1. Introduction and Literature Review

1.1 Introduction:

Bacterial polysaccharides are quite different in function. They are secreted from the cell to form a layer over the surface of the microorganism. Because of their position, they are characterized as exo-polysaccharides (Tombs and Harding, 2003). Alginates are one of these exopolysaccharides which act as extracellular matrix (material) that allows the formation of differentiated biofilms, which restrict diffusion of clinical antibiotics and protect embedded cell against human antibacterial defense mechanisms (Remminghorst and Rehm, 2006).

Alginate is a linear unbranched polymer of D- Mannuronic acid and its C5 epimer, L-guluronic acid which are linked by β-1, 4-glycosidic bonds. In bacteria, alginate is modified by the addition of O-acetyl groups on some D-mannuronic acid residues (Franklin *et al*., 1994; Mathee *et al*., 2002).

The pathogenic roles of alginate can be classified in three general categories; serves as direct barrier against phagocytic cells and effective opsonization, function as an immunomodulatory molecule and serves a role in the biofilm related phenomenon including adhesion and antibiotic resistance (Govan and Deretic, 1996).

Alginate is a commercially important polysaccharide with gelling and colloidal properties. It has a variety of uses, as an ingredient of photographic emulsions and dental impression material and as additive in various food stuff, e.g. ice-cream and jellies (Bushell, 1983). It was also used as thickening agent in the textile and paper industries, wound healing and microencapsulation (Tombs and Harding, 2003).

Alginate is one of the few polymers synthesized by some eukaryotic and prokaryotic organisms. The eukaryotic source of alginate is mainly the marine algae. However, amongst the prokaryotes, two bacterial genera *Azotobacter* and *Pseudomonas* are know to contain species capable of alginate production (Bushell, 1983).

The genetic and biosynthesis pathway of alginate in *Pseudomonas aeruginosa* has been extensively studied due to its role in the disease of cyctic fibrosis which is a cause of morbidity and mortality (Ertesvag and Valla, 1998).

Because of the importance of alginate as a perfect microbial polysaccharide for different uses and applications, this study aimed to alter the alginate producing *P. aeruginosa* H3 using:

1. Physical mutagenesis by UV radiation and LASER.
2. Chemical mutagenesis by MNNG and Mitomycin C.
3. Then, the chemical structure of the alginate produced by overproducer mutants is examined using FTIR spectroscopy.

1.2 Literature Review

1.2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, it is belong to the family pseudomonadaceae which is short, gram negative, aerobic rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum. These bacteria are common inhabitants of soil and water (Todar, 1997). *P. aeruginosa* is an opportunistic human pathogen. It is also an opportunistic pathogen of plants (Iglewski, 1996). *P. aeruginosa* isolates may produce two types of colonies; natural isolates from soil or water typically produce a small, **rough** colony. Clinical isolates, in general, yield one or another of two smooth colony types. One type has a fried-egg appearance which is large, **smooth**, with flat edges and an elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has a **mucoid** appearance, which is attributed to the production of alginate slime. The smooth and mucoid colonies are presumed to play an important role in colonization and virulence (Todar, 1997; Jawetz *et al*., 1998). *P. aeruginosa* strains produce two types of soluble pigments, the fluorescent pigment **pyoverdin** and the blue pigment **pyocyanin**. The latter is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus" which is a characteristic of supporative infections caused by *P. aeruginosa* (Todar, 1997).

P. aeruginosa is a pathogen responsible for many infections including the chronic pulmonary infection in cystic fibrosis patients. The pathogenicity of *P. aeruginosa* may be attributed to the synthesis of the exopolysaccharide alginate. *P. aeruginosa* exists in both mucoid and nonmucoid states; however, it is the mucoid strain which is present in the prolonged infections in the cystic fibrosis lung (Carmichael and Wheeler, 1997). Mucoid *P. aeruginosa* strains owe their appearance to the profuse production and secretion of the exopolysaccharide alginate (Govan and Deretic, 1996).

1.3 Microbial Sources for Polysaccharides

Microbial extracellular polysaccharides are mainly linear molecules to which side chains of varying length and complexity are attached at regular intervals. Microbial polysaccharides and especially those obtained from numerous bacterial species are generally considered to be of uniform structure. Through careful control of culture conditions, through the use of enzymes or through use of nutrients, it has proved possible to prepare microbial polysaccharide with altered structures (Sutherland, 1997).

Exopolysaccharides occur widely, especially among prokaryotic species, both among those that free living and among those that are pathogenic to human, animals and plants. Most microalgae yield some type of exopolysaccharides but they are less common among yeast and fungi. However, some of those isolated from fungi do possess interesting physical and pharmacological properties (Sutherland, 2003).

The functions of exopolysaccharides to the microorganisms are thought to be mainly protective, either as a general physical barrier preventing access of harmful substances, or more specifically as a way of binding and neutralizing bacteriophage and they may prevent dehydration. Also they can prevent phagocytosis by other microorganism or the cells of immune system. They have a role in adhesion and penetration of the host.

Bacterial products are very important in all aspects of polysaccharides biotechnology. Bacteria are known which produce nearly all the major plant polysaccharides such as glucan, alginate and cellulose. Genetic manipulation of bacteria has been studied for longer and as in general much easier than for higher organisms. So they are an obvious target both for manipulation of biosynthetic pathways and for the expression of genes to produce especially desirable enzyme (Tombs and Harding, 2003).

Within the wide diversity of microbial exopolysaccharides both in relation to composition and function, bacterial alginate occupies an unusual and fascinating position (Bushell, 1983).

The microbial sources of important types of polysaccharides were shown as follow in table (1-1).

Table (1-1) Some Microbial Sources of Important Types of Polysaccharides.

Organism Producing Exopolysaccharides	Exopolysaccharides	References
Azotobacter vinelandii	Alginate	Bushell, 1983.
Acetobacter xylinum	Cellulose	Bushell, 1983.
Acinetobacter spp.	Ethapolan	Grinberg *et al.*, 1995.
Acinetobacter calcoacetricus	Emulsan	Grinberg *et al.*, 1995.
Coprinus cinereus	Chitin	Bushell, 1983.
Corynebacterium beticola	Levan	Grinberg *et al.*, 1995.
Escherichia.coli	Colanic acid	Bushell, 1983.
Leuconostoc mesenteroide	Dextran	Bushell, 1983.
Leuconostoc dextranicum	Dextran	Bushell, 1983.
Lactobacillus fermentum	Glucan	Van Geel, 2004.
Lactobacillus reuteri	Levan and inulin	Van Geel, 2004.
Mucor spp.	Chitosan	Bushell, 1983.
Pseudomonas aeruginosa	Alginate	Bushell, 1983.
Pseudomonas elodea	Gellan gum	Bushell, 1983
Pseudomonas fluorescence	Levan	Grinberg *et al.*, 1995.
Sclerotium glucanicum	Scleroglucan	Grinberg *et al.*, 1995.
Xanthomonas campestris	Xanthan	Bushell, 1983

1.4 Different Sources for Alginate Production

Pseudomonas and *Azotobacter* are the only bacterial genera that known to produce alginate. *P. aeruginosa* (a human pathogen causing chronic respiratory infections of cystic fibrosis patients) was first reported to produce this polysaccharide being important for the virulence of this strain and its survival in the lung. Another species of this genus (*P. mendocina* and *P. syringae*) have the ability to produce alginate under several conditions. Many strains of *A. vinelandii* (a nitrogen fixing soil bacterium) were also found to produce this polymer in complex and synthetic media (Franklin *et al.*, 1994).

Bacterial alginate is composed of uronic acid of β-D- mannuronate and its C-5 epimer and α-L- guluronate. These monomers may be arranged in homopolymeric (polymannuronate and polyguluronate) or heteropolymeric block structure as in Figure (1-1). Bacterial alginates are O-acetylated on the D-mannuronate residues. Consequently, bacteria produce a range of alginates with different block structures and degrees of O- acetylation. The high molecular mass of bacterial alginate and the negative change ensure that the polysaccharide is highly hydrated and viscous (Gacesa, 1998).

Whereas, the algae source of alginate consists of an unbranched polysaccharide made of ß-1,4-D-mannumaric acid (M) with its C-5- epimer and α -1,4-L-guluronic acid (G), which are arranged in homopolymeric regions of M and G blocks, interspaced with regions of alternating structure (MG blocks). These features vary according to the nature and the age of the tissue as well as to the season and to the growing area (Nyvall *et al.*, 2003). Thus may affect the physical properties of the polymer in aqueous solution (Franklin *et al.*, 1994).

