic surface properties	Capsules, slime layers, LPS

1.2.2 Alginate-producing Bacteria

The polysaccharide alginate was first isolated from marine macroalgae last century, but it was approximately 80 years later that a bacterial source (*P. aeruginosa*) of the polysaccharide was identified (Linker and Jones, 1966). The association of mucoid forms, i.e.alginate-producing strains, of *P. aeruginosa* with chronic lung infections in patients with cystic fibrosis is now well established, and is recognized as a major cause of morbidity and mortality in these individuals. Mucoid, alginate-producing strains of *P. aeruginosa* have also been isolated from other cohorts of patients, e.g. bronchiectatics and those with urinary tract or middle ear infections, although not normally from individuals with infected burn sites (McAvoy *et al*., 1989).

Alginate is also synthesized by *Azotobacter vinelandii* as part of the encystment process. The mature cysts are surrounded by two discrete alginate rich layers which enable the dormant cells to survive long periods of desiccation (Cote and Krull, 1988).

1.2.3 The Biological Function of Alginate

The role of alginate for the pathogenic *P. aeruginosa*, it serves to protect the bacteria from adversity in its surrounding and also enhances adhesion to solid surfaces. As a result, biofilm develops which is advantageous to the survival of the bacterium in the lung. The same strain, however, produces alginate lyase which cleaves the polymer into short oligosaccharides resulting in increased detachment of the bacteria away from the surface, allowing them to spread and colonize new sites (Boyd and Chakrabarty, 1995).

Alginate provides the bacteria with selective advantages for colonization of the pulmonary tissue, through increased resistance to opsonization and phagocytic engulfment as well as through increased protection from toxic oxygen radicals (Simpson *et al.*,1989).

Since exopolysaccharides are known to chelate heavy metals, it was also reported that alginate formation was induced, in plant-parasitic pseudomonads, by treatment with a bacteriocidal spray containing copper. Thus the secretion of alginate may contribute to some plant-bacterial diseases. Sodium chloride and ethanol were shown to significantly increase alginate production in a variety of fluorescent pseudomonads, suggesting that osmolarity and dehydration may be general signals for production of this polysaccharides (Kidambi *et al*., 1995).

1.2.4 Bacterial and Algal Sources of Alginate

Alginate belongs to a family of unbranched binary copolymers of (1-4) linked ß-D-mannuronic acid (M) and α-L-guluronic acid units (G), the relative amounts of which vary greatly between alginates from different species of algae, or between the different regions in the same algae. Additionally, alginic acid from different sources varies in the arrangement of the uronic acids within the molecule. Except for some bacterial polyuronides, alginates are true block copolymers composed of homopolymeric regions of M and G units, termed M and G blocks interspaced with regions of alternating structures of MG blocks (Figure 1-1) (Moe *et al*.,1995).

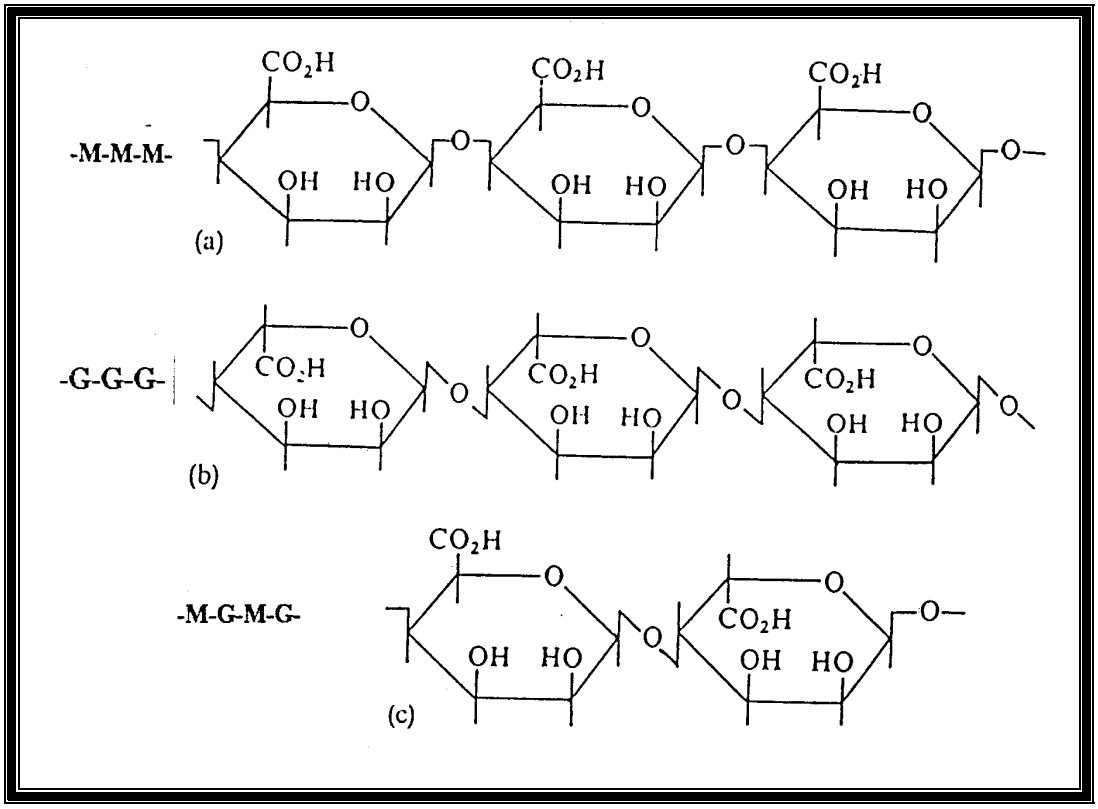


Figure (1-1) Chemical structure of alginate :(a) M blocks, (b) G blocks, (c) MG blocks.

This polyuronide is well known and the present source of commercial alginate is the giant brown algae *Macrocystis pyrifera*. Because only a few species of brown algae are suitable for commercial alginate production, in respect to abundance, location and uniform quality, there is a present interest in an alternative bacterial alginate. Microbially derived alginates are under development and their future is very promising (Davidson *et. al.*, 1977).

Species of *Pseudomonas* and *Azotobacter* are the only prokaryotic sources for this algal like polymer. *P. aeruginosa* was first reported to produce this polysaccharide being important for the virulence of this strain and its survival in the lung. Also several species of the genus *Pseudomonas*

(*P. mendocina* and *P. syringae*) have the ability to produce alginate under several conditions. Many strains of *Azotobacter vinelandii* (a nitrogen fixing soil bacterium) were also found to produce this polymer in complex and synthetic media (Franklin *et al*. 1994).

Unlike algal alginates, bacterial alginates are partly o-acetylated, with the acetyl groups being located solely on the mannuronic acid residues. These acetyl groups are suggested to play a role in protecting certain mannuronic acid residues from epimerisation to guluronic acid (Davidson *et. al*., 1977).

1.2.5 Physical Properties of Alginate

1.2.5.1 Ion Binding

The ion binding properties of alginates are the basis for their gelling properties. Alginates show characteristic ion-binding properties so that their affinity for multivalent cations depends on their composition. Characteristic affinities were shown to be a property exclusive to polyguluronate, while polymannuronate was almost without selectivity.

The high selectivity between ions as similar as the alkaline earth metals indicates that the mode of binding could not be by non-specific electrostatic binding only, but some chelation caused by structural features in the G-blocks must contribute to the selectivity. This characteristic property was explained by the so-called ‘egg-box’ model based upon the linkage conformations of the guluronate residues. Nuclear magnetic resonance studies suggested a possible binding sites for Ca^{2+} ions in a single alginate chain as shown in Figure(1-2)(Franklin *et al*.,1994; Moe *et al*.,1995).

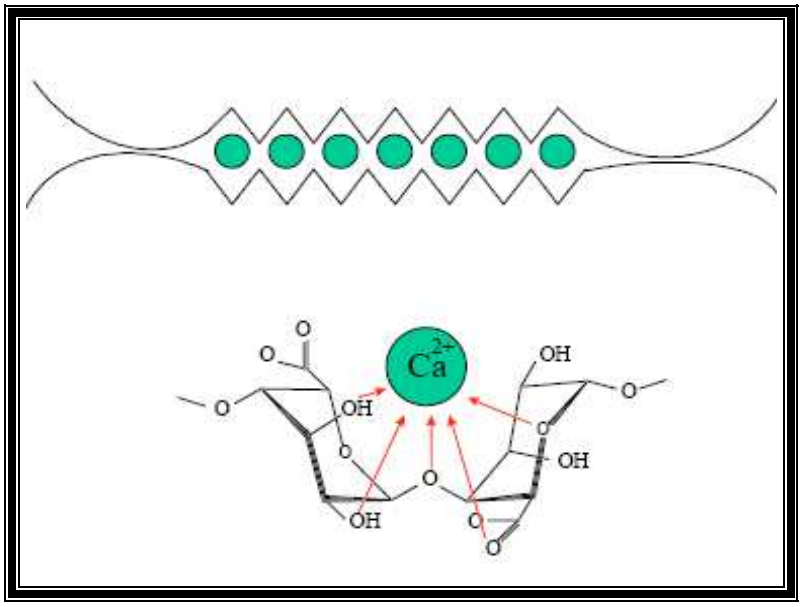


Figure (1-2) The egg-box model for binding of divalent cations to alginate and the possible chelation of ions by GG sequences (Moe *et al*., 1995).

1.2.5.2 Solubility

There are three essential parameters which determine the solubility of alginate in water are:

- **The pH of the medium**. Lowering the pH of the medium leads to a precipitation of the alginate within a relatively narrow pH range depending on the molecular weight of the alginate.

- **The ionic strength of the medium**. Alginate may be precipitated and fractionated by high concentrations of inorganic salts like potassium chloride. On the other hand, salt concentration of less than 0.1M is sufficient to slow down the kinetics of the dissolution process and hence limits the solubility.

- **Effect of gelling ions**. At Ca^{2+} concentrations below 3mM, almost all alginate is found within the supernatant, whereas almost no alginate is present in solution when the free Ca^{2+} ion concentration exceeds 3mM (Moe *et al*., 1995).

1.2.5.3 Viscosity

Alginate solutions are in general highly viscous. This, however, is caused by the extended conformation of the alginate molecule, giving alginate a large hydrodynamic volume and high ability to form viscous solutions. The intrinsic viscosity of alginates is shown to be dependant on the conformation (their molecular weight, compositions, and sequence of M and G units) and on the ionic strength of the solution (Lebrun *et al*., 1994).

1.2.6 Applications of Alginate in Biotechnology

Alginic acid is a commercially important polysaccharide which has many applications in biotechnology and as follow:

A. Food industry

Alginate is used mainly in food industry, which currently consumes about 50% of the alginate produced. It is used, for example, in ice-creams, frozen custards, as well as cream and cake mixtures. It also found application in beer manufacture to enhance the foam and fruit drinks to assist the suspension of fruit pulp, which makes the product more appealing to the consumer (Neidleman, 1991).

B. Medicines

Alginate is active in stimulating immune cells to secrete cytokines, e.g. tumor necrosis factor -α (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) (Otterlei *et al*. 1991). Surprisingly, the response of the immune system appears to depend upon the sequential structures of alginates, giving the highest response with M-rich polymers, while the G-blocks appear to be non-stimulating. In fact, guluronic acid residues cannot be accepted in therapeutic preparations because it triggers unwanted effects such as antibody generation (Skjak-Braek,1992).

C. Industrial processes

In recent years, entrapment within spheres of calcium alginate gel has become the most widely used technique for immobilising living cells (bacteria,cyanobacteria, algae, fungi, yeast, plant protoplasts, plant and animal cells). Alginate immobilised cell systems are used as biocatalysts in several industrial processes ranging from ethanol production by yeast cells to the production of monoclonal antibodies from hybridoma cells. Alginate gel also has a potential as implantation material for hormone-producing cells,and encapsulated langerhans islets are currently being evaluated as a bio-artificial endocrine pancreas (Skjak-Braek, 1992; Clementi, 1997).

Textile and paper industries use alginate along with other materials as ‘sizes’ to improve the surface properties of cloths and paper. This is important prior to printing to enable deposition and adherence of dyes and ink substances (Sutherland and Ellwood, 1979).

Alginates are also used in water treatment processes since they help in increasing the aggregate sizes in the flocculation processes (Rehm and Valla, 1997).

D. Pharmaceuticals

Alginates are also used in pharmaceutical preparations (to form stable emulsion) and as dental impression material (Morris,1987).

1.2.7 Biosynthesis Pathway of Alginate from *P.aeruginosa*

The alginate biosynthetic pathway has been under scrutiny for a number of years, and several excellent reviews have been published. The initial steps in the alginate biosynthesis pathway are essentially those of general carbohydrate metabolism and the intermediates are widely utilized. In particular,the steps up to and including GDPmannose are common to alginate and LPS biosynthesis (Figure 1-3) (Goldberg *et al*., 1993).

In brief, the *algA*, *algC* and *algD* genes (Table 1-2) encode the enzymes required for synthesis of the alginate precursor guanosine diphosphate (GDP)-mannuronic acid. Once GDP-mannuronic acid is synthesized, the precursor is polymerized and transported across the inner membrane by a hypothesized combination of the *alg8* and *alg44* gene products (Maharaj *et al*., 1993; Monday and Schiller, 1996).

After polymerization, some of the mannuronate residues are epimerized to guluronate residues by a C-5-epimerase (AlgG). A scaffold model has been proposed where AlgG interacts in the periplasm directly with AlgK in order to protect the growing alginate polymer from degradation by alginate lyase (AlgL) (Jain and Ohman, 1998).

Recent evidence suggests that AlgX may be part of this periplasmic scaffold (Robles-Price *et al*., 2004). After epimerization, some of the mannuronate residues are acetylated at the O2 and/or O3 positions by the *algF*, *algJ* and *algI* gene products, which might form a complex in the membrane that serves as a reaction centre for O-acetylation (Franklin and Ohman, 2002). After O-acetylation, the copolymer is transported out of the cell through the outer membrane protein AlgE (Rehm and Valla, 1997).