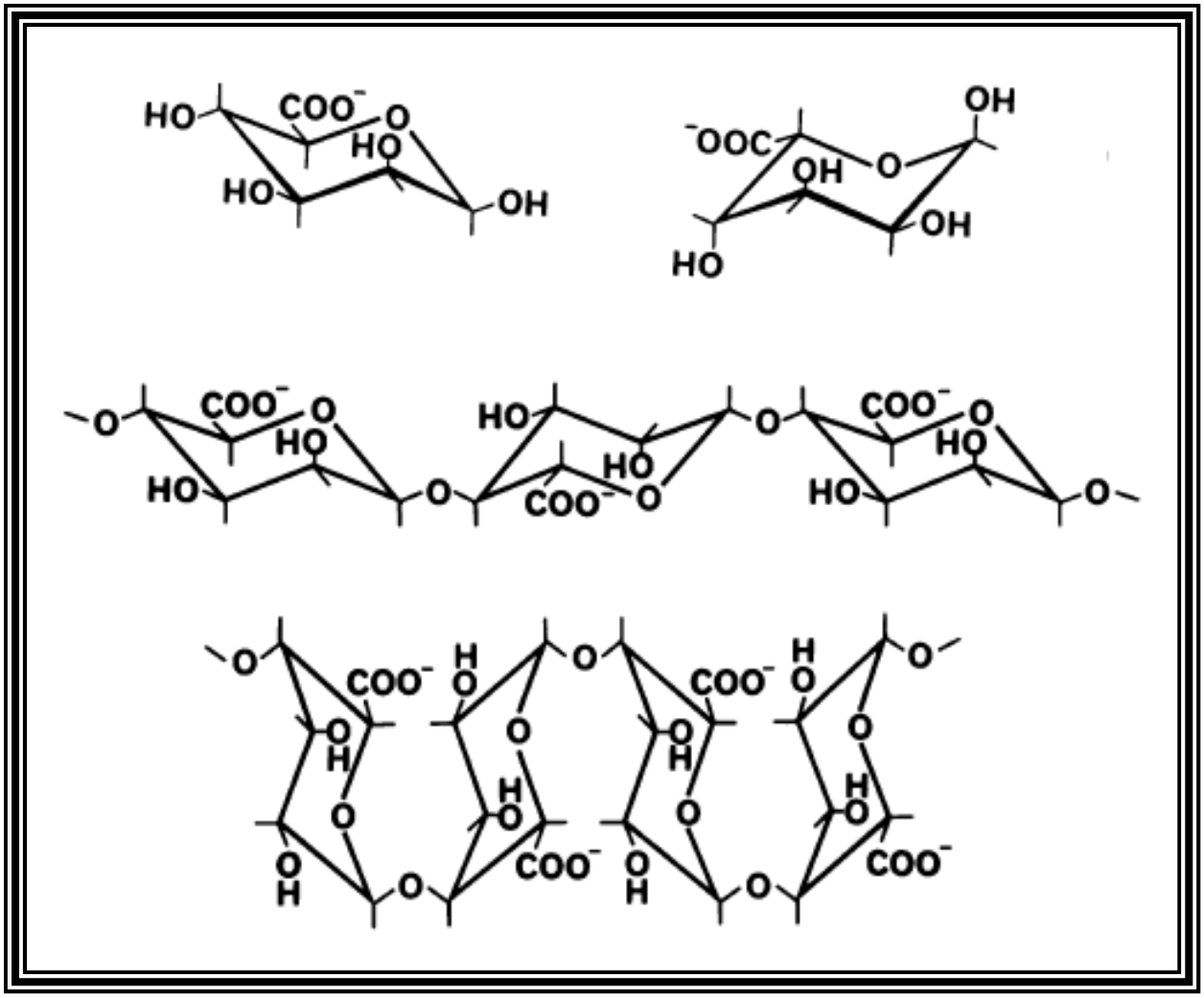

Figure (1-1) Structure of Alginate. The uronic acids β-D-mannuronate (top left) and α-L-guluronate (top right) are assembled into (1–4)-linked blocks of polymannuronate (centre), polyguluronate (bottom) (Gacesa, 1998).

1.5 Physical Properties of Alginate

1.5.1 Ion Binding

Ion binding characteristic are the basis for the gelling properties of alginate, it shows that their affinity for multivalent cations depends on their composition. The alginate binding affinity increased markedly with increasing content of α -L-guluronate residues in the chain. Poly-mannuronate blocks and alternating blocks were almost without selectivity (Draget *et al*., 1997).

The high selectivity between ions as similar as the alkaline earth metals indicates that the mode of binding could not be by specific electrostatic binding only, but that some chelation caused by structural features in the G blocks must contribute to the selective attempts were made to explain this phenomenon by the so- called "egg-box" model, based upon the linkage conformations of the guluronate residue as in Figure (1-2). Nuclear magnetic resonance studies suggested a possible binding site for Ca^{2+} ions in a single alginate chain (Franklin *et al*., 1994; Draget *et al*., 1997).

1.5.2 Solubility

There are three essential parameters determining and limiting the solubility of alginate in water (Moe *et al*., 1995) 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.

1.5.3 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).

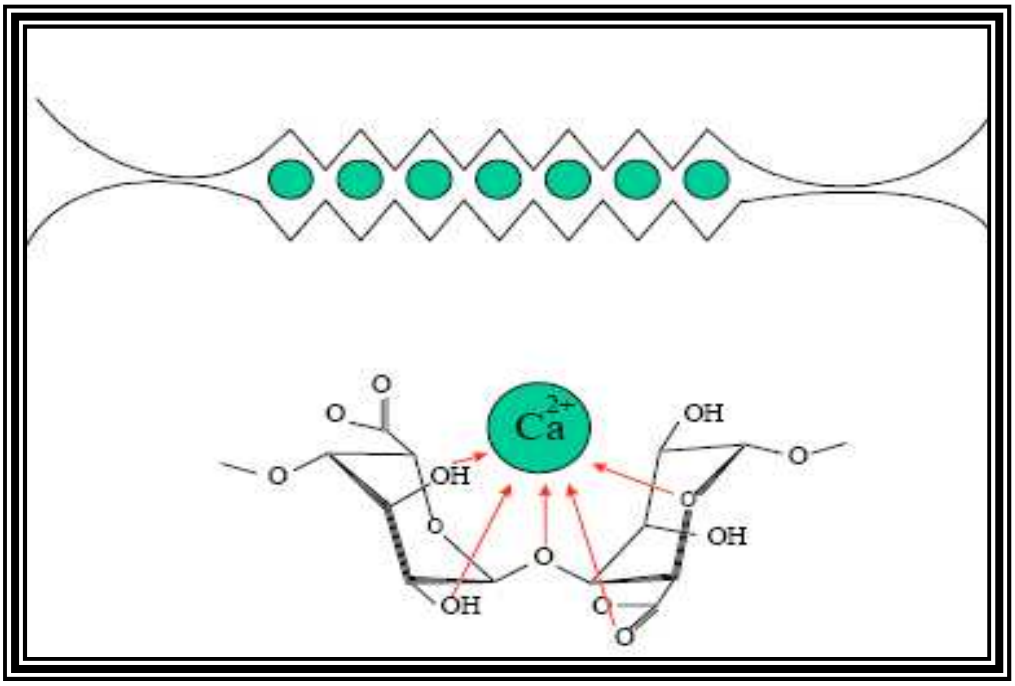

Figure (1-2) The Egg-box Model for Binding of Divalent Cations (Kvam, 1987).

1.6 Biological Properties of Alginate

It has been established that the alginate molecule itself has different effects on biological systems. This is because of the large variety of possible chemical composition and molecular weights of alginate preparations. A biological effect of alginate initially was hinted at in the first animal transplantation trials of encapsulated Langerhans islets for diabetes control. Overgrowth of alginate capsules by phagocytes and fibroblasts, resembling a foreign body inflammatory reaction (Soon-Shiong *et al.,* 1991). In bioassays, induction of tumor necrosis factor and interleukin 1 showed that the infusibility depended upon the content of mannuronate in the alginate sample (Soon-Shiong *et al*., 1993). This result directly explains the observed capsule overgrowth; mannuronate- rich fragments, which do not take part in the gel network, will leach out of the capsules and directly trigger an immune response (Stokke *et al.*, 1993). This observed immunologic response can be linked in part to (1-4) glycosidic linkage. The immunologic potential of polymannuronates have been observed *in vivo* in animals models as for protection against lethal bacterial infections and irradiation and for increase non- specific immunity (Espevik and Skjak, 1996).

1.7 Applications of Alginate

Alginates are one of the most versatile polysaccharides which have a large use of different applications:

1.7.1 Medicine and Pharmacy

Alginate had been used for decades as helping agents in various human health applications. Some examples include the traditional wound dressing, in dental impression material, and some formulations preventing gastric reflex.

Alginate's increasing in popularity as an immobilization matrix in various biotechnological processes. Entrapment of cells within Ca- alginate spheres has become the most widely used technique for the immobilization of living cells. This immobilizing procedure can be carried out in a single step process under very mild conditions and is therefore compatible with most cells. The possible uses for such systems in industry, medicine, and agriculture are numerous, production of ethanol by yeast, production of monoclonal antibodies by hybirdoma cells and mass production of artificial seed by entrapment of plant embryos (Smidsrod and Skjak- Braek, 1990).

Major interest has been focused on insulin- producing cell for the treatment of type I diabetes. Alginate poly-L- lysine capsules containing pancreatic Langerhans islets have been showed to reverse diabetes in large animals and currently are being clinically tested in humans (Soon- Shiong *et al.*, 1994).

1.7.2 Technical Utilization

Alginates are used for paper coating to obtain surface uniformity and as binding agents in the production of welding rods. Ammonium alginate is used for can sealing because of its very low ash content. In textile printing, alginate has gained a high popularity because of the resulting color yield, brightness, and print level (Onsoyen, 1996).

1.7.3 Foods

Alginates are used as food additives to improve, modify, and stabilize the texture of foods. This is valid for such properties as viscosity enhancement, gel-forming ability, and stabilization of aqueous mixtures, dispersions, and emulsions (McHugh, 1987).

For applications in jams, jellies, fruit fillings and ice cream, the synergetic gelling between alginates high in guluronate and highly esterified pectins may be utilized (Toft *et al.*, 1986).

1.8 Genetics and Biochemistry of Alginate Synthesis from *P. aeruginosa*

Most of alginate biosynthesis genes shown in table (1-2), including ***alg****A*, ***alg****D*, ***alg****8*, ***alg****44*, ***alg****K*, ***alg****E*, ***alg****G*, ***alg****X*, ***alg****L*, ***alg****I*, ***alg****J*, ***alg****F*, are found in a chromosomal gene cluster which functions as an operon controlled by the alg D protein. alg C, which is also involved in lipopolysaccharide biosynthesis is located outside of this cluster and it is expressed from its own promoter (Albrecht and Schiller, 2005).

Alginate biosynthesis begins in the cytoplasm by the gene product of which is convert fructose -6- phosphate to GDP- Mannuronic acid and then transported a cross the periplasmic membrane, by alg 8 and alg 44 (Shanker *et al.*,1995). A periplasmic polymer is subject to the action of C-5 epimerase encoded by ***alg*** *G*, and alginate lyase encoded by ***alg*** *L* (Mathee *et al.*, 2002). ***Alg*** *I* and ***alg*** *J* are two newly identified gene required for alginate acetylation; ***alg*** *F* encoding an acetylase (Govan and Deretic, 1996). The product of ***alg*** *E* may be involved in polymer export to the bacterial surface (Chu *et al.*, 1991; Rehm *et al.*, 1994). ***Alg*** *8*, is considered to be a good candidate for the polymerization of GDP- mannuronic acid residues into a poly (M) chain (Jain and Ohman, 1998). ***Alg*** *K* plays an important role in the polymerization and transport of mannuronate to alginate (Aarons *et al.*, 1997; Jain and Ohman, 1998).

The later stages of alginate synthesis occur via a protein complex or scaffold composed of alginate protein algG, algK, and algX. This scaffold is believed to assist in polymer formation by bring the enzymes and mannuronic acid residues together in one location, facilitating the modification of these residues and guiding the movement of the developing polymer to the outer membrane secretion (Albrecht and Schiller, 2005). Alginate biosynthetic pathway shown in Figure (1-3).

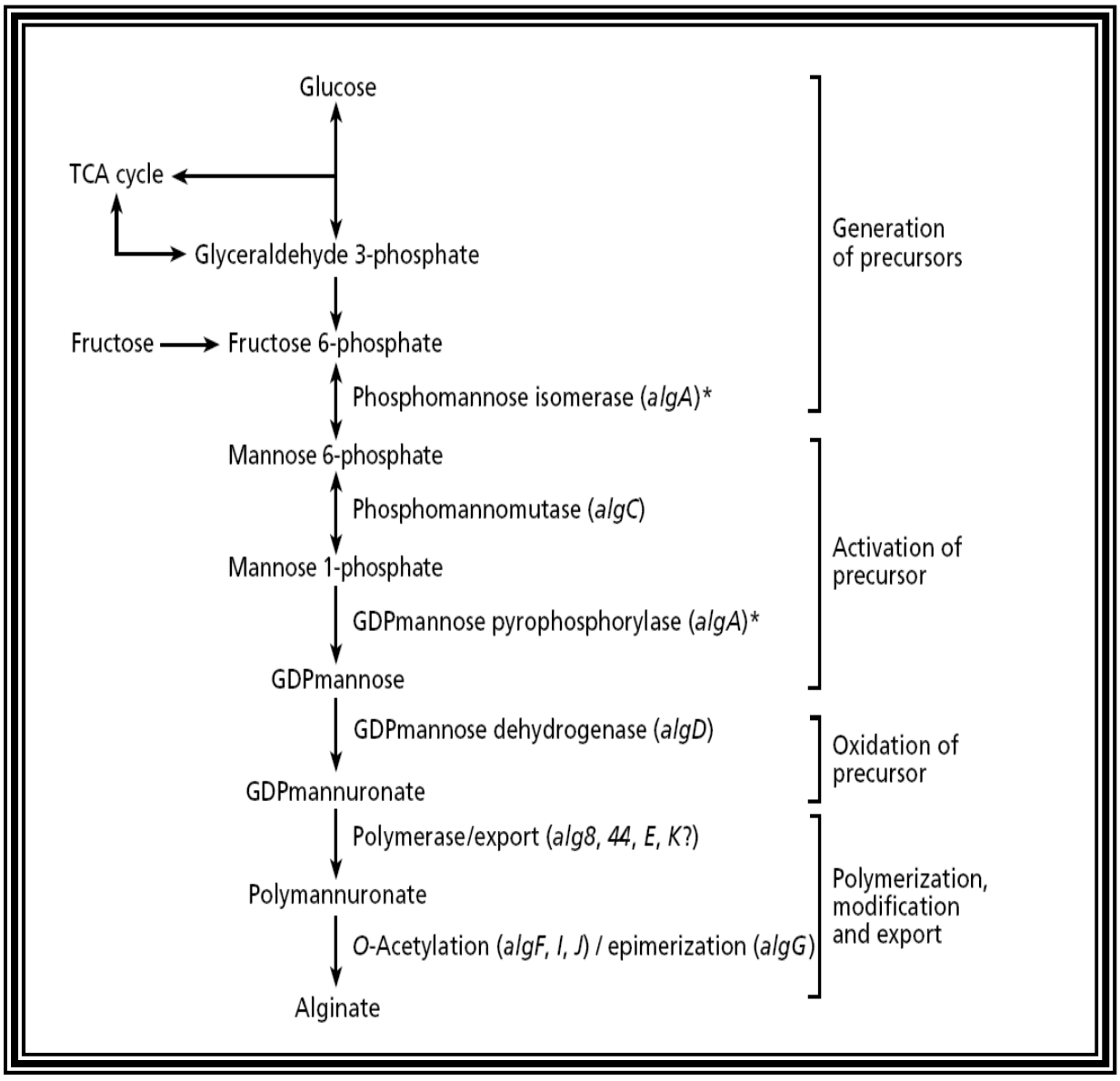

Figure (1-3) Alginate Biosynthetic Pathway (Gacesa, 1998).

Table (1-2) Alginate Genes and Their Products in *P. aeruginosa*

Gene	Gene product	Reference
algA	Phosphomannose isomerase and GDP-mannose pyrophosphorylase	Shinabarger *et al.*, 1991.
algC	Phosphomannomutase	Coyne *et al.*, 1994.
algD	GDP-mannose dehydrogenase	Deretic *et al.*, 1987.
algE	Outer membrane protein	Rehm *et al.*, 1994.
algF	O-Acetylation	Franklin and Ohman, 1993.
algG	Mannuronan C-5-epimerase	Jain *et al.*, 2003.
algI	O-Acetylation	Franklin and Ohman, 1996.
algJ	O-Acetylation	Franklin and Ohman, 1996.
algK	Alginate translocator	Aarons *et al.*, 1997.
algL	Alginate lyase	Schiller *et al.*, 1993.
algX	Periplasmic protein required for proper polymer formation	Robles-Price *et al.*, 2004.
alg8	Polymerase / export function	Maharaj *et al.*, 1993.
alg44	Polymerase / export function	Maharaj *et al.*, 1993.

1.9 Regulation of Alginate Biosynthesis

The regulatory genes in *P. aeruginosa* map at 10 minutes and 13 minutes and those genes responsible for a genotypic switch to alginate production are found at 68 minutes. This genotypic switch region consists of ***alg*** *U*, ***muc*** *A*, ***muc*** *B*, ***muc****C* and ***muc****D*. The genes which encode the enzymes responsible for alginate biosynthesis comprise the ***alg*** operon. The transcription of this operon is controlled from the ***alg*** *D* promoter. The ***alg*** *D* promoter is activated by conditions of high osmolarity, N or P starvation, dehydration or the presence of phosphorylcholine which activates a cascade of regulatory proteins (Rehm and Valla, 1997).