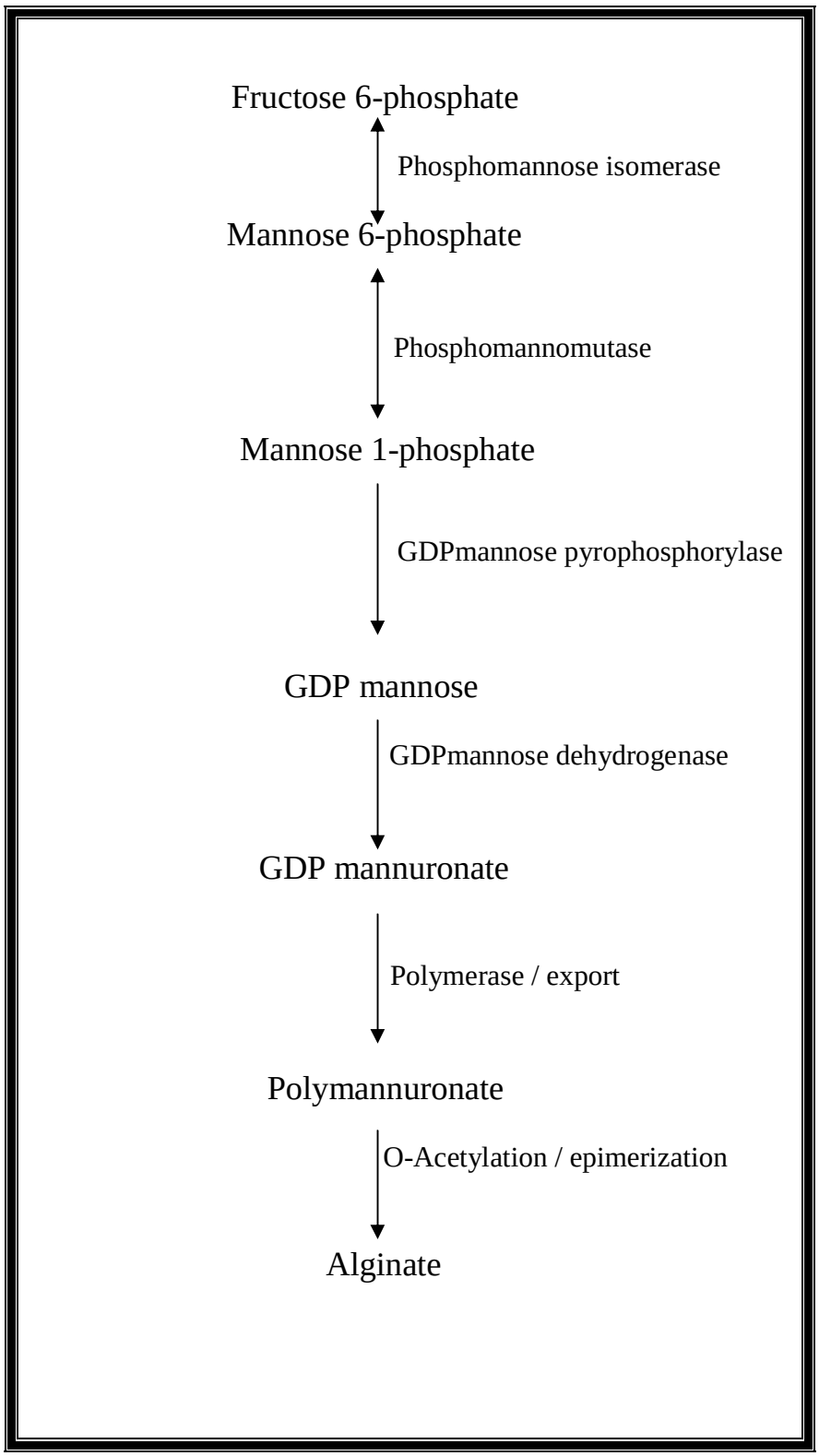


Figure (1-3) Alginate biosynthetic pathway (Gacesa, 1998).

Table (1-2) Alginate genes of *P. aeruginosa*

Gene	Gene product	Reference
algA	Phosphomannose isomerase and GDP-mannose	Shinabarger *et al.*,1991.
algC	pyrophosphorylase	Coyne *et al.*,1994.
algD	Phosphomannomutase	Tatnell *et al.*,1994.
algE	GDP-mannose dehydrogenase	Rehm *et al.*,1994.
algF	Outer membrane protein	Franklin and Ohman,2002
algG	Mannuronan C-5-epimerase	Jain *et al.*,2003.
algI	O-acetylation	Franklin and Ohman,2002
algJ	O-acetylation	Franklin and Ohman,2002
algK	Periplasmic protein required for proper polymer formation	Jain and Ohman,1998.
algL	Alginate lyase	Schiller *et al.*,1993.
algX	Periplasmic protein required for proper polymer formation	Robles-Price *et al.*,2004.
alg8	Polymerase / export function	Mejia-Ruiz *et al.*,1997.
alg44	Polymerase / export function	Mejia-Ruiz *et al.*,1997.

1.2.8 Optimum Conditions for Alginate Production

1.2.8.1 Optimum Nutrition Factors

Microorganisms differ in their needs to carbon sources according to their nutrient nature; the use of pure carbon sources like (glucose, sucrose and fructose) is expensive from the economical case, so the industrial fermentation try to use cheap carbon sources especially industrial and agricultural by-products such as date extract, beet molasses and cane molasses (Anderson *et al*., 1987; Horan *et al*., 1983).

It was found that higher alginate production achieved when use sucrose as a sole carbon source and alginate production reach to (5.6 g/L) and (5.0 g/L) when use fructose comparing with other sources such as glucose, manitol and sorbitol (Horan *et al*., 1981).

It is possible for most microorganisms to use organic and inorganic nitrogen sources, inorganic nitrogen can be supplied as ammonia , ammonium salts or nitrate while organic nitrogen can be supplied as amino acid, proteins or urea and usually the growth will be faster when use organic source of nitrogen. Alginate production reach to (3.8 mg/L) when use the organic source peptone comparing with the use of ammonium nitrate which gives low production (1.4 mg/L) (Brivonese and Sutherland, 1989).

Mineral salts has an effect on the production of alginate , and there are several studies find that the use of low concentration of inorganic phosphate(8 – 32 mg/L) lead to increase alginate production comparing with the use of high concentration(1 g/L) of it (Horan *et al*., 1981), the presence of phosphate in the media may have negative or positive effect on the enzymes that involve in the biosynthesis of alginate, it was found that an increase in phosphate concentration lead to inhibit some of these enzymes (Jarman *et al*., 1978).

1.2.8.2 Optimum Temperature, PH and Incubation period for Alginate Production

Preservation the temperature of specific growth medium is one of the most important factors in alginate production from one side and in the growth of the microorganism from another side, it was found that 37°C was the optimum temperature for production of alginate from *P. aeruginosa* (Theilacker *et al.*, 2003; Stapper *et al*., 2004).

Optimum pH represents one of the important parameters which affect the alginate production. It was found that the optimum pH for alginate production from *P. aeruginosa* was 7.5 (Brivonese and Sutherland, 1989) and 7.2 (Stapper *et al*., 2004).

The optimum incubation time of alginate production was studied to determine in which phase of bacterial growth the alginate was produced, so it was found that 72 hr was the optimum incubation period for production of alginate from *P. aeruginosa* (Theilacker *et al.*, 2003).

1.2.9 Purification of Alginate

The step of purification represent one of the important and essential steps in the production of biological materials, and in case of exopolysaccharides which the problems of isolation are decreases due to use of centrifugation methods in the separation procedures, but the basic problem in such compounds is the high viscosity which conflict with the displacement of cells (Cerning, 1990).

Alginate can be precipitated from culture medium by the use of organic solvents such as ethanol, isopropanol or methanol in a volume 3:1 of alginate (Jarman *et.al.*,1978), after that the precipitated alginate dissolved in

water at 45°C and reprecipitate by ethanol to get more pure sample (Wistler and Murphy, 1973).

The precipitated polysaccharide by alcohol can be purified by using ion-exchange chromatography by Dowex column or DEAE – cellulose column or by using gel filtration by Sephacryl S – 1000 column or Sephadex G 200 column (Cohen and Johnstone, 1964; Doco *et al.*, 1990).

The molecular weight of polysaccharide can be determined by gel filtration chromatography technique (Doco *et al.*, 1990).

3. Results and Discussion

3.1 Isolation of *Pseudomonas* spp.

In order to isolate *P. aeruginosa,* thirty seven samples were collected from different environments and clinical cases from Al-Yarmouk hospital and Al-Karama hospital in Baghdad governorate during the period from 10/2004 to 12/2004.

Results in table (3-1) showed that 41 isolates were obtained from blood, Cerebrospinal fluid, ears, sputum, cystic fibrosis patients and burns, while 16 isolates obtained were from water and soil samples.

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

Source of sample	No. of samples	No. of isolates	Growth on Cetrimide agar
Blood	4	7	1
Cerebrospinal fluid	2	2	2
Ear	2	4	1
Sputum	9	13	1
Cystic fibrosis	1	1	1
Burns	12	14	6
Water	4	6	0
Soil	3	10	1
Total	37	57	13

Among the total isolates, only 13 isolates were able to grow on cetrimide agar plates , which gives an indicator that these isolates belong to *Pseudomonas* spp. These 13 isolates were further characterized and identified according to the cultural, morphological and biochemical tests.

From the results mentioned in table (3-1) we can notice that there are other 44 isolates from clinical and environmental samples may belong to other pathogenic or nonpathogenic bacteria from different genera.

3.2 Identification

Local isolates able to grow on cetrimide agar plates, were further identified according to morphological characteristics and biochemical tests. For the former, colonies of each isolate plated on nutrient agar showed different morphological characteristics of *P. aeruginosa* such as mucoidal growth, smooth in shape with flat edges and elevated center, whitish or creamy in color and has fruity odor.

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

Some biochemical tests were done to ensure that these 13 isolates are *P. aeruginosa*. Results indicated in table (3-2) showed that all of these isolates give positive results for catalase, oxidase and pyocyanin production. On the other hand, results indicated in table (3-2) showed that three of these isolates termed (H2, H5, H9) were unable to utilize citrate, while (H1, H13) and (H10, H11) isolates were unable to hydrolyze gelatin or produce fluorescein respectively.

From these results it can conclude that six of the total isolates grown on citrimide may belong to *P. aeruginosa*, they are (H3, H4, H6, H7, H8, H12). Further more, these results were agreed with those observed by (Palleroni, 1985; Hawkey and Lewis, 1998).

Table (3-2) Biochemical tests of the locally isolated *Pseudomonas* spp.

Isolate / Test	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	-	+	+	-	+	+	+	-	+	+	+	+
Gelatin hydrolysis	-	+	+	+	+	+	+	+	+	+	+	+	-
Pyocyanin production	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluorescein production	+	+	+	+	+	+	+	+	+	-	-	+	+

Further identification of the isolates was performed using Api 20 E system figure (3-1). The isolates were able to utilize arginine, citrate, gelatin and glucose. While they gave negative results for β-galactosidase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease, 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; Collee *et al*., 1996).

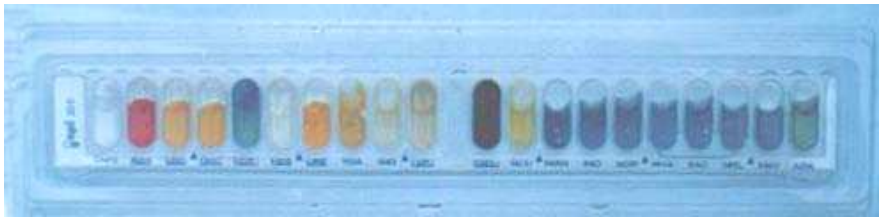


Figure (3-1) Api 20 E system for identification of *P.aeruginosa.*

3.3 Screening of *P. aeruginosa* Isolates for Alginate Production

In order to select the efficient isolate in alginate production, the ability of these six isolates were examined in alginate production medium by determination the dry weight of crude alginate in culture medium after isopropanol precipitation method mentioned in (2.2.7).

As shown in table (3-3), *P. aeruginosa* H3 gave the maximum production of alginate (1.2 g/ L) while H4, H6, H7, H8, H12 isolates gave 0.4, 0.9, 0.5, 1 and 0.8 g/L respectively.

From these results it was concluded that the *P. aeruginosa* H3 was the efficient in alginate production, because it was isolated from cystic fibrosis tissue and in this tissue there is necessary need for alginate to invade the

tissue, because of the role of alginate in the biofilm formation and resistance to antibiotics and phagocytosis (Simpson *et al.*,1989).

Table (3-3) Ability of *P. aeruginosa* isolates in alginate production in culture medium after incubation in shaker incubator at 150 rpm for 48hrs at 37°C.

Isolate	Dry weight of crude alginate(g/L)
P. aeruginosa H3	1.2
P. aeruginosa H4	0.4
P. aeruginosa H6	0.9
P. aeruginosa H7	0.5
P. aeruginosa H8	1
P. aeruginosa H12	0.8

3.4 Optimum Conditions for Alginate Production

3.4.1 Carbon source

Five carbon sources (sucrose, fructose, maltose, glucose and date extract) were used as a sole source of carbon and energy to determine the optimum in alginate production by the locally isolated *P. aeruginosa* H3, these carbon sources were added in a concentration of (2%) .

Results in figure (3-2) showed that the maximum production of alginate was obtained when the culture medium was supplemented with date extract. Using this carbon source, the dry weight of crude alginate extracted from

culture medium was (2.3 g/L), while the productivity of alginate in the media containing sucrose, maltose, fructose and glucose under the same conditions was 1.2, 0.8, 1.9 and 1.7 g/L respectively.