A two-component signal-transduction pathway consisting of the sensor proteins alg Q and alg Z as well as regulator proteins such as alg R and alg B, controls transcription of the ***alg*** operon. The positive regulators bind upstream of the ***alg*** *D* promoter resulting in formation of a suprahelical structure with the aid of the histone-like alg P protein causing activation of transcription As well, the sigma-like factor Alg U is responsible for the initiation of ***alg*** D transcription (Gacesa, 1998).

The transcription of the ***alg*** operon in response to environmental stress results in only low level alginate production. In order for copious production of alginate there must be inactivation of the negative regulators (Muc A and Muc B) of alg U activity through mutations. It is the mutational inactivation of Muc A and Muc B which leads to full activity of alg U and hence transcription of the ***alg*** operon (Deretic *et al.*, 1990; Martin *et al.*, 1993) Figure (1-4) illustrates the regulatory circuit controlling the expression of alginate.

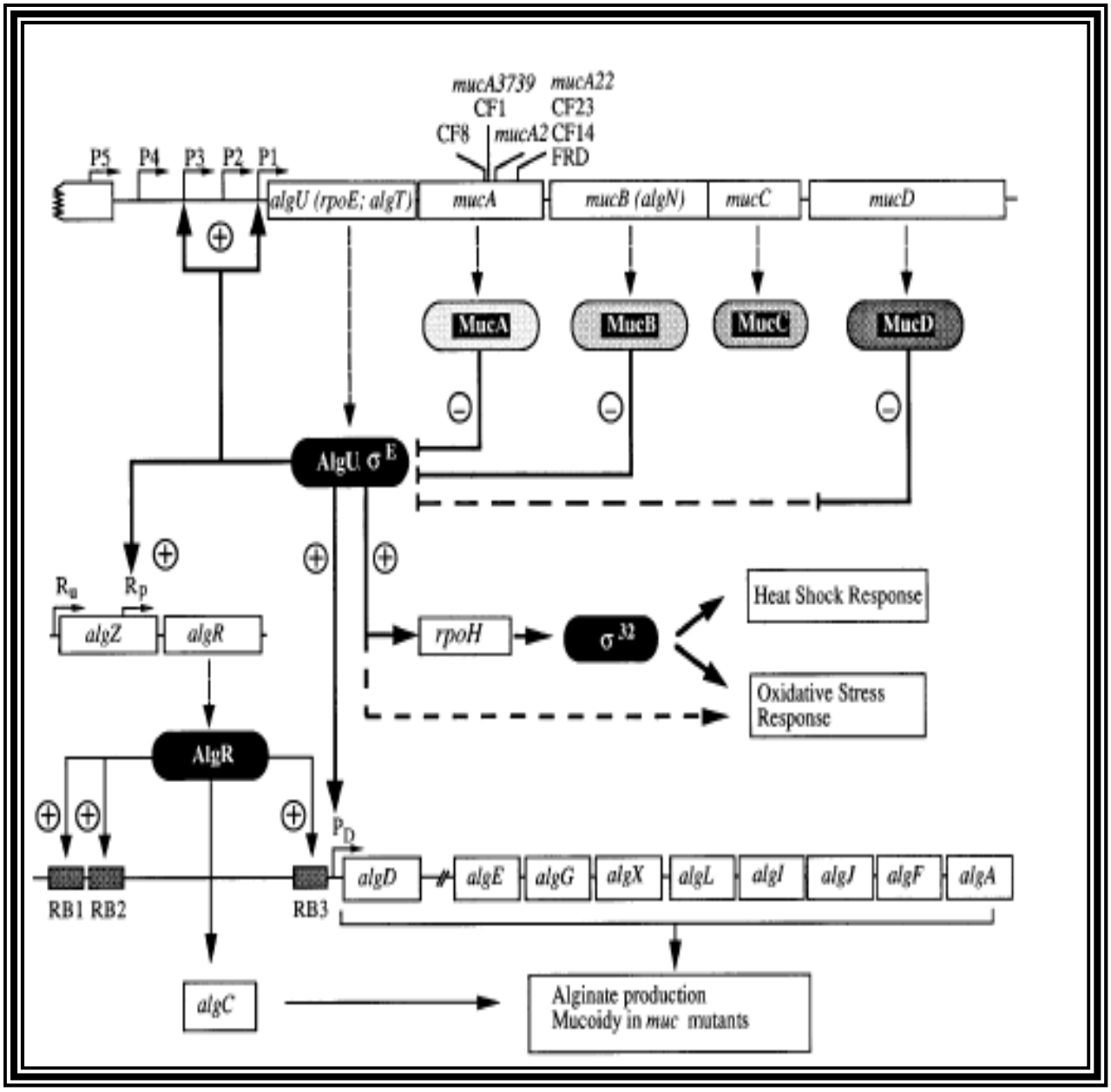

Figure (1-4) The Regulatory Circuit Controlling the Expression of Alginate, Mutations Causing Mucoidy in *P. aeruginosa* (Govan and Deretic, 1996).

1.10 Mutagenesis

Mutagenesis, the creation or the formation of a mutation can be used as a powerful genetic tool. By inducing mutations in specific ways and then observing the phenotype of the organism, the function of the genes and even individual nucleotides can be determined. Mutagenesis is used to enhance strains with desired properties, such as the ability to overproduce a desired metabolite or enzyme. So now the study of mutagenizing microorganisms is important, interesting and potentially profitable. The mutation of a gene or genes under study can be achieved by first altering the DNA of the microorganism in some fashion and then screening or selecting for the desired phenotype (Maki, 2002).

Three general treatments can be used to mutagenize microorganisms: radiation, chemical mutagens, and transposons. Mutation by radiation involves exposing the microbe to high energy waves (UV light, Laser or X- ray). This procedure damages the target DNA and sometimes, during repair, an improper base pair (or pairs) is incorporated in the DNA, causing a mutation. Chemical mutagens are also employed. These compounds are added to a growing culture of an organism for a given time period and interfere with the replication of the DNA. Some mutagens achieve this by serving as base analogs, others chemically modify the DNA, and yet another class can insert or intercalate in between the base pairs of DNA causing DNA polymerase to make mistakes. In all cases, the mutagen causes incorrect copying of the DNA resulting in base substitutions (exchange of one base pair for another), insertions (addition of one or more base pairs), or deletions (removal of one or more base pairs) (Paustian and Kurtz, 1994).

The third method of mutagenesis involves the use of mobile genetic elements. The most commonly employed of these are a sub-class called transposons. Transposons are relatively short pieces of DNA that replicate by

inserting into other pieces of DNA (plasmids, chromosomes, viruses). They encode two sets of functions. One set is involved in regulating and performing the movement of the transposon from one piece of "host" DNA to the next (transposition functions). The other set of functions encode genes that may provide an advantage for the host of the transposon- antibiotic resistance and Hg^{+2} resistance, for example (Paustian and Kurtz, 1994).

1.10.1 Physical Mutagens:

Physical mutagens are different types of radiations having mutagenic properties such as UV light and Ionizing radiation. The energy content of a radiations depends upon its wavelength i.e.: shorter the wavelength, the greater the energy value of the radiation. While the ionizing radiation which is one of the physical mutagens, has the greater penetration power than non Ionizing radiation. Ionizing radiation causes single strand breaks in DNA and produces deletion. Ultraviolet rays are the only non-ionizing rays with mutagenic properties (Setty and Sreekrishna, 2004).

1.10.1.1 Mutagenic Properties of Ultraviolet Radiation:

UV light is the portion of the spectrum with wavelengths of 100-400 nm, which is just shorter than visible light (Miller *et al.*, 1999). UV radiation (UVR) is lethal and potentially mutagenic to all organisms greatly dependant on the source of radiation and the time exposure. UV can be classified into UV-A (320-400nm), UV-B (290-320nm) and UV-C (<290). Photons of UVB and UVC wavelengths cause direct DNA damage by inducing the formation of DNA photoproducts such as cyclobutyl pyrimidine dimers (CPD) and the Pyrimidine (6-4) pyrimidinione. The accumulation of DNA photoproducts can be lethal to cells through the blockage of DNA replication and RNA

transcription. The UVA typically cause only indirect damage to cellular DNA through catalyzing the formation of chemical intermediates such as reactive oxygen species. Distinct differences between far- UVC and near- UV (UVB and UVA) damage have been observed in bacteria and bacteriophage, UVC has the most potential for directly damage DNA (Eisenstark, 1989; Miller *et al*., 1999; Kim and Sundin, 2001; Qiu *et al*., 2004).

UV light is widely used mutagen that generates a broad spectrum of lesions in DNA. The most important mutagenic effect of UV irradiation is believed to be stimulation of misrepair (Goodenough, 1984).

1.10.1.2 LASER

A LASER from the acronym of "**L**ight **A**mplification by **S**timulated **E**mission of **R**adiation" is an optical source that emits photons in a coherent beam. Laser light is a typically near- monochromatic, i.e., consisting of a single wavelength or color and emitting in a narrow beam (Cheba, 2000).

In 1916, Albert Einstein laid the foundation for the invention of the laser and its predecessor, but the first laser was demonstrated in 1960. Later in 1960 Ali Javan made the first gas laser using helium and neon gas (Stean, 1998).

Laser consists of three parts (Bhawalkar and Kukreja, 1992):-

1. Active medium.
2. Source of pumping energy.
3. Optical Resonance.

The active medium could be solid like (Nd:YAG or Er:YAG), semiconductor (Diode), liquid (Dye laser) or gas (He-Ne laser, CO_2 laser, N laser and Argon laser). The properties of laser differ from the common light by

including: - coherence, monochromaticity, collimation, high intensity, ultra short pulses, brightness and tenability (Chopra and Chawla, 1992).

Laser can be classified according to the active medium or to the operation power (continuous wave or pulsed wave) and can be classified according their power (High, mid and low or soft power). (Merza, 1988; Al-Emarra, 1990)

Applications of laser in life science and medicine depends on laser biosubstances interaction, because the laser beam may be reflected or scattered or transmitted or absorbed, this is depends on the physical properties of the biosubstances, laser type, wavelength and its power. Using the absorption property, laser used in mutation, inactivation, photosensitization for bacteria and tumor cell (Cheba, 2000).

It is well known that 633 nm laser radiations has the wide spread use in biology and medicine, therefore, the elucidation of mechanisms and regularities of these radiations action on cells is of a big interest (Voskanyan, 1990). So He-Ne laser is a type of small gas laser which is typical example for the low power laser. He-Ne lasers are constructed to produce laser action in red with 632.8 nm, in the green at 543.5 nm and in the infrared at 1523 nm (Kandela, 1988).

1.10.2 Chemical Mutagenesis:

A chemical mutagen is a substrate that can alter a base that is already incorporated in DNA by change its hydrogen – bonding specifically (Freifelder, 1987). Among the most widely used mutagenic reagent with microorganisms are the alkalating agents, ethyl methane sulfonate (EMS) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Mitomycin C (MMC) also used as chemical mutagen which causing cross-link formation in cellular DNA by deletion (Szybalski and Iyer, 1964).

1.10.2.1 N-methyl-N-nitro-N-nitrosoguanidine (MNNG)

The first chemicals determination and use of MNNG was in 1947. MNNG as in Figure (1-5) is a member of N-nitroso group which has mutagenic and carcinogenic activities. MNNG has been shown to inhibit the synthesis of macromolecules such as DNA, RNA and protein in bacterial cells (Bagewadikear and Bhattacharya, 1982).

MNNG is stable at pH=5 but at low acidity it hydrolyzes to nitrous acid and at alkaline pH hydrolyzes to diazomethane (Cerada- Olmedo and Hanawalt, 1967). MNNG this mutagen tend to prefer guanosin-rich (G- rich) regions, reacting to form a variety of modified G residues, the result often being depurination. Some of these modified G- residues have the ability to error prone repair. This stimulation of error prone repair allows all sorts of mutation types to occur as a result of these mutagens, also that alkalated bases can mispair during replication MNNG transfer a methyl group to guanine, to O^6 position. The additional methyl or alkyl group causes a distortion in the helix (Roberts, 2003).

Depurination may be remedied by the action of the DNA repair system, but in the event of a replication will cease. The action of an error prone repair system knows as SOS repair allows replication to proceed past the gap, with the incorporation of an incorrect base in the new DNA strand. This mechanism accounts for the known prosperity of MNNG to cause multiple closely linked mutations in the vicinity of the position of the replication fork at the time of treatment (Dale, 1998).

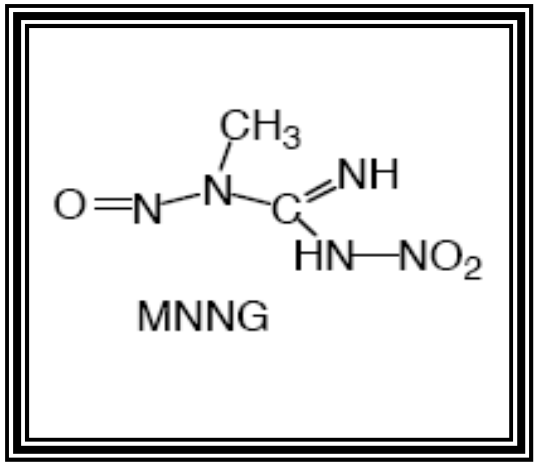

Figure (1-5) N-methyl-N-nitro-N-nitrosoguanidine (MNNG) Structure.

1.10.2.2 Mitomycin C

Mitomycin C was identified in 1956 as an antibiotic produced by S*treptomyces lavendulae* and subsequently established as an important antitumor agent. Mitomycin C functions as a producing and requires enzymatic and chemical reduction to become a highly reactive alkylating agent (Tomasz *et al.,* 1988; Henderson, 1993).

It has been reported that the antibiotic mitomycin C has a specific effect on cellular DNA, but has little or no effect on either RNA and protein formation (Suzuki and Kilgore, 1966).The ability of mitomycin to inhibit bacterial cell growth involves the combined action of DNA alkylation and the formation of reactive oxygen species (Sheldon *et al.*, 1996).

The primary action of mitomycin C is believed to be associated with either the inhibition of DNA biosynthesis (Sekiguehi and Takagi, 1960) or the breakdown of the nuclear apparatus (Reich *et al.,* 1961). Mitomycin C seem to form covalent bonds with DNA, mitomycin C can cross-link the complementary strands of DNA *in vivo* and under appropriate conditions, *in vitro* as well. It was proposed that the bacteriocidal effect of mitomycin C is the indirected

consequence of the cross-links (Mereado and Tomasz, 1972) which cause the deletion mutation type. Mitomycin Structure shown in Figure (1-6).

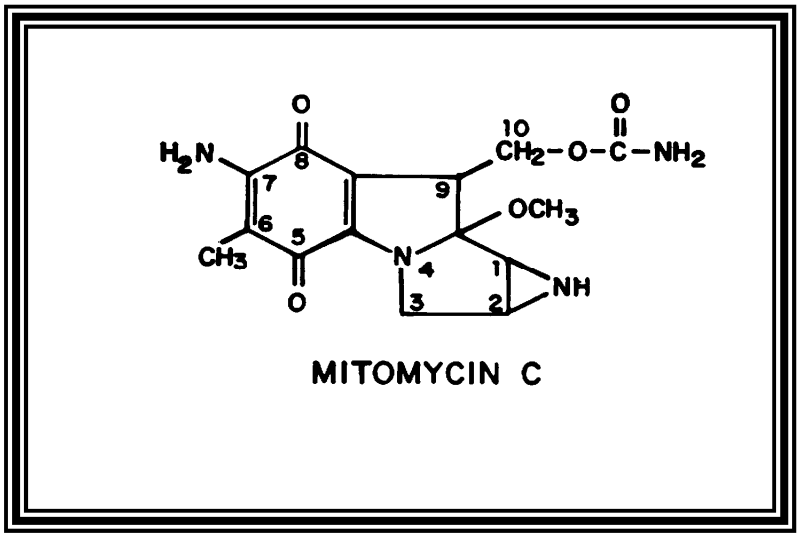

Figure (1- 6) Mitomycin C Structure (Mercado and Tomasz, 1972).

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)
Cooling centrifuge	Harrier (U.K.)
Distillator	Gallenkamp (England)
Oven	Gallenkamp Sanyo (U.K.)
Incubator	Gallenkamp (England)
Shaker incubator	GFL (Germany)
Millipore filter unit (0.22 µm)	Millipore Corp.(U.S.A.)
Micropipettes	Brand (West Germany)
pH-meter	Mettler-GmpH Toledo (U.K.)
Sensitive balance	Sartorius (Germany)
UV- transiluminator	Ultraviolet products (U.S.A.)
Helium: Neon gas laser	MWK Industries (U.S.A.)
UV-V is Spectrophotometer	Aurora instrument Ltd.(U.K.)
Laminar air flow	Memmert (West Germany)
Vortex mixer	Stuart scientific (U.K.)
Fourier Transform- Infrared (FTIR)	Shimadzu (Japan).