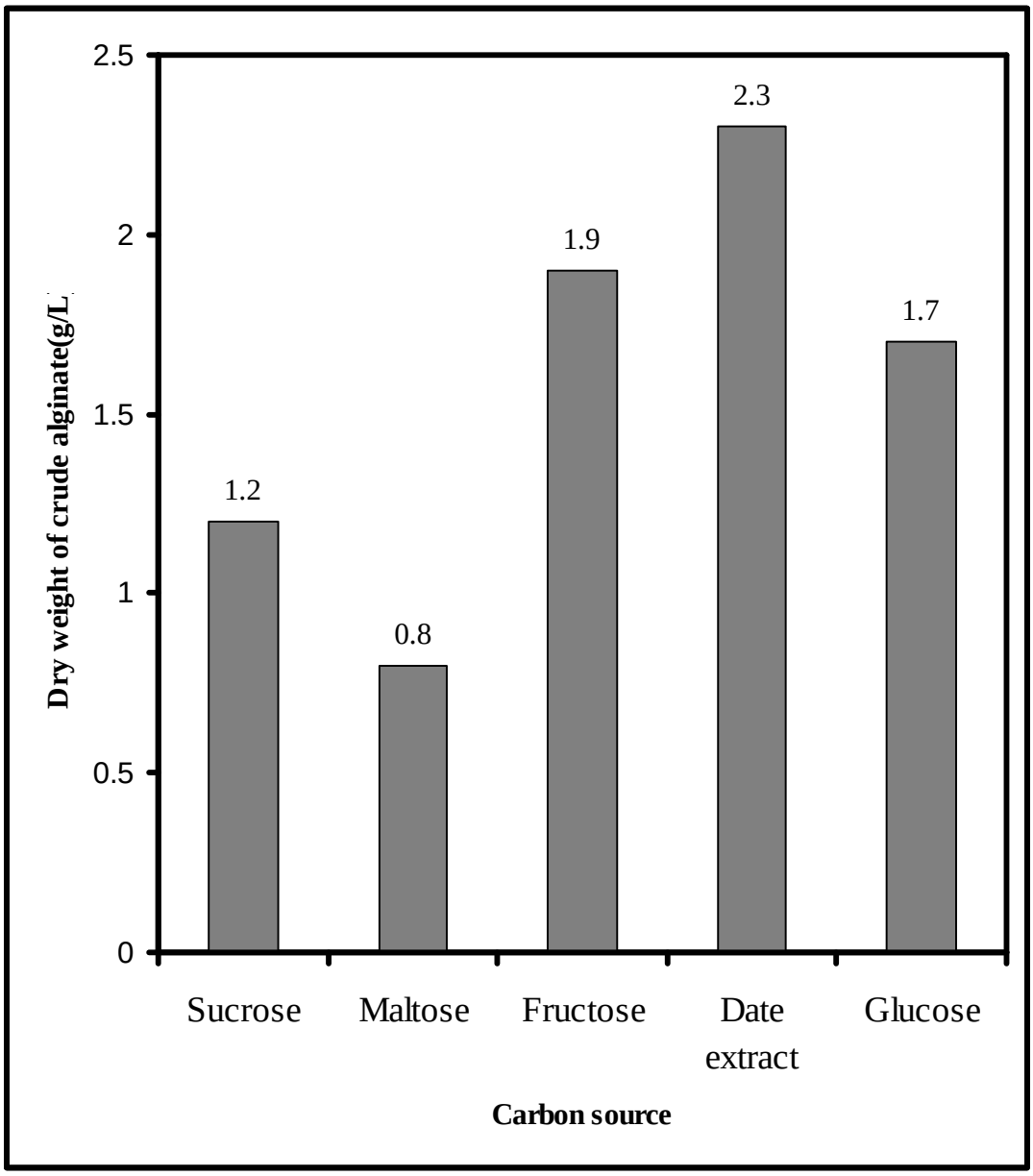


Figure (3-2) Effect of carbon source on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph 7 for 48hrs at 37°C.

From these results we conclude that date extract was the best carbon source because it contain many nutrients, growth factors and variety of vitamins that supplements the growth requirements for bacteria, Further more the date extract contain reduced sugar (55.8%) protein (1.2%) and several mineral salts such as (K, Cl, Ca, P, Mg, S, Na, Fe, Cu), the date also contain vitamin A, B1, B2, B7 and low percent of vitamin C (Sutherland and Ellwood, 1979).

This optimum carbon source (date extract) was used in the next experiments of optimization for alginate production.

3.4.2 Concentration of carbon source

The optimum carbon source (date extract) was used in a concentrations of (2, 4, 6, 8, 10% v/v) to determine the optimum concentration for production of alginate by the *P.aeruginosa* H3.

As shown in figure (3-3) the maximum production of alginate was obtained when the date extract was used in a concentration of (4%), at this concentration, the dry weight of crude alginate was (3.7 g/L). The decrease in the production of alginate in the concentrations (6, 8 and 10%) may be attributed to the increase of sugar concentration in growth medium that inhibits bacterial metabolism and then alginate production as it was mentioned by Sutherland and Ellwood, (1979) .

This optimum concentration of date extract (4%) was used in the next experiments of optimization for alginate production.

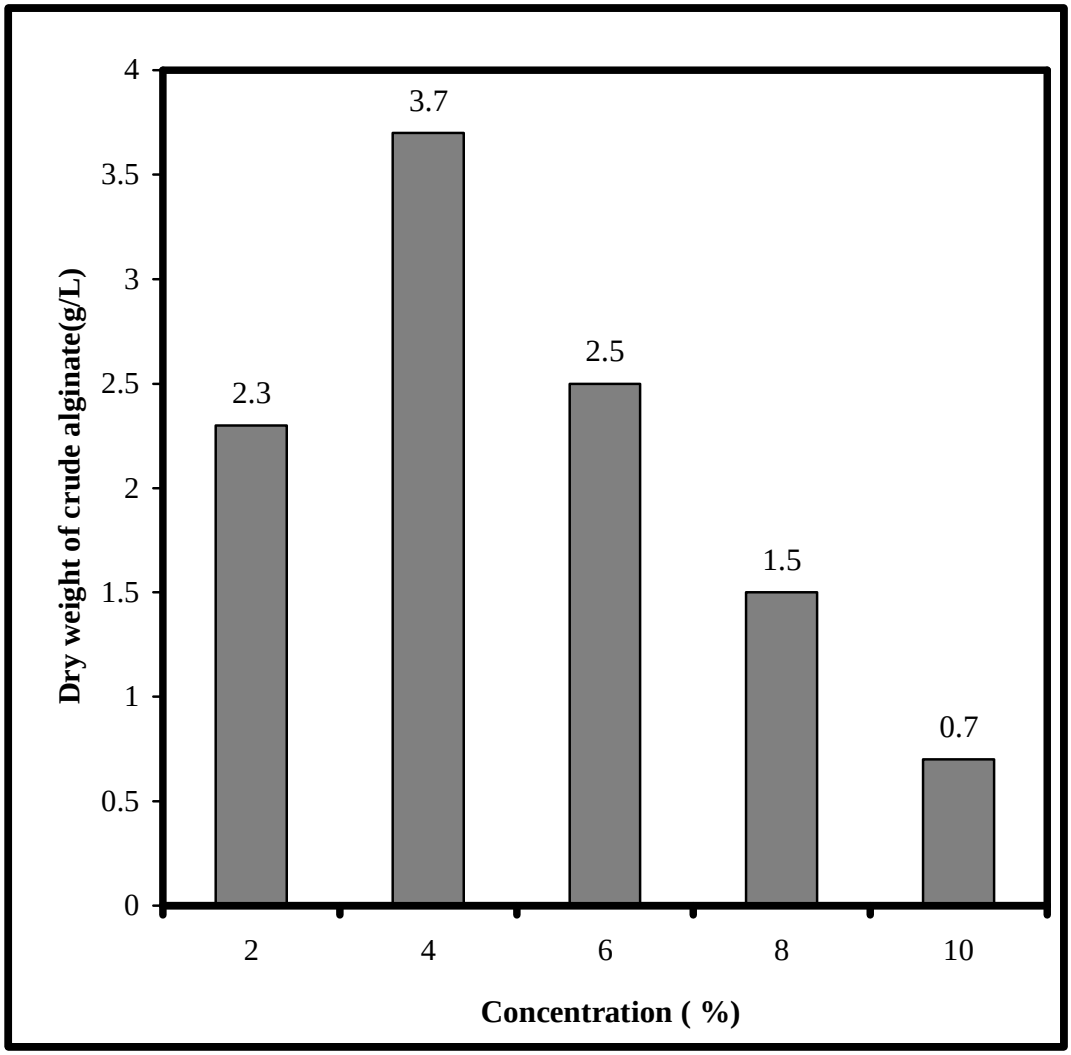


Figure (3-3) Effect of date extract concentration on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph7 for 48hrs at 37°C.

3.4.3 Nitrogen source

Six nitrogen sources (yeast extract, peptone, tryptone, ammonium sulphate, ammonium chloride and commercial baker's yeast) were used to examine the ability of locally isolated *P.aeruginosa* H3 in alginate production. These sources were added to the production medium in a concentration of (0.2 % w/v).

Results mentioned in figure (3-4) showed that the maximum production of alginate was obtained when the production medium was consist of commercial baker's yeast, the dry weight of alginate using this nitrogen source was (4.3g/L). This result may be attributed to the high nitrogen and growth factors contents of commercial baker's yeast that supplements the bacterial requirements for growth, production and secretion of alginate to culture medium, so this nitrogen source was used in the next experiments of optimization.

From the other results, figure (3-4) that there was lower production of alginate when yeast extract, peptone, tryptone, ammonium sulphate and ammonium chloride were used respectively as a nitrogen source in the production medium. Further more, the production of alginate in culture medium by *P.aeruginosa* H3 using organic nitrogen sources (commercial baker's yeast, yeast extract, peptone and tryptone) was better than the alginate production using inorganic nitrogen sources (ammonium sulphate and ammonium chloride) under the same condition. These results were agreed with Brivonese and Sutherland, (1989).

The increase in the production of alginate using the commercial baker's yeast may attributed to it's natural component that provided the medium with nitrogen source which contributed in the supporting of bacterial biomass, also it contain Ca, Mg, and carbohydrates that provided the

optimum condition for enzymatic activities especially those enzymes responsible for biosynthesis of alginate. Sikyte, (1983) refers that the use of yeast as a nitrogen source supported the medium with (75-105 mg of nitrogen/g of yeast), (0.1g Ca /g of yeast), (2g Mg/g of yeast) and carbohydrates (82mg /g of yeast) .

This optimum nitrogen source (commercial baker's yeast) was used in the next experiments of optimization for alginate production.

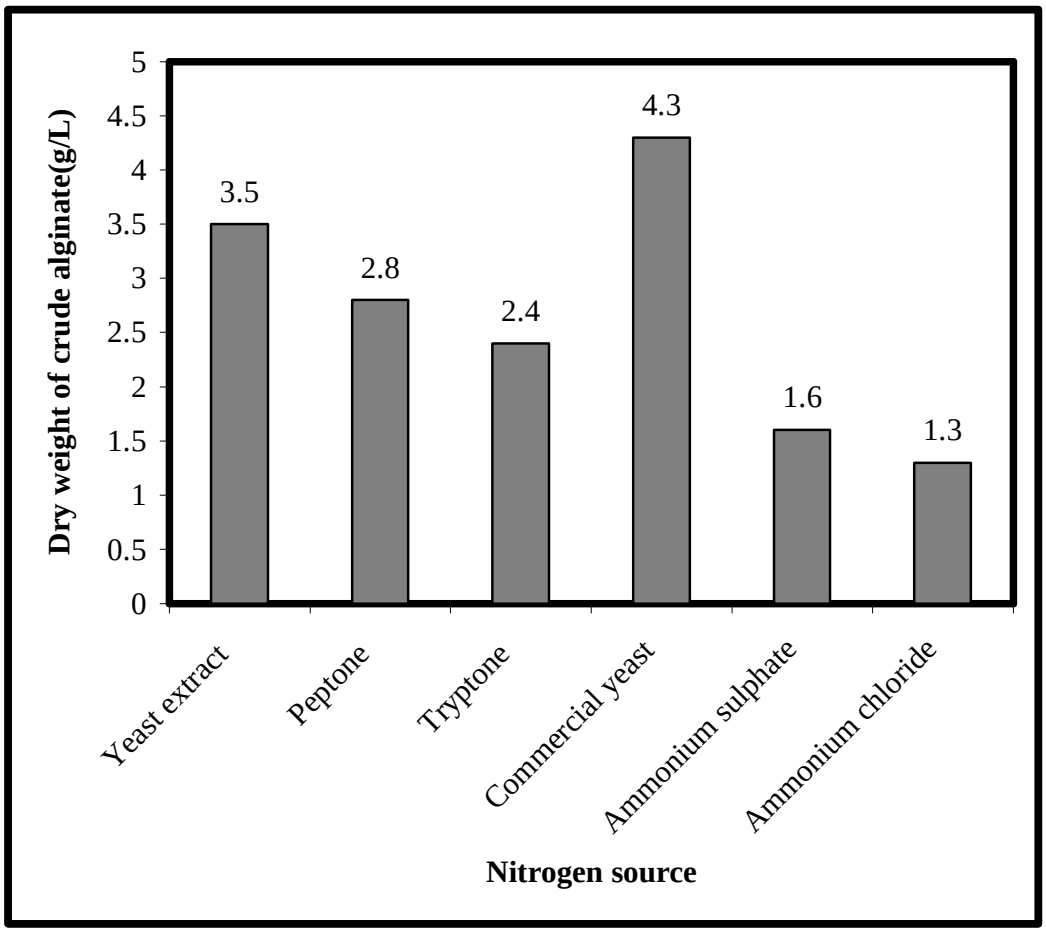


Figure (3-4) Effect of nitrogen source on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph7 for 48hrs at 37°C.

3.4.4 Concentration of nitrogen source

To determine the optimum concentration of commercial baker's yeast as it was the optimum nitrogen source for alginate production, six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, 2.0 % w/v) of commercial baker's yeast were used by the locally isolated *P.aeruginosa* H3.

Results in figure (3-5) showed that the maximum production of alginate was obtained when commercial baker's yeast was added to the production medium in a concentration of (1 %), the dry weight of crude alginate extracted from culture filtrate was (5.1g/L), while the increase or decrease the concentration of commercial baker's yeast above or below the optimum concentration causing a decrease in alginate production, this may be due to low concentrations of commercial baker's yeast necessary for heavy growth and production, while the high concentrations above the optimum may lead to change the bacterial metabolism especially those responsible for alginate production as it was mentioned by Brivonese and Sutherland, (1989).

This optimum concentration of commercial baker's yeast (1%) was used in the next experiments of optimization for alginate production.