2.1.2 Chemicals:-

The following chemicals were used in this study:

Chemicals	Company(origin)
Nutrient agar	Biolife (Italy)
Nutrient broth	Biolife (Italy)
Yeast extract	Biolife (Italy)
Cetrimide, Glycerol	Riedel-DeHaen (Germany)
NaCl	Merck (Germany)
N-methyl-N-nitro-N-nitrosoguanidine (MNNG)	Fluka(Switzerland)
Mitomycin C	Kyowa Hakko Kogyo (Japan)
KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.9H_2O$, $Na_2MO_4.2H_2O$, Na_2HPO_4, NaH_2PO_4, K_2SO_4, Peptone, $MgCl_2$	BDH (England)
Date extract	Kerbala (Iraq)
Agar, Hydrogen peroxide, , N,N,N,N-tetramethyl-p-phenylene-diamine dihydrochloride, Simmon citrate agar	Difco (U.S.A)
Isopropanol	Riedel-DeHaen-(Germany)
NaOH	Merck (Germany)
HCl	BDH (England)

2.1.3Media:-

2.1.3.1 Commercial Media

The following media were prepared as recommended by manufacturing Company, pH was adjusted to 7.0 and sterilized by autoclaving.

- Nutrient agar.
- Nutrient broth.
- Simmon citrate agar.

2.1.3.2 Laboratory Prepared Culture Media

1. **Luria- Bertani Broth** (Maniatis *et al*., 1982)

   This broth medium contained the following components:

Component	Weight (g)
Peptone	10g
NaCl	10g
Yeast extract	5g

All components were dissolved in 950ml D.W, pH was adjusted to 7.5, then the volume was completed to one liter and sterilizing by autoclaving.

2. **Luria- Bertani Agar (**Maniatis *et al*., 1982)

   Agar (15%) was added to LBB (2.1.3.2.1) and autoclaved.

3.Cetrimide agar (Stolp and Gadkari, 1984).

This medium was prepared consisting of the following components:

Component	Weight (g)
Peptone	20g
$MgCl_2$	4.5g
K_2SO_4	10g
Cetrimide	0.3g
Agar	15g

All components were dissolved in 950ml D.W, pH was adjusted to 7.5, then the volume was completed to one liter and sterilized by autoclaving.

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

This medium was prepared consisting of the following components:

Component	Weight (g)
Peptone	20g
K_2SO_4	10g
$MgCl_2$	1.4g
Glycerol	10ml
Agar	15g

All components were dissolved in 950ml D.W, pH was adjusted to 7.5, then the volume was completed to one liter and sterilized by autoclaving.

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

This medium was prepared consisting of the following components:

Component	Weight (g)
Peptone	20g
$MgSO_4.7H_2O$	3.5g
K_2SO_4	1.5g
Glycerol	10ml
Agar	15g

All components were dissolved in 950ml D.W, pH was adjusted to 7.5, then the volume was completed to one liter and sterilized by autoclaving.

6. Mineral salt medium (production media) (Al-Janabi, 2006)

This medium was prepared consisting of the following components:

Component	Weight (g)
KH_2PO_4	0.05g
$MgSO_4.7H_2O$	0.2g
$CaCl_2.2H_2O$	0.1g
$FeSO_4.9H_2O$	0.05g
$Na_2MO_4.2H_2O$	0.007g
Date extract	4%
Commercial baker's yeast	1g

All components were dissolved in 950ml D.W, pH was adjusted to 7.2, then the volume was completed to one liter and sterilized by autoclaving.

2.1.4 Reagents:-

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

This reagent was prepared to be consisting of (3%) hydrogen peroxide.

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

This reagent was prepared by dissolving one gram of N,N,N,N-tetramethyl-p-phynylene-diamine dihydrochloride in 100 ml distilled water and kept in a dark bottle at 4°C.

2.1.5 Buffers and Solutions:-

1. Phosphate Buffer Solution:

This solution was prepared by dissolving 9.52g of Na_2HPO_4 and 6g of NaH_2PO_4 in 950 ml of D.W., pH was adjusted to 7, then the volume was completed to 1000ml, and sterilized by autoclaving.

2. N-methyl-N-nitro-N-nitrosoguanidine (MNNG) stock solution:

Stock solution of MNNG (500 µg/ml) was prepared by dissolving 10 mg of MNNG in 20 ml of phosphate buffer solution pH 7.2.

3. Mitomycin C stock solution:

Stock solution of Mitomycin C (1000 µg/ml) was prepared by dissolving 10 mg of Mitomycin C in 10 ml of distilled water.

2.1.6 Bacterial Isolate

P.aeruginosa H3 isolate was obtained from the Department of Biotechnology/ College of Science/ Al-Nahrain University.

2.2 Methods

2.2.1 Sterilization methods

- **Autoclaving**

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

- **Oven sterilization**

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

2.2.2 Identification of *P. aeruginosa* H3 isolate

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

Single colony of the bacterial isolate was transferred and smeared on a clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with 70% alcohol, and counterstained with safranine, then examined under light microscope.

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

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

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

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

- **Citrate utilization** (Atlas *et al*., 1995)

This test was used to examine the ability of *P. aeruginosa* H3 to utilize citrate as a sole source of carbon and energy. In this test, a colony of the bacterial isolate was inoculated on to the surface of simmon citrate slant and the medium was incubated overnight at 35°C. An appearance of blue color indicates a positive result.

- **Growth on King A** (Cruikshank *et al.*, 1975)

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

- **Growth on King B** (Cruikshank *et al.*, 1975)

Single colony of the bacterial isolate was streaked 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 isolate ability in fluorescent pigment production.

- **Growth on Cetrimide agar** (Greenwood *et al.*, 1997)

This medium was used as a selective medium for *Pseudomonas* spp. The plates were inoculated with bacterial isolate by streaking and incubated at 37°C for 24 hrs.

2.2.3 Maintenance of Bacterial Isolate

Maintenance of *P. aeruginosa* H3 was performed according to (Maniatis *et al*., 1982) and as follows:

- **Short term storage**

Bacterial isolate was maintained for few weeks on Nutrient agar plates, the plates were tightly wrapped in parafilm and stored at 4°C.

- **Medium term storage**

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

- **Long term storage**

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

2.2.4 Mutagenesis of *P. aeruginosa* H3

P. aeruginosa H3 was subjected to physical and chemical mutagenesis as follows:-

2.2.4.1 Physical mutagenesis

P. aeruginosa H3 was subjected to two types of physical mutagens (UV radiation and LASER). First of all, over night culture of *P. aeruginosa* H3 was used to inoculate LB medium containing- flasks and incubated at 37 °C till mid log phase, then cells were precipitated at 6000 rpm for 15 min., washed and resuspended by 5 ml phosphate buffer and subjected to physical mutagens as follow:-

A. UV Irradiation:

UV irradiation was achieved according to Kidambi *et al.* (1996) by subjecting *P. aeruginosa* H3 suspension with phosphate buffer to UV radiation in a dark place using UV photoelectric cell detects actual intensity. The UV source was UV-transiluminator [Flou-Link FLX]. The tray for irradiation approximately 15*25 cm, exposes sample in glass petri dish and the distance between UV source and irradiated suspension was 11 cm. The dose rate of UV irradiation was 2.5 $J/m^2/s$.

Five ml of bacterial cell suspension in sterilized petri dish prepared as (2.2.4.1) was subjected to different doses (0, 2, 4, 6, 8 and 10 J/m^2) of UV radiation in UV transiluminator. Then 0.1 ml aliquots of cell suspension was taken after each treatment, diluted properly and spread on LB agar plates, then plates were incubated at 37°C for 24 hrs. viable count and survival of *P. aeruginosa* H3 after irradiation were determined.

B. LASER Irradiation:

Eppendorf tubes containing cell suspension of *P. aeruginosa* H3 prepared in (2.2.4.1) was subjected in a dark place to laser source (He: Ne gas laser) with 1mW power at a wave length of 632.8 nm which emits a red light of laser. The distance between laser source and the tubes was 20 cm.

Laser irradiation was achieved according to Al-Khafaji (2002) by subjecting the cell suspension containing- eppendorf tubes to laser for different periods (0, 30, 60, 90 and 120 sec.), then 0.1 ml aliquots of irradiated cell suspension was taken after each time of irradiation and spread on LB agar plates, and incubated at 37° C for 24 hrs. viable count and survival of *P. aeruginosa* H3 after irradiation were determined.

2.2.4.2 Chemical mutagenesis

P. aeruginosa H3 was also subjected to two types of chemical mutagens (MNNG and Mitomycin C). In the beginning, over night culture of *P. aeruginosa* H3 was used to inoculate LB medium containing tube and incubated at 37 °C till mid log phase, then cells were precipitated at 6000 rpm for 15 min., washed and resuspended by 5 ml phosphate buffer and incubated with the chemical mutagens as follow:-

A. Mutagenesis by MNNG:

Mutagenesis by MNNG was achieved according to McBeth (1988) by incubating the cell suspension of *P. aeruginosa* H3 with the mutagen. This was performed by adding 0.3 ml of MNNG stock solution to test tube containing 5 ml of cell suspension of *P. aeruginosa* H3 prepared in (2.2.4.2) to give a final

concentration of 30 µg/ml, and placed in a shaker incubator at 37 °C for 60 min. During this period, 0.1 ml aliquots of cell suspension was taken every 15 min. of incubation with the mutagen, diluted and spread on LB agar plates and incubated at 37 °C for 24 hrs. viable count and survival of *P. aeruginosa* H3 after treatment were determined.

B. Mutagenesis by Mitomycin C:

Mutagenesis by Mitomycin C was achieved according to Bowring and Morris (1985) by incubating the cell suspension of *P. aeruginosa* H3 with the mutagen. This was performed by adding 0.15 ml of Mitomycin C stock solution to test tube containing 5 ml of cell suspension of *P. aeruginosa* H3 prepared in (2.2.6.2) to give final concentration of 10 µg/ml, and placed in shaker incubator of 37 °C for 60 min. During this period, 0.1 ml aliquots of cell suspension was taken every 15 min. of incubation with mutagen, diluted and spread on LB agar plates and incubated at 37 °C for 24 hrs. survival *P. aeruginosa* H3 after treatment were determined.

2.2.5 Extraction of Alginate

Alginate was extracted from culture medium by alcohol precipitation method (Jarman *et al.*, 1978), which was first achieved by inoculation the production medium prepared as in (2.1.3.2.6) with (100µl) of fresh culture of *P. aeruginosa* H3 (wild type) and mutants, the cultures were incubated at 37°C for 48 hrs. with shaking at 150 rpm. After that cells were precipitated by centrifugation (6000 rpm for 20 min.), and the alginate was extracted from the supernatant by adding 3 ml portion of isopropanol containing 1 ml of supernatant; mixed vigorously and left to stand for 10 min., then centrifugation

(6000 rpm for 20 min.), supernatant (isopropanol layer) was discarded, while the pellet (precipitated alginate) was kept to determine the concentration of crude alginate.

To determine the ability of *P. aeruginosa* H3 in alginate production, the alginate precipitation was dried in an oven at 45°C for 16 hrs. and weighed by sensitive balance to determine the alginate production (mg/L).

2.2.6 Characterization of Alginate Using FTIR

Chemical structure of alginate produced by *P. aeruginosa* H3 (wild type) and the alginate produced by the over producer mutants (after physical and chemical mutagenesis) were examined using Fourier Transform-Infrared Spectrometry (FTIR) at the Chemistry Department/ College of Science/ Baghdad University. The FTIR spectrum which is an advanced type of infrared (IR) spectrometry, was utilized to detect the functional groups of chemical structure of alginate. This was done under FTIR spectrometry in a wave length ranged between (400-4000 cm^{-1}). This spectrum was used to determined the functional chemical groups that are found in the alginate structure (O-H bond range from 3600-3200 cm^{-1}, C-O-C bond range from 1260-1000 cm^{-1} and C=O bond 1760-1670 cm^{-1}) in order to compare between them and identify if there is a qualitative differences between them.

3. Results and Discussion

3.1 Identification of *P. aeruginosa* H3

P. aeruginosa H3 which was isolated and identified in a previous study (Al-Janabi, 2006) was further identified and examined for its ability in alginate production.

Results showed that colonies of this isolate on nutrient agar were mucoid, smooth in shape with flat edges and elevated center, creamy color and have fruity odor. The microscopical examination showed that it was single cells, non-spore forming, gram negative and rod shape, these results refer that this isolate may belongs to *Pseudomonas* spp.

Some biochemical tests were performed for more validation. Results indicated in table (3-1) showed that this isolate gave a positive result for oxidase, catalase, citrate utilization and capable to grow on cetrimide agar which is a selective media for *Pseudomonas* spp and grow on King A producing green pigment; hence able to grow on King B without producing any pigment.

From these results, it can be concluded that this isolate is a strain of *P. aeruginosa.* The results mentioned above were in agreement with Palleroni (1985); Hawkey and Lewis (1998).

Table (3-1) Some Morphological Characteristics and Biochemical Tests for *P. aeruginosa* H3

Test	Result
Oxidase	+
Catalase	+
Citrate utilization	+
Gram's Stain	-
Growth on:	
Cetrimide agar	+
King A	+/ Green
King B	+/ without pigment

3.2 Ability of *P. aeruginosa* H3 to Alginate Production

The ability of *P. aeruginosa* H3 to produce alginate is well known in previous study (Al-Janabi, 2006), the alginate dry weight was (70 mg/L) in comparison with (120 mg/L) mentioned by (Al-Janabi, 2006) and (35.5 mg/L) as it was described by Govan and Deretic (1996). The high productivity may be due to the nature of this isolate which was isolated from patient suffering from cystic fibrosis; hence *P. aeruginosa* need the alginate polysaccharide as a virulence factor to accumulate as a biofilm. *P. aeruginosa* is usually accompanied by a phenotypic changes resulting in an increased expression of bacterial genes coding for the production of alginate (Simpson *et al.*, 1989; Carmichael and Wheeler, 1997).

3.3 Mutagenesis of *P. aeruginosa* H3

In order to enhance the ability of *P. aeruginosa* H3 in alginate production, it was subjected to mutagenesis by different types of mutagens included physical mutagens (UV radiation and Laser) and chemical mutagens (MNNG and Mitomycin C).

According to Abbas *et al*. (2004); colonies screened to investigate the genetic alteration on the chromosomal DNA after mutagen treatments caused 90% death (10% survival) which may lead to over production mutants.

By observing the morphological differences, the mucoidal growth and colonies size, thirty candidate mutant colonies were selected after each mutagenic treatment. Alginate was extracted, precipitated and dry weight was determined as in (2.2.4) for each mutant.

3.3.1 Physical Mutagenesis:

3.3.1.1 Mutagenesis by UV radiation

The results demonstrated in a figure (3-1) shows the mutagenic and lethal UV radiation effect on *P.aeruginosa* H3, there was a reduction of total viable count of the bacterial cells from 1.31x 10^9 CFU/ml in the zero time to 0.35x10^9 CFU/ml after the first exposure to (2 J/m^2) of UV radiation. Survival percent was 26.7% after the first exposure followed by sever reduction to 5.34%, 4% and 2% for the next different UV irradiation doses.

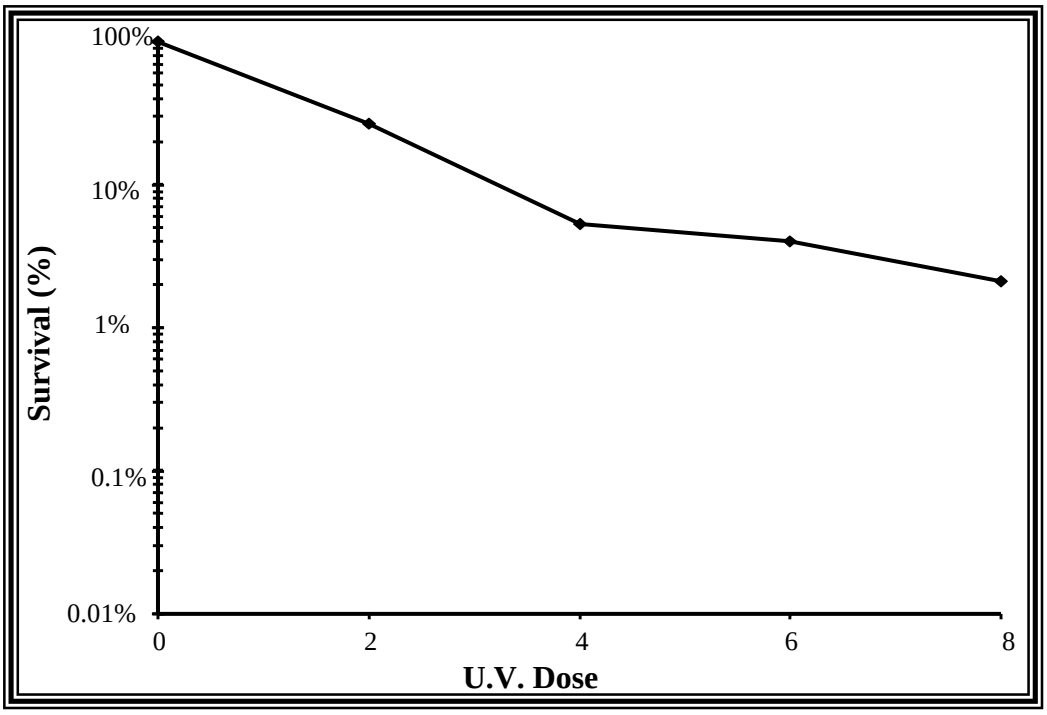

Figure (3-1) Effect of Different Doses of UV. Radiation on the Survival of *P. aeruginosa* H3.