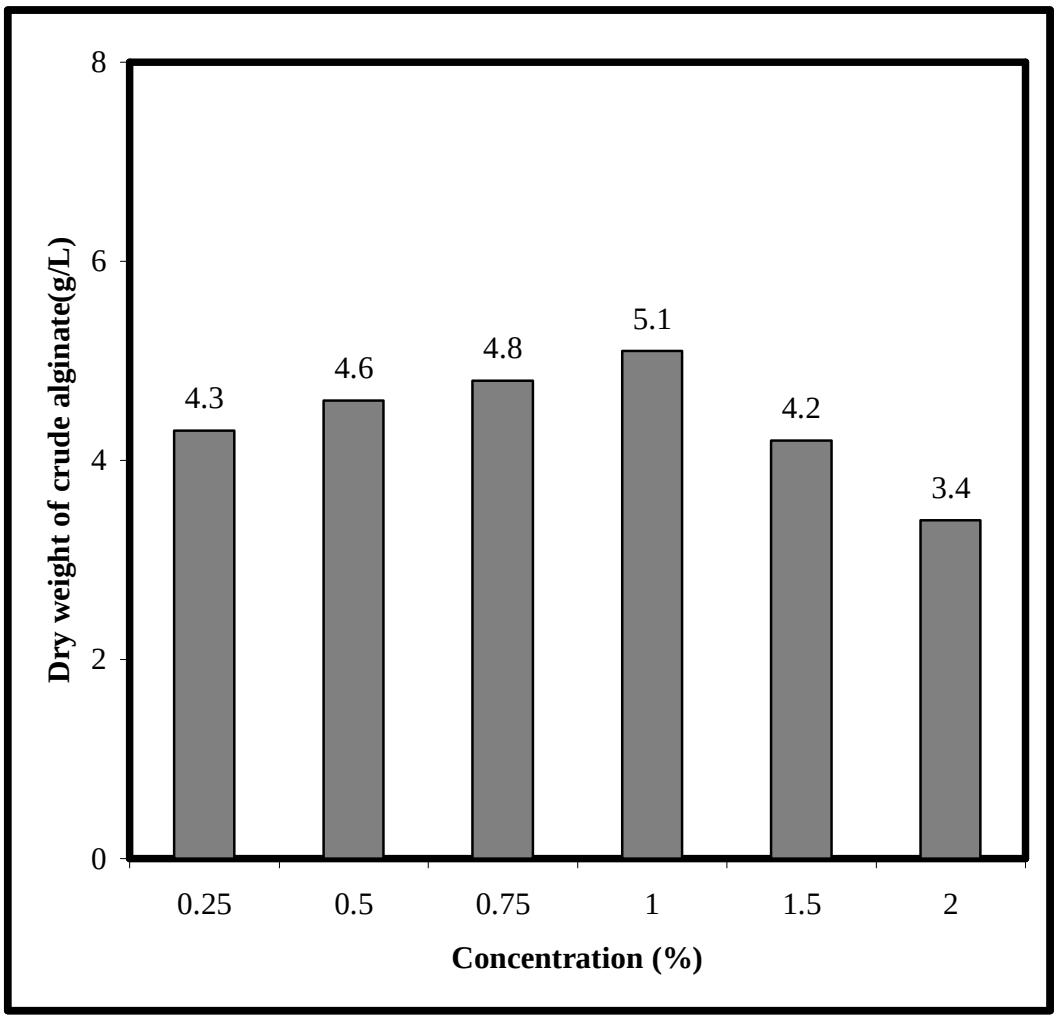


Figure (3-5) Effect of commercial baker's yeast concentration on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph7 for 48hrs at 37°C.

3.4.5 Phosphate source

In order to determine the optimum phosphate source for alginate production by the locally isolated *P.aeruginosa* H3, two types of phosphate sources KH_2PO_4 and K_2HPO_4 and a mixture of them (0.07% of KH_2PO_4 and 0.03% of K_2HPO_4) was used in a concentration of (0.1% w/v) in addition to control treatment (without phosphate source).

Results indicated in figure (3-6) showed that the maximum production of alginate was obtained when the production medium was consists of KH_2PO_4, which causes an increase in alginate production to (5.8g/L) in comparison with K_2HPO_4 or the mixture of them.

Horan *et. al*. (1981) found that a mixture of K_2HPO_4 (1g/L) and KH2PO4 (8mg/L) in the production medium gave a significant amount of alginate (2.2g/L) excreted to the culture medium.

On the other hand, results mentioned in figure (3-6) showed that the production of alginate in the control (4g/L) was better than the alginate production (3.2g/L) when K_2HPO_4 was used as a phosphate source. This result was previously mentioned by Jarman, (1979) who found that the alginate production was increased in the culture medium under limited phosphate concentrations.

The presence of phosphate in the culture medium works as a buffering capacity when the medium become acidic because of the biosynthesis of alginate (Brivonese and Sutherland, 1989) .

This optimum phosphate source (KH_2PO_4) was used in the next experiments of optimization for alginate production.

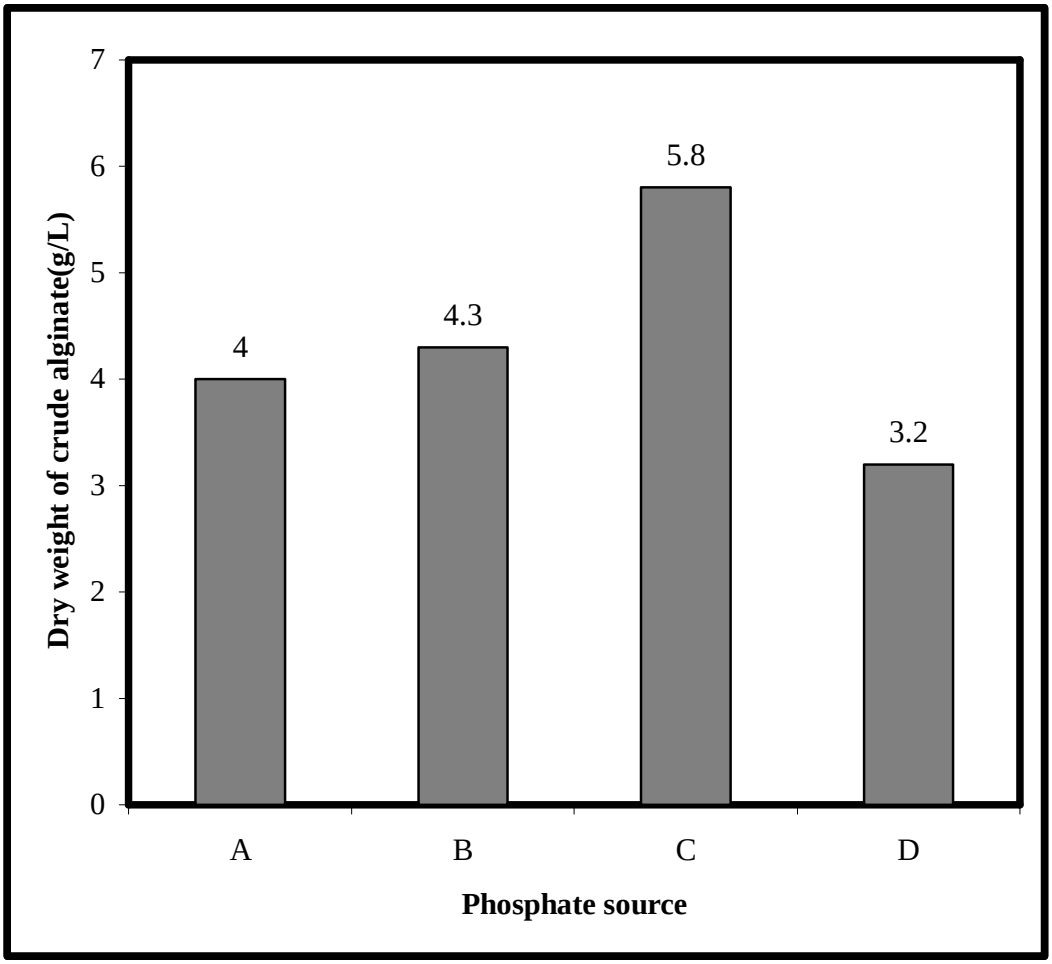


Figure (3-6) Effect of phosphate source on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 Rpm, Ph7 for 48hrs at 37°C.

(A) Control (without phosphate source).

(B) KH_2PO_4 (0.07%) + K_2HPO_4 (0.03%).

(C) KH_2PO_4 (0.1%).

(D) K_2HPO_4 (0.1%).

3.4.6 Concentration of phosphate source

Six concentrations (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3%) of KH_2PO_4 were used to determine the optimum for the alginate production by the locally isolated *P. aeruginosa* H3.

Results in figure (3-7) showed that the maximum production of alginate was obtained when KH_2PO_4 was used in a concentration of 0.05%, the dry weight of crude alginate extracted from the culture filtrate was 6.4 g/L .

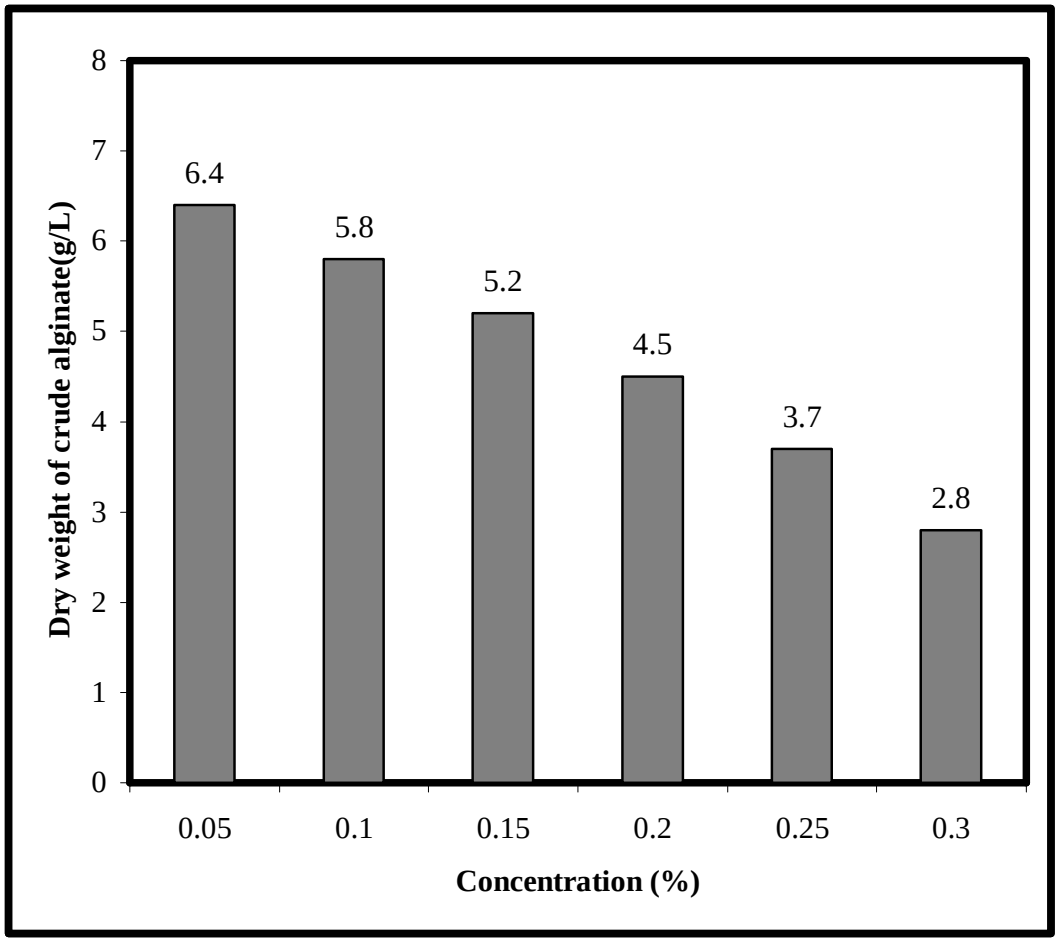


Figure (3-7) Effect of KH_2PO_4 concentration on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph7 for 48hrs at 37°C.

At this concentration of KH_2PO_4, the buffering capacity may makes the growth conditions suitable for alginate production. On the other hand, results in table (3-7) showed that there was a reversible relationship between KH_2PO_4 concentration and alginate production. This result proved with that obtained by Jarman, (1979) who found that the alginate production was increased under limited phosphate concentrations. Further more Horan *et al*., (1981) found that the high concentration of phosphate source may lead to decrease in alginate production because of the inhibition effects of high phosphate concentration on alginate biosynthesis enzymes.

This optimum concentration (0.05%) of KH_2PO_4 was used in the next experiments of optimization for alginate production.

3.4.7 Temperature

In order to determine the optimum temperature for alginate production by the locally isolated *P.aeruginosa* H3, different temperatures (30, 35, 37, 40°C) were used for this purpose.

As shown in figure (3-8) it was found that the maximum production of alginate was obtained when the temperature was (37˚C), at this temperature the dry weight of crude alginate extracted from culture filtrate was (6.4g/L), this may be because that this temperature was the optimum for growth and production of alginate, while the high temperature (40°C) led to decrease the bacterial growth rate and made the conditions unsuitable for alginate production, as it was mentioned by Stapper *et.al.,* (2004) who found that the optimum temperature for alginate production by *P. aeruginosa* was (37˚C).

This optimum temperature (37°C) was used in the next experiments of optimization for alginate production.

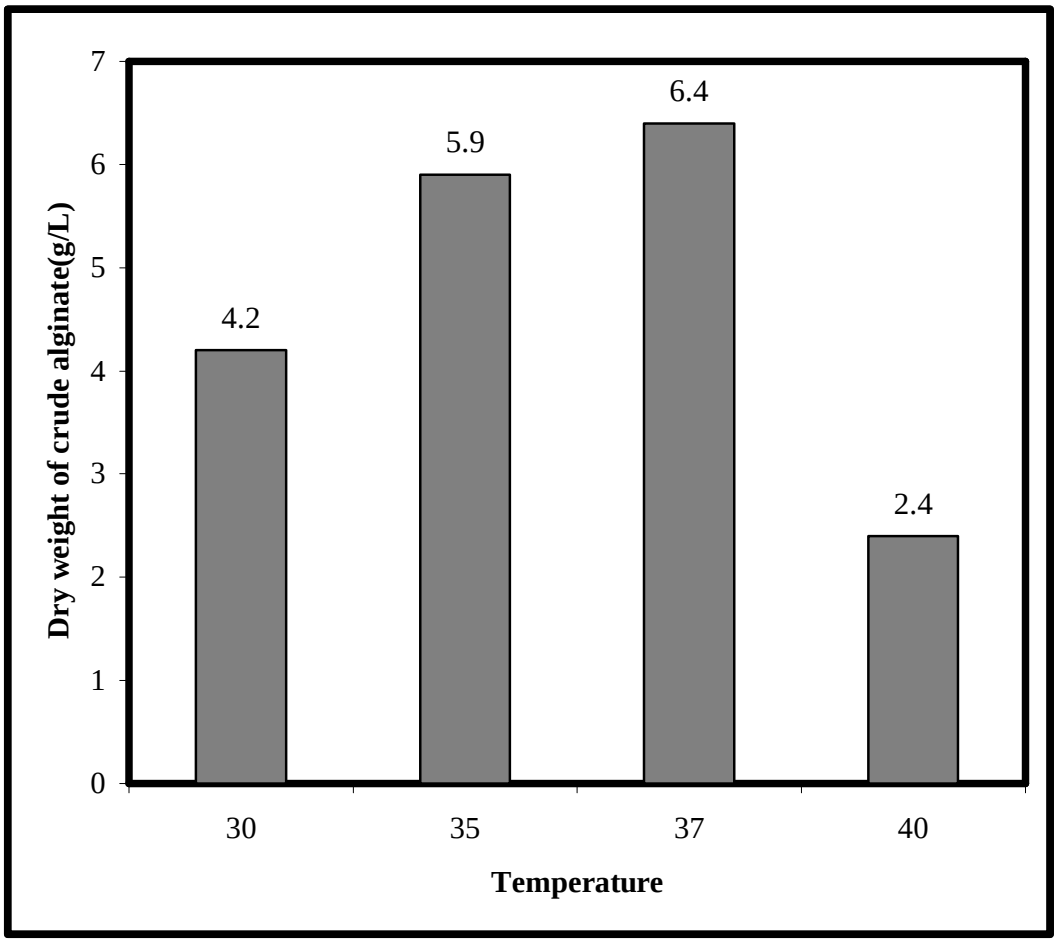


Figure (3-8) Effect of temperature on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph7 for 48hrs.

3.4.8 pH

In order to determine the optimum pH for production of alginate by the locally isolated *P.aeruginosa* H3, the following pH values were used for this purpose: (6.0, 6.5, 7.0, 7.5, 8.0).

As shown in figure (3-9) it was found that the maximum production of alginate was obtained when the pH value of the growth medium was adjusted to 7.0, the dry weight of crude alginate extracted from the culture filtrate was (6.4 g/L). This may because of this pH value may achieve the optimum conditions for bacterial growth and alginate production, especially the optimum pH for the activity of enzymes involved in alginate biosynthesis as it was mentioned by Haug and Larsen,(1971), and Brek and Larsen,(1985), while the increase or decrease of the pH value of the production medium above or under the optimum pH causes a significant decrease in alginate production, and this may because of the alteration of the activities of all enzymes responsible for alginate biosynthesis.

On the other hand, it was found that the optimum pH value for production of extracellular polysaccharides depends on the bacterial species, but it was almost near from neutral for most bacteria (Williams and Wimpenny, 1977) .

This optimum pH value (7.0) was used in the next experiments of optimization for alginate production.

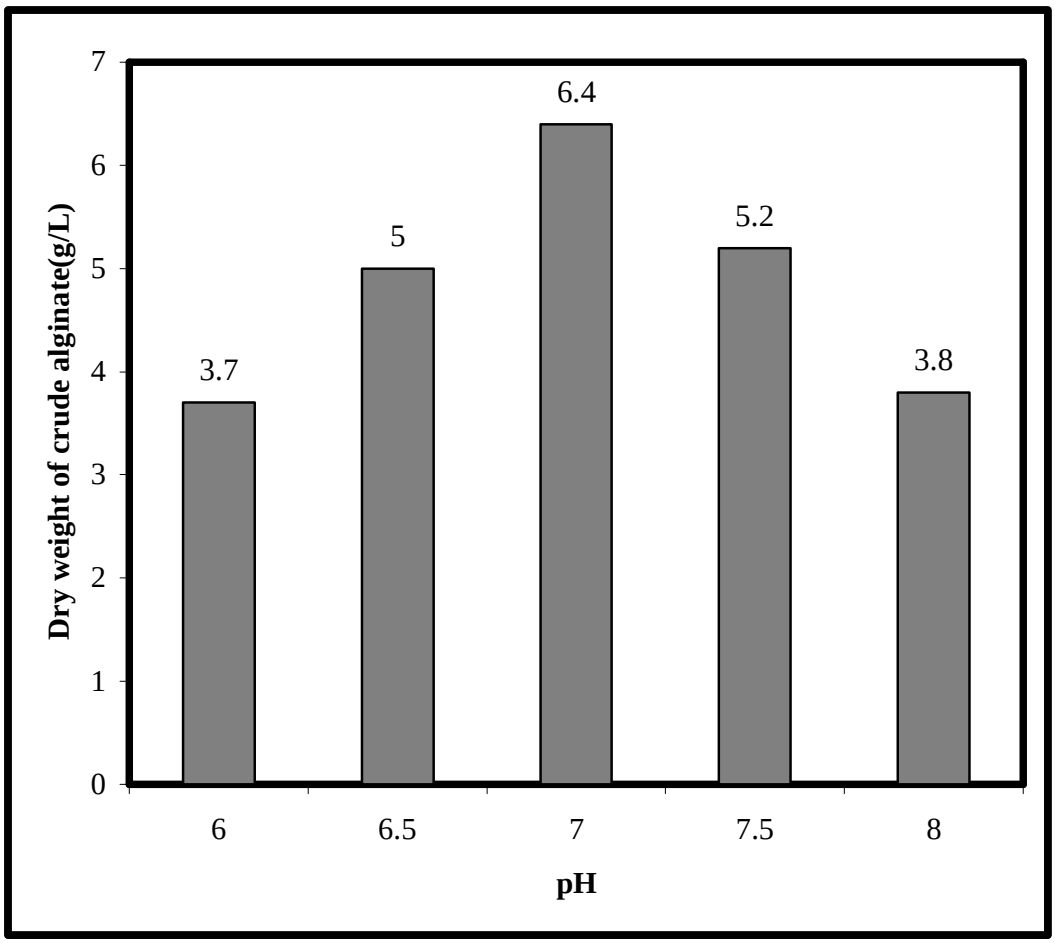


Figure (3-9) Effect of pH on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm for 48hrs at 37°C.

3.4.9 Inoculum size

Different inoculum sizes were used to determine the optimum for alginate production by the locally isolated *P.aeruginosa* H3.

Results in figure (3-10) showed that the maximum production of alginate was obtained when the growth medium was containing about (8×10^7 cell/100mL) of the inoculum, Using this count of viable cells in growth medium, the productivity of crude alginate reached (7.6 g/L). While the increase of the inoculum size over the optimum (8×10^7 cell/100mL) lead to decrease the alginate production under the same conditions, this may because of the competition between bacterial cells on the limited nutrients in growth medium that made the conditions unsuitable for alginate production.

The same thing was observed when the inoculum size was less than the optimum. In this case the lower production of alginate may be attributed to cell divisions to build-up more biomass for alginate production (Stapper *et.al.*,2004).

This optimum inoculum size (8×10^7 cell/100mL) was used in the next experiments of optimization for alginate production.

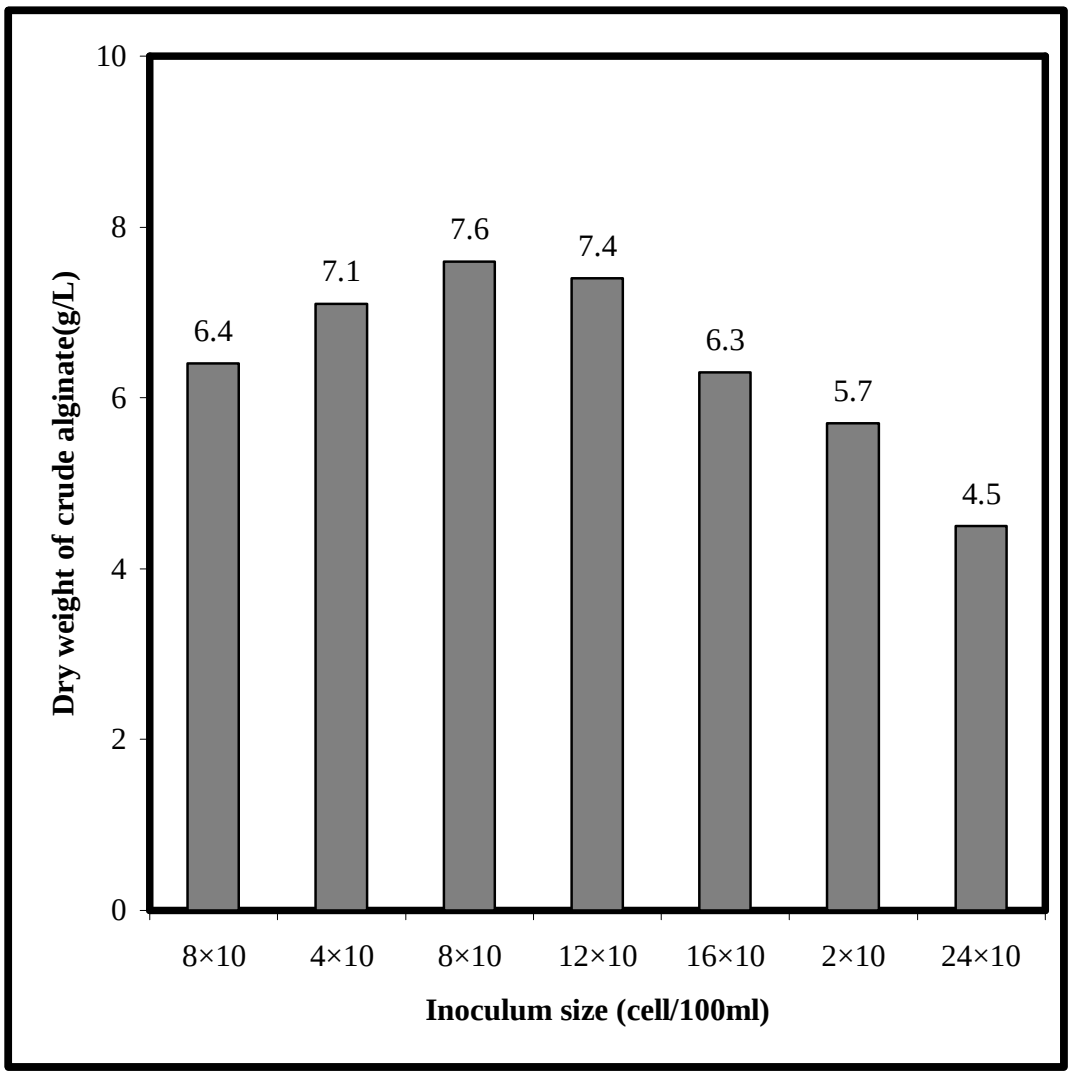


Figure (3-10) Effect of inoculum size on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph7 for 48hrs at 37°C.

3.4.10 Incubation time

To determine the optimum incubation time for alginate production by the locally isolated *P.aeruginosa* H3, six time periods (24, 48, 72, 96, 120, 144 hrs.) were used for this purpose.

As shown in figure (3-11) it was found that the maximum production of alginate was obtained when the culture medium was incubated for 96 hrs., after this period the dry weight of crude alginate produced in culture filtrate was (8.5 g/L). On the other hand, results indicated in figure (3-11) showed that the increase of the incubation period above the optimum period causes a significant decrease in alginate production, this may because of the exhausting of the nutrients in growth medium in addition to entrance the death phase, also the presence of alginate lyase which cleaves the polymer into short oligosaccharides result in the decrease of alginate production, while the decrease of the incubation period under the optimum also causes a significant decrease in alginate production, and this may because of these periods were insufficient to utilize all the nutrients for biomass and alginate production as it was mentioned by Pecina and Pancque, (1994).

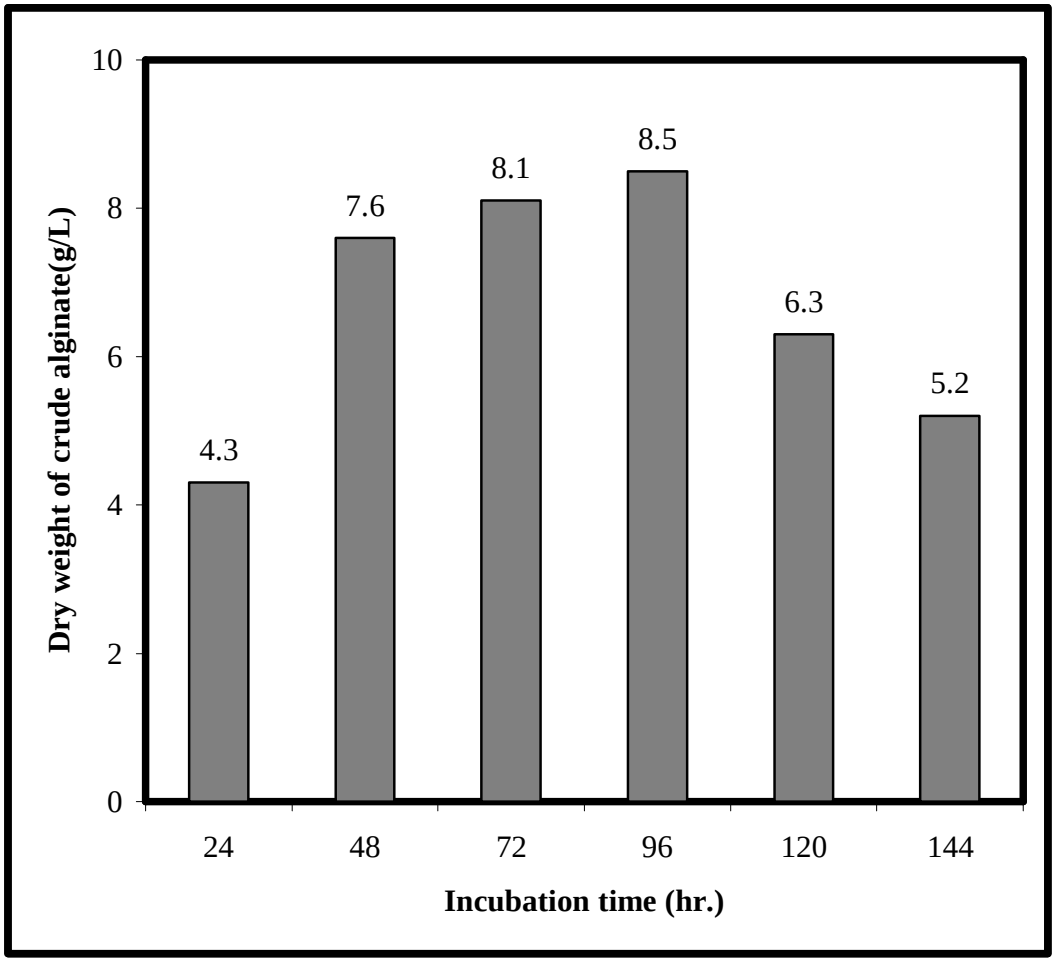


Figure (3-11) Effect of incubation time on alginate production by *P.aeruginosa* H3 after incubation in shaking incubator at 150 rpm, Ph7 and 37°C.

3.5 Purification of Alginate

Crude alginate produced by the locally isolated *P.aeruginosa* H3 was partially purified using gel filtration technique. First the crude alginate was dialyzed against distilled water with three increments of substitutions in order to remove different molecules of impurities, then the alginate solution was lyophilized and prepared to further purification by gel filtration technique. For this purpose 3ml of alginate solution was added to sepharose CL-6B 200 column prepared previously as in (2.2.10), and eluted by phosphate buffer saline.

Results in figure (3-12) showed that there was only one peak for alginate that refers to the first steps of purification by alcohol precipitation and dialysis of alginate solution.

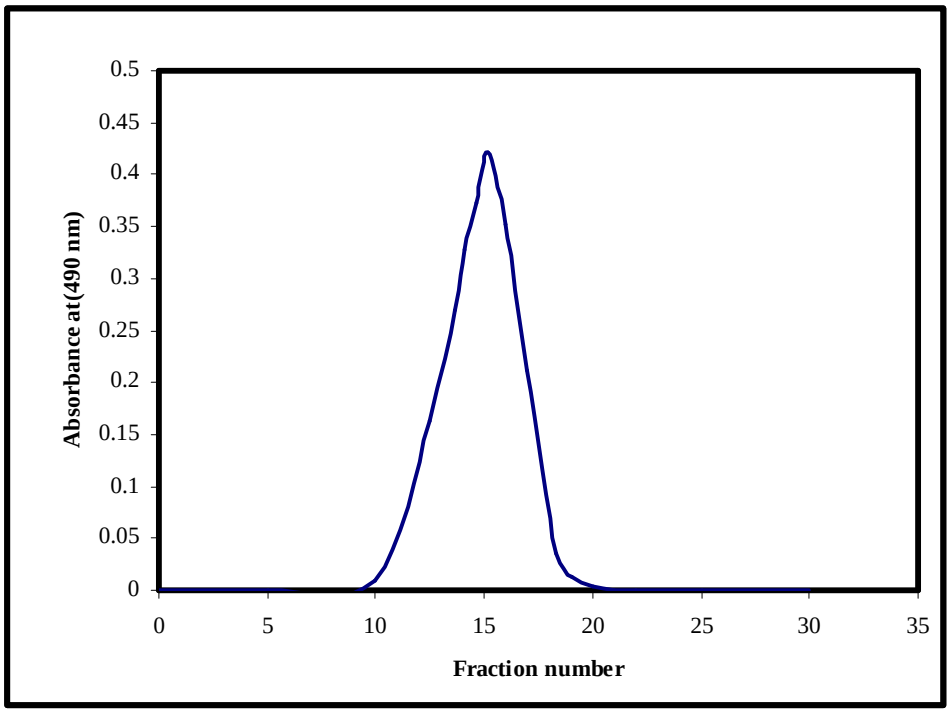


Figure (3-12) Gel filtration of alginate on sepharose CL-6B 200 column (2.7×15 cm), eluted with phosphate buffer saline (pH 7.3) at a flow rate (1ml/min.).

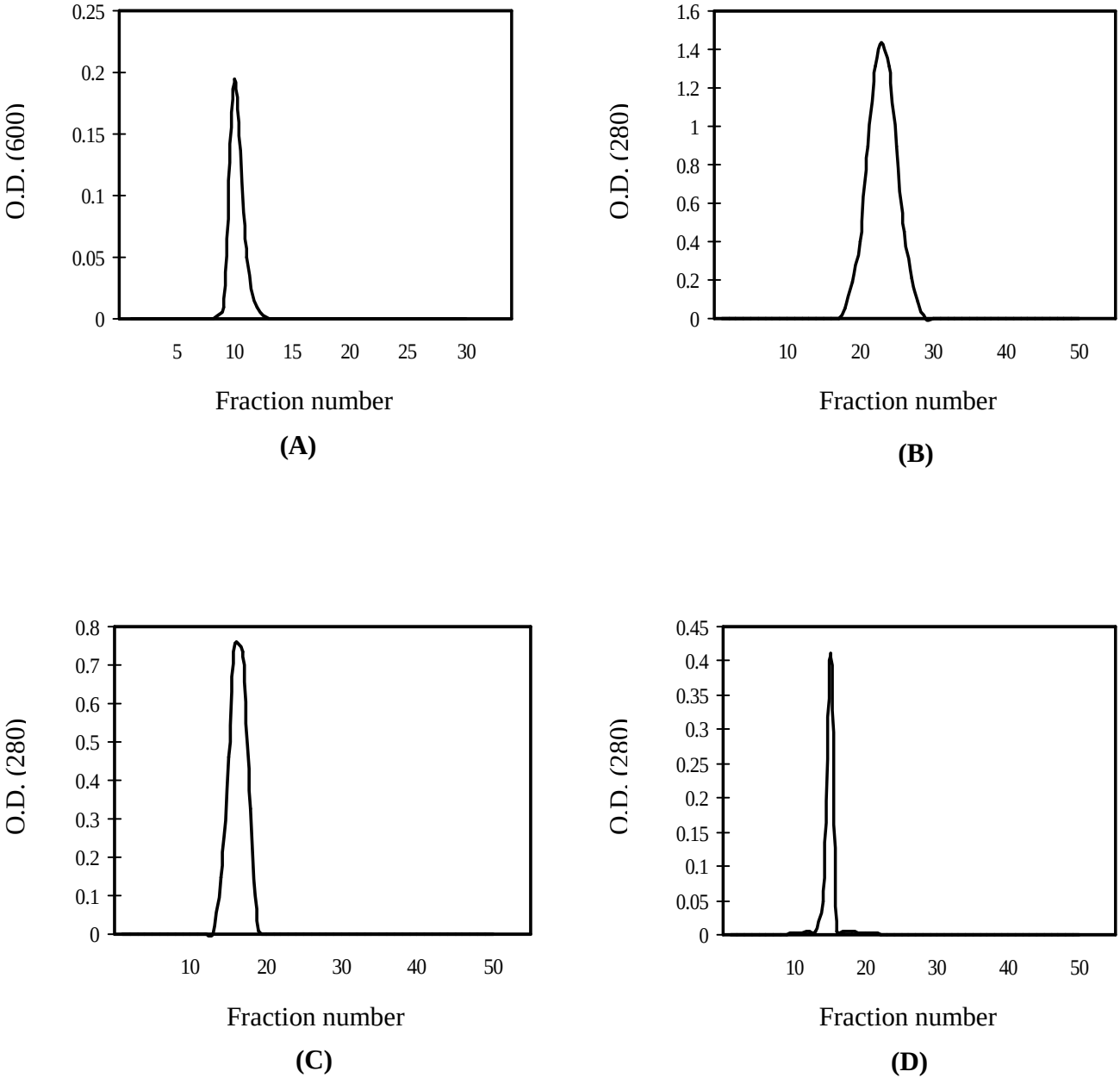
3.6 Molecular Weight Determination of Alginate by Gel filtration chromatography technique

Molecular weight of alginate was determined using gel filtration chromatography technique.The purified alginate and three standard proteins (lysozyme, bovine serum albumin and choline esterase) were transferred to sepharose CL-6B 200 column separately and eluted by phosphate buffer saline .

Void volume (V_o) of the column was calculated using blue dextran 2000 which was also transferred to the same column and eluted under the same conditions.

Results in figure (3-13) showed the diagram of elution of blue dextran and each standard protein, from this figure, column void volume (V_o) and elution volume (V_e) for each standard proteins was calculated.

In order to calculate alginate molecular weight, elution volume of alginate and other standard proteins was estimated to calculate the V_e/ V_o ratio for each of them (table 3-5), then the molecular weight of alginate was calculated throughout the selectively curve which represent the relation between the V_e/ V_o ratio and logarithm of molecular weight.



Figure(3-13) Gel filtration of (A) Blue dextran,(B) Lysozyme,(C)Human serum albumin and (D) Choline esterase on sepharose CL-6B 200 column(2.7×15 cm), eluted with phosphate buffer saline (pH 7.3) at a flow rate (1ml/min.).

Table (3-4) (V_e/V_o) ratio of standard proteins and purified alginate.

Standard proteins	Molecular weight (Dalton)	V_e	(V_e/V_o)
Lysozyme	14,000	69	2.3
Bovine serum albumin	67,000	54	1.8
Choline esterase	260,000	40	1.3
Purified alginate	-	45	1.5

Results obtained from the selectivity curve (figure 3-14) indicated that the molecular weight of alginate was about 141,253 dalton. This result was very close to that result obtained by Greenwood and Munro, (1979) who found that the molecular weight of alginate was 150,000 Dalton.

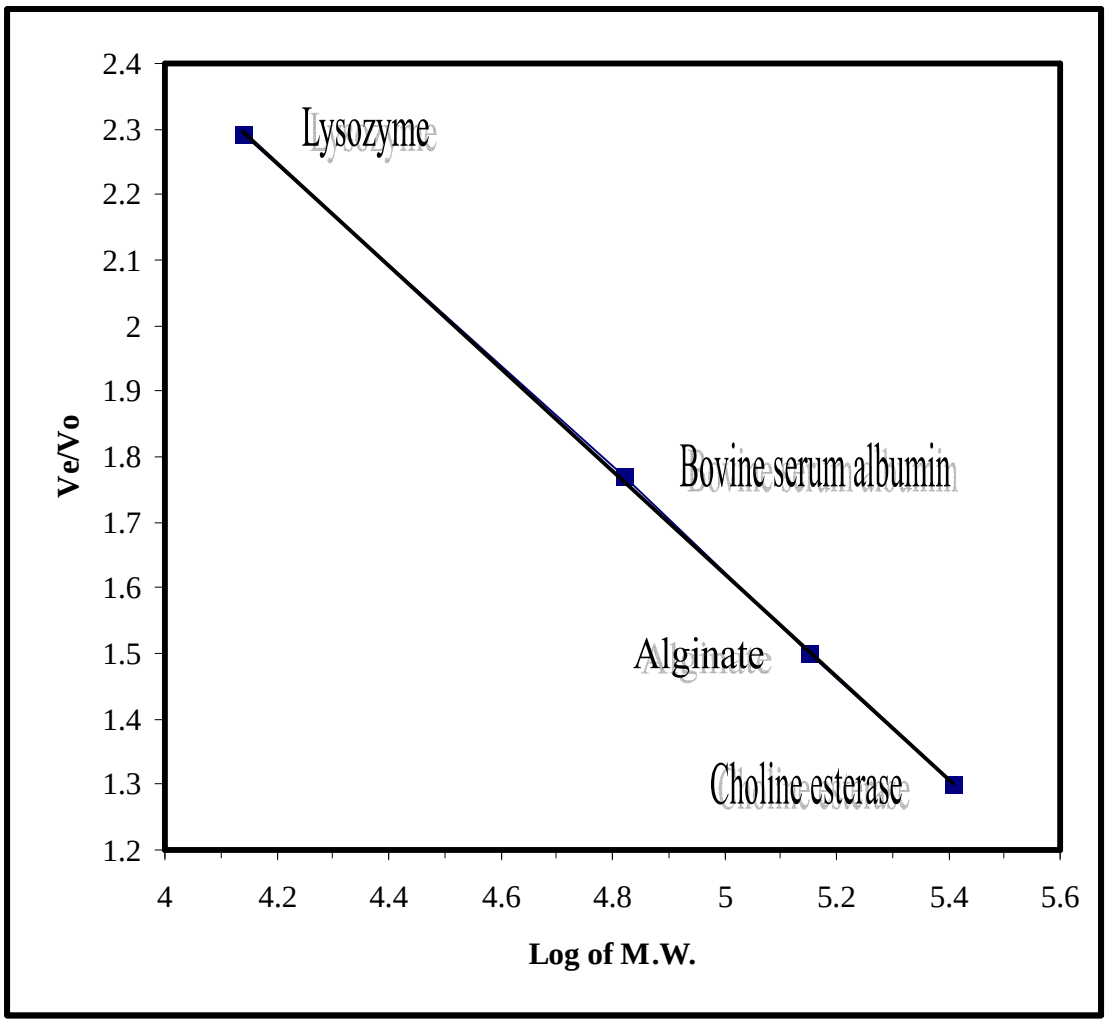


Figure (3-14) Selectivity curve for molecular weight determination of alginate from *P. aeruginosa* H3 by gel filtration technique.

Conclusions

1) Locally isolated *P.aeruginosa* H3 from cystic fibrosis patient was the efficient one in the production of alginate.
2) Optimum conditions for alginate production by the locally isolated *P.aeruginosa* H3 was obtained when the production medium was consists of 4% date extract, 1% commercial baker's yeast, 0.05% KH_2PO_4, pH 7 and incubated for 96 hrs. at 37°C with inoculum size of 8×10^7.
3) Crude alginate produced by *P.aeruginosa* H3 was simply purified throughout isopropanol precipitation, and gel filtration chromatography.
4) Molecular weight of purified alginate produced by the locally isolated *P.aeruginosa* H3 was about 141,253 Dalton.

Recommendations

1) Improvement of *P.aeruginosa* H3 ability in alginate production by physical and chemical mutagenesis.
2) Large scale production of alginate by *P.aeruginosa* H3 throughout the application of optimum conditions for production.
3) Cloning of alginate genes in suitable vector and transferred in *E. coli* for large scale production.

2. Materials and Methods

2.1 Materials

2.1.1 Equipment and Apparatus

The following equipments and apparatus were used in this study:

Equipment	**Company (origin)**
Autoclave	Gallenkamp (England)
Compound microscope	Olympus (Japan)
Cooled centrifuge	Harrier (U.K.)
Distillator	Gallenkamp (England)
Freeze-dryer(Lyophilizer)	Virtis (U.S.A.)
Hot plate with magnetic stirrer	Gallenkamp (England)
Incubator	Gallenkamp (England)
Oven	Gallenkamp sanyo (U.K.)
pH-meter	Metter-GmpH Toledo (U.K.)
Sensitive balance	Sartorius (Germany)
Shaker incubator	GFL (Germany)
Spectrophotometer	Aurora instrument Ltd.(U.K.)
UV lamp	Vilber Lourmat (France)
Vortex	Stuart scientific (U.K.)
Water bath	Memmert (Germany)

2.1.2 Chemicals

The following chemicals were used in this study:

Material	Company (origin)
K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.9H_2O$, Na_2HPO_4, $Na_2Mo_4.2H_2O$, Peptone, $MgCl_2$, K_2SO_4, Glucose, Sucrose, Fructose, Na_2CO_3, $CaCl_2$, NaOH, Maltose, Ammonium sulphate, KCl	BDH (England)
Gelatin, Yeast extract	Biolife (Italy)
Agar, Hydrogen peroxide, N,N,N,N-tetramethyl-p-phynylene-diamine dihydrochloride	Difco(U.S.A)
NaCl, crystal violet	Fluka(Switzerland)
H_2SO_4, HCl,	Hopkin and Williams(U.K)
Tryptone	Oxoide (U.K)
Cetrimide, Glycerol, Isopropanol, Phenol, Ammonium chloride	Riedel-Dehaeny-(Germany)
Sepharose CL-6B 200, Blue dextrane, Choline esterase, Lysozyme, Bovine serum albumin,	Sigma (U.S.A)
Date extract	Kerbala canning co. (Iraq)
Commercial yeast (Saf-instant)	S.I.Lesaffre (France)

2.1.3 Api 20E Kit (Api Bio Merieux, Lyon, France)

Api 20E Kit consist of :

(A) Galleries: the gallery is a plastic strip with 20 microtubes containing dehydrated reactive ingredients.

(B) Api 20E reagents :

- Oxidase reagent (1% tetra-methyl-p-phenyle-diamine)
- Kovac's reagent (p-dimethyl aminobenzaldehyde at 4% in HCl isoamyl alcohol).
- Voges-Proskauer reagent:
  - Vp1 (40% potassium hydroxide).
  - Vp2 (6% alpha-naphthol).
- Ferric chloride 10%.

2.1.4 Media

2.1.4.1 Ready to Use Media

These media were prepared as recommended by manufacturing company. The pH was adjusted to 7.0 and autoclaved at 121°C for 15 min.

- MacConkey agar (Oxoid-England).
- Nutrient agar (Oxoid-England).
- Nutrient broth (Oxoid-England).
- Simmon citrate agar (Difco-U.S.A).

2.1.4.2 Synthetic Media

- Cetrimide agar (Stolp and Gadkari, 1984).

Peptone	20g
$MgCl_2$	4.5g
K_2SO_4	10g
Cetrimide	0.3g
Agar	15g
Distilled water	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving.

- King A (Starr *et al*., 1981).

Peptone	20g
K_2SO_4	10g
$MgCl_2$	1.4g
Glycerol	10ml
Agar	15g
Distilled water	1000ml

pH was adjusted to 7.2 and sterilized by autoclaving.

- King B (Starr *et al*., 1981).

Peptone	20g
$MgSO_4.7H_2O$	3.5g
K_2SO_4	1.5g
Glycerol	10ml
Agar	15g
Distilled water	1000ml

pH was adjusted to 7.2 and sterilized by autoclaving..

- Gelatin medium (Stolp and Gadkari, 1984).

Gelatin	4g
K_2HPO_4	0.5g
KH_2PO_4	0.5g
Glucose	0.05g
Distilled water	1000ml

pH was adjusted to 7.0 and sterilized by autoclaving.

- Alginate production medium (Thompson and Skerman, 1979)

K_2HPO_4	0.3g
KH_2PO_4	0.7g
$MgSO_4.7H_2O$	0.2g
$CaCl_2.2H_2O$	0.1g
$FeSO_4.9H_2O$	0.05g
$Na_2Mo_4.2H_2O$	0.007g
Sucrose	10g
Yeast extract	5g
Distilled water	1000ml

pH was adjusted to 7.0 and sterilized by autoclaving.

2.1.5 Reagents

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

  This reagent composed of (3%) hydrogen peroxide.

- **Oxidase reagent (Atlas *et al*., 1995)**

One gram of N,N,N,N-tetramethyl-p-phynylene-diamine dihydrochloride was dissolved in 100 ml distilled water and kept in dark bottle at 4°C.

2.1.6 Solutions and Buffers

- **Phosphate buffer saline (pH 7.3)**

This solution was prepared by dissolving (8g) NaCl, (0.2g) KCl, (0.2g) KH_2PO_4, (1.15g) Na_2HPO_4 in (1000 ml) of distilled water, pH was adjusted to (7.3) and sterilized by autoclaving (Cruikshank *et al.*, 1975).

- **Phenol solution (5%)**

This solution was prepared by dissolving (5g) of phenol in (100 ml) distilled water.

- **Calcium chloride solution (0.25 M)**

This solution was prepared by dissolving (0.27g)of calcium chloride in (20 ml) distilled water.

- **Blue dextrane 2000 solution (4 mg / ml)**

This solution was prepared by dissolving (0.02g) of blue dextrane in (5 ml) of phosphate buffer saline (pH 7.3).

- **Solutions of standard proteins**

1. Lysozyme solution (3mg / ml)

This solution was prepared by dissolving (9mg) of lysozyme in (3 ml) of phosphate buffer saline (pH 7.3).

2. Bovine serum albumin solution (3mg / ml)

This solution was prepared by dissolving (9mg)of bovine serum albumin in (3 ml) of phosphate buffer saline (pH 7.3).

3. Choline esterase solution (3mg / ml)

This solution was prepared by dissolving (9mg) of choline esterase in (3 ml) of phosphate buffer saline (pH 7.3).

2.2 Methods

2.2.1 Sterilization methods

- **Autoclaving**

Media and solutions were sterilized by autoclaving at 121°C for 15 min.

- **Oven sterilization**

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

2.2.2 Isolation of *P. aeruginosa*

2.2.2.1 Sample collection

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

- **Clinical samples**

Blood samples from patients suffering from septicemia, sputum samples, samples from burns, in addition to samples from ears and cystic fibrosis infections were collected from Al-Yarmouk hospital and Al-Karama hospital in Baghdad governorate.

- **Environmental samples**

Water and soil samples were collected from Al-Yarmouk hospital and Al-Karama hospital in Baghdad governorate.

2.2.2.2 Sample preparation

Clinical samples were grown 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:

- **Water samples**

One-milliliter of each sample was diluted by (9ml) of sterilized distilled water in test tubes, mixed thoroughly, serial dilutions for each tube were done separately, then (100µl)aliquots from the appropriate dilution (10^{-3}) were taken out and spreaded on MacConkey agar plates and incubated at 37°C for 18 hours.

- **Soil samples**

One-gram of each soil sample was added to (9ml) of sterilized distilled water in test tubes, mixed thoroughly, serial dilution for each tube were done separately, then (100µl) aliquots from the appropriate dilution (10^{-3}) 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 were selected and streaked on cetrimide 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 staining ability, shape, color, size, production of pigments, transparency and mucoid properties of the colonies growing on nutrient agar and MacConkey agar plates, and some biochemical tests were achieved as follows:

- **Gram's stain (Harely and Prescott, 1996)**

Single colony of each isolate was transferred and smeared on a clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with absolute alcohol, and counterstained with safranine, then examined under light microscope.

- **Catalase Test**

This test was performed by adding few drops of hydrogen peroxide (3%) on a single colony of each isolate grown on nutrient agar. The production of gaseous bubbles indicates a positive result.

- **Oxidase Test**

Filter paper was saturated with oxidase reagent, then single colony of each isolate was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates a positive result.

- **Citrate utilization**

This test was used to examine the ability of *P. aeruginosa* to utilize citrate as a sole source of carbon and energy. In this test, a colony of each

isolate was inoculated on to the surface of simmon citrate slant and the medium was incubated overnight at 37°C. The appearances of blue color indicates a positive result.

- **Gelatin hydrolysis test**

This test was performed to demonstrate the ability of bacterial isolate to hydrolyze gelatin. Tubes of gelatin media were inoculated with each isolate by stabbing, and then the tubes were incubated at 37°C for five days. Liquification of gelatin indicates a positive result.

- **Growth on King A**

Single colony of each isolate was streaked separately on King A agar medium and incubated at 37°C for 24 hrs. to examine the isolates ability in pyocyanin pigment production.

- **Growth on King B**

Single colony of each isolate was streaked separately on King B agar medium and incubated at 37°C for 24 hrs. Then the plates were exposed to U.V. light to examine the isolates ability in fluorescent pigment production.

2.2.4 Identification of *P. aeruginosa* Isolates using Api 20 E system

Local isolates that have the 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.

- **Preparation of the strip**

Five-milliliter of distilled water dispensed into the honeycombed wells of the tray in order to provide a humid atmosphere during incubation.

- **Preparation of the inoculum**

A single, pure isolated colony was picked up from plating medium of nutrient agar. This colony was suspended in a test tube containing 5 ml of sterilized distilled water and mixed thoroughly.

- **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 inoculations, the plastic lid was placed on the tray and inoculated at 37°C for 24 hr.

- **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 10% 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 Bacterial Isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al*.,(1982) as following:

- **Short term storage**

  Bacterial isolates were maintained for period of few weeks on MacConkey agar plates . The plates were tightly wrapped in parafilm and stored at 4°C.

- **Medium term storage**

  Bacterial isolates were maintained in stab culture for period of few months. Such cultures were prepared in screw-capped bottles containing (5-8 ml) of agar medium and stored at 4°C.

- **Long term storage**

Bacteria can be stored for many years in nutrient broth containing 15% glycerol at low temperature without significant loss of viability. This was done by adding (1.5 ml) of sterilized glycerol to an exponential growth of bacterial isolates in a screw-capped bottle with final volume (10 ml) and stored at -20°C.

2.2.6 Screening the ability of *P. aeruginosa* Isolates in Production of Alginate

To examine the ability of local isolates of *P. aeruginosa* in alginate production, the production medium described by (Thompson and Skerman, 1979) was used for this purpose, by inoculating (100 ml) of the production

medium with (100 µl) of fresh culture of each isolate separately and incubated in shaker incubator (150 rpm at 37°C for 48 hrs).

2.2.7 Extraction and Dry Weight Determination of Crude Alginate

Separation of alginate from culture medium was performed by centrifugation of 10ml from the culture medium after incubation for 48hrs. in cooled centrifuge at 6000 rpm in 4°C for 20 min., the supernatant was then used as a crude alginate.

The dry weight of crude alginate was determined by alcohol precipitation method as described by (Jarman *et al*., 1978) by adding 3 volumes of isopropanol to 1 volume of crude alginate, then they were mixed vigorously and left to stand for (10 min.), after that the contents was filtered using filter paper (Whatman No.1), then the filter paper containing the crude alginate precipitate was dried in oven at (40-45°C) for 16 hrs, and the dry weight of crude alginate was determined.

2.2.8 Qualitative Detection of Alginate

For qualitative detection of alginate, the following method was performed as described by (British pharmacopeia, 1980).

Ten-milliliter of distilled water was added to (0.1g) of crude alginate and (0.25g) of sodium carbonate, then the mixture was filtered by filter paper (Whatman No.1). After that (0.5ml) of calcium chloride (0.25 M) was added to the filtrate, the formation of gelatinous precipitate indicates the presence of alginate.

2.2.9 Optimum Conditions for Alginate Production

In order to determine the optimum conditions for alginate production, the selected isolate termed *P. aeruginosa* H3 which was the best among the different isolates in alginate production, was used for production. Optimum conditions includes carbon source and it's concentration, nitrogen source and it's concentration, phosphate source and it's concentration, temperature, pH, inoculum size and incubation time.

2.2.9.1 Carbon source

Five different carbon sources were used to determine the optimum for alginate production (sucrose, fructose, maltose, glucose and date extract).These carbon sources were added in a concentration of (2% w/v) for sucrose, fructose, maltose and glucose, and (2% v/v) for date extract.

2.2.9.2 Concentration of carbon source

Date extract was selected as a good carbon source for alginate production by *P. aeruginosa* H3 therefore it was used at different concentrations (2, 4, 6, 8, 10 % v/v) to determine the optimum concentration for production of alginate.

The total carbohydrate ratio in date extract was determined by phenol–sulfuric acid method (Dubois *et al.,* 1956) according to the standard curve of glucose as following:

1. Standard curve of glucose by phenol–sulfuric acid method

Stock solution of glucose (80 µg /ml) was prepared by dissolving (0.008 g) of glucose in (50 ml) of distilled water, then the volume completed to (100 ml) in a volumetric flask.

Then the following volumes of stock solution were added in a test tubes, and appropriate volumes of distilled water were added as the following table:

Tube no.	Stock glucose solution (ml)	Added distilled water (ml)	Glucose concentration (µg / ml)
1	0	1.0	0
2	0.1	0.9	8
3	0.3	0.7	24
4	0.5	0.5	40
5	0.7	0.3	56
6	1.0	0	80

Then (1 ml) of (5 %) phenol solution was added to each tube and mixed well, followed by the addition of (5 ml) of sulfuric acid and mixed vigorously and left to stand at room temperature. The absorbance at (490nm) for each tube was measured and the first tube was used as blank.

The standard curve was drown according to the relationship between the absorbance at (490nm) and glucose concentration (µg / ml) as shown in Figure (2-1).

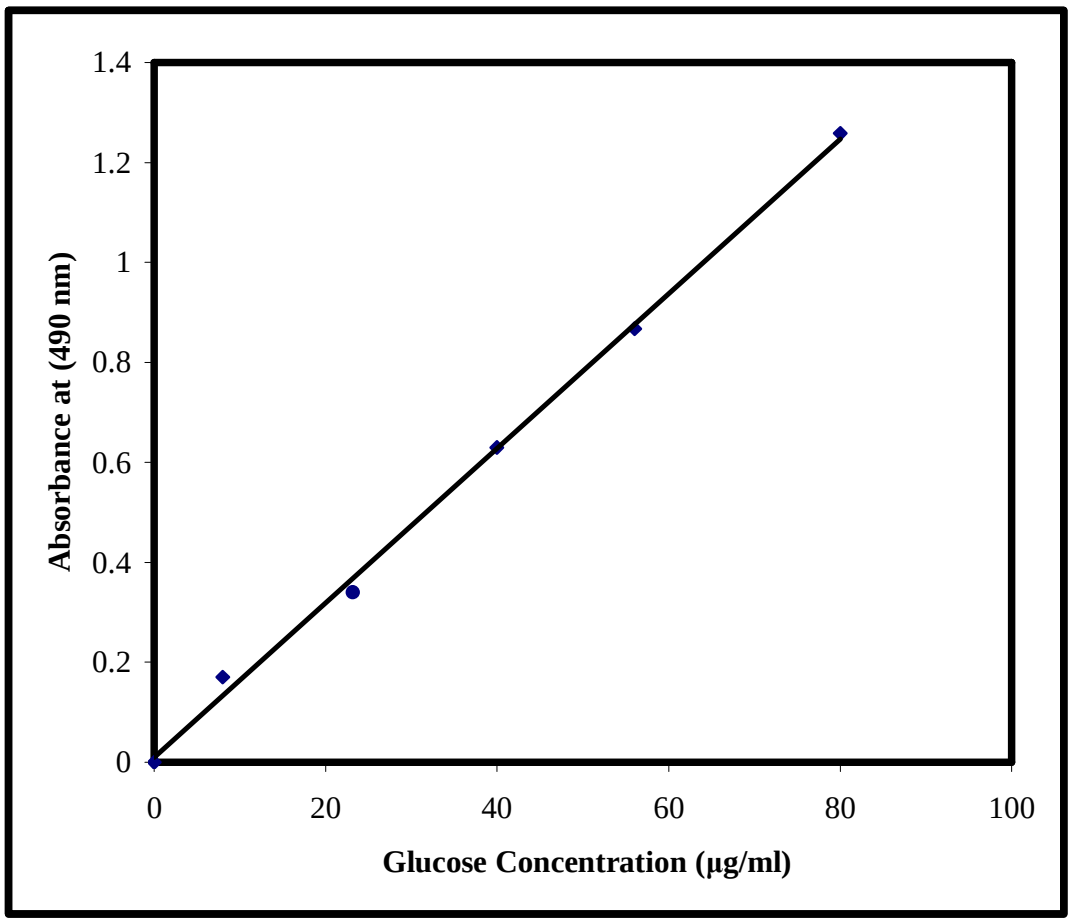


Figure (2-1) Standard curve of glucose by phenol–sulfuric acid method.

2. Total carbohydrate ratio determination

The total carbohydrate ratio in date extract was determined by phenol–sulfuric acid method that mentioned in the above steps, the concentration of total carbohydrate was measured according to the standard curve of glucose, also this method was used in the molecular weight determination of alginate (Horan *et al.*, 1981).

2.2.9.3 Nitrogen source

Six nitrogen sources were used (yeast extract, peptone, tryptone, ammonium sulphate,ammonium chloride and commercial baker's yeast(Saf-instant)).These sources were added at a concentration of (0.2 % w/v) at 37ºC, pH 7 for 48 hr.

2.2.9.4 Concentration of nitrogen source

Six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, 2.0 % w/v) of the appropriate nitrogen source were used to determine the optimum concentration for alginate production at 37ºC, pH 7 for 48 hr.

2.2.9.5 Phosphate source

In order to determine the optimum phosphate source for alginate production, two types of phosphate sources (KH_2PO_4 and K_2HPO_4) and a mixture of them (0.07% of KH_2PO_4 and 0.03% of K_2HPO_4) were used in a concentration of (0.1% w/v), also in the absence of phosphate source (control) at 37ºC, pH 7 for 48 hr.

2.2.9.6 Concentration of phosphate source

Six concentrations (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 % w/v) of the appropriate phosphate source were used to determine the optimum concentration for alginate production at 37ºC, pH 7 for 48 hr.

2.2.9.7 Temperature

In order to determine the optimum temperature for alginate production, four different temperatures (30, 35, 37, 40°C) were used for this purpose at pH 7 for 48 hr.

2.2.9.8 pH

In order to determine the optimum pH for production of alginate, the following pH values were used for this purpose: (6.0, 6.5, 7.0, 7.5, 8.0) at 37ºC for 48 hr.

2.2.9.9 Inoculum size

Different inoculum sizes were used to determine the optimum inoculum size for alginate production and as follow: One-milliliter of fresh culture was diluted by (9ml) of sterilized distilled water in test tube, mixed thoroughly, serial dilutions were done, then (100µl) aliquots from each dilution was taken out and spreaded on nutrient agar plates and incubated at 37°C for 18 hours, then viable count was done from the appropriate dilution and multiply by inverse dilution.

(8×10^6, 4×10^7, 8×10^7, 12×10^7, 16×10^7, 2×10^8, 24×10^7) cell / 100ml of production medium at 37ºC, pH 7 for 48 hr.

2.2.9.10 Incubation time

In order to determine the optimum incubation time for alginate production, six time periods (24, 48, 72, 96, 120, 144 hrs.) were used for this purpose at 37ºC and pH 7.

2.2.10 Purification of Alginate

After determined the optimum conditions for alginate production, the following purification steps were performed to purify alginate produced by the locally isolated *P. aeruginosa* H3.

- **Alcohol Precipitation**

Alginate in crude filtrate was precipitated according to alcohol precipitation method described by (Jarman *et al.*, 1978) by adding three volumes of isopropanol to one volume of crude filtrate, then the mixture was mixed vigorously and filtered by filter paper (Whatman No.1), after that the sample was dried in an oven at (40-45°C).

The precipitate was dissolved in a suitable volume of distilled water and dialyzed against distilled water with three increments of substitutions at (4°C)for 24 hrs.

After that crude alginate was lyophilized by using a Lyophilizer, and kept for the next step of purification (Anderson *et al.*, 1987).

- **Gel Filtration Chromatography**

Crude alginate was purified by gel filtration chromatography technique using Sepharose CL-6B 200 which was prepared according to the instructions of manufacturer (Sigma). Gel matrix was poured in a column to give a dimensions of (2.7×15 cm), the column was washed and equilibrated for (24 hrs.) with phosphate buffer saline (pH 7.3) at a flow rate of (1ml/min.), then (3 ml) of crude alginate (3mg/ml) was added to the column accurately and eluted with phosphate buffer saline (pH 7.3) at a flow rate of (1 ml/min..).

Fractions of (3 ml) were collected and the absorbency at (490nm) for each fraction was measured by phenol-sulfuric acid method to determine the elution volume(V_e) of alginate fraction (Brek and Larsen, 1985).

2.2.11 Molecular Weight Determination of Alginate by Gel filtration chromatography technique

- **Determination of the void volume(V_o) of the column**

The column was equilibrated and washed for (24 hrs.) with phosphate buffer saline (pH 7.3)at a flow rate of (1 ml/min.). A 5 ml of blue dextrane 2000 solution was passed through the column, then eluted with phosphate buffer saline (pH 7.3). Fractions of (3 ml) were collected and the absorbency at (600nm) for each fraction was measured. The void volume was determined by estimation of total volume of the fractions as characterized with start point movement of the blue dextrane that of the climax of absorbency of the blue dextrane (Cote and Krull, 1988).

- **Determination of standard proteins elution volume (V_e)**

Three ml of the following standard proteins were applied through the column separately, then eluted with phosphate buffer saline (pH 7.3) (Table 2-1).

Table (2-1) Standard proteins.

Standard proteins	Molecular weight(Dalton)
Lysozyme	14,000
Bovine serum albumin	67,000
Choline esterase	260,000

The elution volume (V_e) was estimated for each standard protein by measuring the absorbency of the separated fractions at (280nm).

The (V_e /V_o) ratio was calculated for each standard protein and for the purified alginate, then standardization was done by plotting the elution volume (V_e) of each standard protein to the void volume (V_o) of the blue dextrane 2000 (V_e/V_o) versus the log value of molecular weight (Stellwagen, 1990).The molecular weight of alginate accordingly was calculated.

Chapter One

Introduction and Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Contents

Chapter Two: Materials and Methods

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Mean
O. D.	Optical Density
G	α-L-glucuronic acid
M	β-D-mannuronic acid
N. M.	Nanometer
TNF-α	Tumer necrosis factor-α
IL-1	Interleukin-1
LPS	Lipopolysaccharide
GDP	Guanosine diphosphate
M.W.	Molecular weight
V_e	Elution volume
V_o	Void volume
Rpm	Round per minute
Sp.	Species
Spp.	Specieses
U.V.	Ultraviolet

Ministry of Higher Education
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Production and Characterization of Alginate Produced by the Locally Isolated *Pseudomonas aeruginosa*

A Thesis
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By

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Summary

A total of 37 samples were collected from different clinical cases (ear, blood, sputum, burns, cerebrospinal fluid and cystic fibrosis samples) and environments (water and soil samples)from different locations in Baghdad governorate. The total isolates obtained from these samples were 57 isolates,13 of them were identified as *Pseudomonas* spp. after detecting it's ability to grow on cetrimide agar. Biochemical tests were carried out on these 13 isolates. Results showed that 6 of these isolates were identified as *Pseudomonas aeruginosa*, furthermore it has been proved using Api 20 E system.

The ability of these isolates in alginate production was examined. Results showed that *P.aeruginosa* H3 was the efficient one in alginate production, the productivity of alginate from this isolate was 1.2 g / L.

Optimum conditions for alginate production by locally isolated *P.aeruginosa* H3 were studied. Results showed that the optimum conditions for alginate production were achieved using the production medium that contains 4% of date extract (as a carbon source), 1% of commercial baker's yeast (as a nitrogen source), 0.005% of KH_2PO_4 (as a phosphate source), at pH 7, the production medium was inoculated with 8×10^7 cell/100 ml and it was incubated at 37° C for 96 hrs. the productivity of alginate under these conditions was 8.5 g/ L.

Alginate produced by *P.aeruginosa* H3 was purified by two steps, the first step included isopropanol precipitation and dialysis, the second was gel filtration through Sepharose CL-6B 200. The molecular weight of the purified alginate was determined using gel filtration technique through Sepharose CL-6B 200. The results showed that the approximate molecular weight of alginate produced by locally isolated *P.aeruginosa* H3 was 141,253 Dalton.

الإهداء

إلى الروح التي كانت ترعاني ولا زالت
إلى الروح التي في الجنة سكنت
إلى الروح التي للقياها قد اشتقت
روح أبي
إلى منبع التضحية والحب والحنان
أمي
إلى سنين عمري
إخوتي وأخواتي

اهدي ثمرة جهدي المتواضع

حسن

بسم الله الرحمن الرحيم

قَالُوا۟ سُبْحَٰنَكَ لَا عِلْمَ لَنَآ إِلَّا مَا عَلَّمْتَنَآ ۖ إِنَّكَ أَنتَ ٱلْعَلِيمُ ٱلْحَكِيمُ

صدق الله العظيم

سورة البقرة
الآية:٣٢

الخلاصة

جمعت ٣٧عينة من مختلف الحالات المرضية (إصابات الأذن، الدم، البلغم، الحروق، سائل النخاع الشوكي والتكيس الرئوي) والبيئية (مياه المجاري والتربة) من مواقع مختلفة في محافظة بغداد. وقد تم الحصول على ٥٧ عزلة شخصت ١٣ عزله منها على إنها .*Pseudomonas* spp بعد التحري عن قابليتها على النمو على وسط الستريمايد. وبعد إجراء الفحوصات الكيموحيوية على هذه العزلات (١٣عزلة) تم تشخيص ٦ عزلات منها على انها *Pseudomonas aeruginosa* وقد تم التأكد من هذه العزلات المشخصة باستخدام نظام التشخيص Api 20 E.

اختبرت قابلية العزلات المشخصة على إنتاج الالجينيت وقد أظهرت النتائج أن العزلة البكتيرية *P.aeruginosa* H3 كانت هي الأكفأ في الإنتاج، إذ بلغت كمية الالجينيت المنتج منها ١,٢ غم \ لتر.

درست الظروف المثلى لإنتاج الالجينيت بوساطة العزلة البكتيرية *P.aeruginosa* H3، وقد أظهرت النتائج أن الظروف المثلى للإنتاج تضمنت استخدام الوسط الإنتاجي الحاوي على مستخلص التمر بتركيز ٤٪ (مصدرا كاربونيا)، خميرة الخبز التجارية بتركيز ١٪ (مصدرا نايتروجينيا)، فوسفات البوتاسيوم ثنائية الهيدروجين بتركيز ٠,٠٠٥٪ (مصدرا فوسفاتيا)، برقم هيدروجيني ٧، وقد لقح وسط الإنتاج بعدد لقاح ٨×١٠^{٧} خلية \ ١٠٠ مل، وحضن بدرجة حرارة ٣٧ درجة مئوية لمدة ٩٦ ساعة، وقد بلغت إنتاجية الالجينيت تحت هذه الظروف ٨,٥ غم \ لتر.

تم تنقية الالجينيت المنتج بوساطة العزلة *P.aeruginosa* H3 بخطوتين تضمنت الخطوة الاولى الترسيب بالكحول الايزوبروبيلي ثم الديلزة، والثانية الترشيح الهلامي باستخدام هلام sepharose CL-6B 200 .

قدر الوزن ألجزيئي للالجينيت المنقى بتقنية الترشيح الهلامي أيضا خلال عمود sepharose CL-6B 200 وقد أشارت النتائج إلى أن الوزن ألجزيئي التقريبي للالجينيت المنتج من العزلة المحلية *P.aeruginosa*H3 هو ١٤١,٢٥٣دالتون.