From the selection and screening, table (3-2) shows the results of UV radiation: sixteen mutants out of thirty (53.33%) showed an increasing in alginate production 80mg/L for (mutants H3R10, H3R17, H3R23 and H3R26) to 170mg/L for (mutant H3R1) compared with the productivity of the wild type. There were seven mutants 23.33% with lower alginate production than the wild type. The alginate dry weight produced by these mutants ranged between 30mg/L for (mutant H3R5) to 60 mg/L (mutants H3R7, H3R15, H3R18, H3R27 and H3R28). Finally, the rest seven mutants 23.33% maintained their ability of alginate production 70mg/L.

UV irradiation is affected via missrepair of damaged DNA by SOS repair system and termed indirect mutagen it was applied as mutagens for the halotolerant *Micrococcus* sp., cell survival and mutability of *P.aeruginosa* and *P. syringae* and the survival of *Shewanella oneidensis* were determined after UV radiation (Al-Bakri and Umran, 1994; Kim and Sundin, 2001; Qiu *et al*., 2004).

Table (3-2) Alginate production by *P. aeruginosa* H3 After Irradiation with UV Radiation.

Bacterial Isolate	Alginate concentration (mg/L)
P. aeruginosa **H3** wild type	70
P. aeruginosa **H3R1**	170
P. aeruginosa **H3R2**	160
P. aeruginosa **H3R3**	70
P. aeruginosa **H3R4**	100
P. aeruginosa **H3R5**	30
P. aeruginosa **H3R6**	90
P. aeruginosa **H3R7**	60
P. aeruginosa **H3R8**	100
P. aeruginosa **H3R9**	100
P. aeruginosa **H3R10**	80
P. aeruginosa **H3R11**	90
P. aeruginosa **H3R12**	140
P. aeruginosa **H3R13**	120
P. aeruginosa **H3R14**	40
P. aeruginosa **H3R15**	60
*P. aeruginosa***H3R16**	140
P. aeruginosa **H3R17**	80
P. aeruginosa **H3R18**	60
P. aeruginosa **H3R19**	90
P. aeruginosa **H3R20**	100
P. aeruginosa **H3R21**	70
P. aeruginosa **H3R22**	70
P. aeruginosa **H3R23**	80
P. aeruginosa **H3R24**	70
P. aeruginosa **H3R25**	70
P. aeruginosa **H3R26**	80
P. aeruginosa **H3R27**	60
P. aeruginosa **H3R28**	60
P. aeruginosa **H3R29**	70
P. aeruginosa **H3R30**	70

3.3.1.2 Mutagenesis by Laser radiation

The survival of *P. aeruginosa* H3 after Laser exposure is shown in figure (3-2), results indicated that *P. aeruginosa* H3 was Laser sensitive. There was a reduction of total viable count from $6.3x10^{10}$ CFU/ml in the zero time to $2.2x10^{10}$ CFU/ml after the first time of exposure (30 sec) to Laser. Then reduction in the survival percent 34.9% after the first exposure when be sever reduction 26.9%, 9.68% and 2% survival for the next periods exposure of Laser.

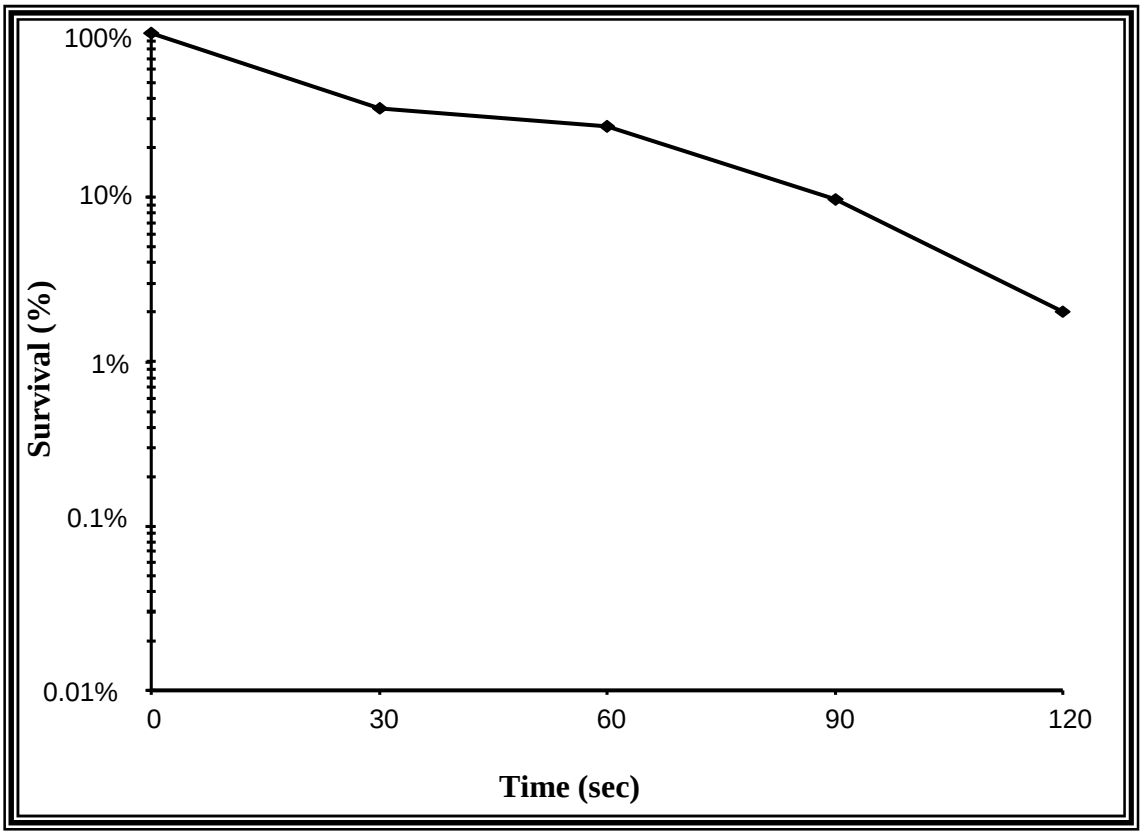

Figure (3-2) Effect of Laser Radiation on the Survival of *P. aeruginosa* H3, After Exposure for Different Periods.

Table (3-3) shows the results after laser radiation: seventeen mutants out of thirty (56.66%) were with high alginate production ranged between 80 mg/L for (mutants H3R34, H3R140, H3R47 H3R53 and H3R56) to 130 mg/L for (H3R42 mutant) compared with the productivity of wild type (70 mg/L). There were another five mutants (16.66%) showed decreasing in alginate production. The alginate dry weight produced by these mutants ranged between 50 mg/L for (mutant H3R35) to 60 mg/L for (mutants H3R37, H3R45, H3R48 and H3R57). There were also eight mutants (26.66%) expressed the same alginate production (70 mg/L for the mutants H3R33, H3R44, H3R51, H3R52, H3R54, H3R55, H3R59 and H3R60) after exposure to Laser radiation.

He-Ne laser induced cell inactivation is due to the induction of DNA damage. Laser is applied on *E. coli* to study its effect on the viability of the cells and its genomic contents (Cheba, 2000), photosensitization effected of laser was studied on *P. aeruginosa* (Al-Khafaji, 2002) the bacteriocidal effect of laser on *staphylococcus aureus* and *P. aeruginosa* (De Simone *et al*., 1999).

Table (3-3) Ability of *P. aeruginosa* H3 in Alginate Production After Irradiation with Laser.

Bacterial Isolate	Alginate concentration (mg/L)
P. aeruginosa **H3** wild type	70
P. aeruginosa **H3R31**	100
P. aeruginosa **H3R32**	90
P. aeruginosa **H3R33**	70
P. aeruginosa **H3R34**	80
P. aeruginosa **H3R35**	50
P. aeruginosa **H3R36**	90
P. aeruginosa **H3R37**	60
P. aeruginosa **H3R38**	100
P. aeruginosa **H3R39**	100
P. aeruginosa **H3R40**	80
P. aeruginosa **H3R41**	90
P. aeruginosa **H3R42**	130
P. aeruginosa **H3R43**	120
P. aeruginosa **H3R44**	70
P. aeruginosa **H3R45**	60
P. aeruginosa **H3R46**	110
P. aeruginosa **H3R47**	80
P. aeruginosa **H3R48**	60
P. aeruginosa **H3R49**	90
P. aeruginosa **H3R50**	100
P. aeruginosa **H3R51**	70
P. aeruginosa **H3R52**	70
P. aeruginosa **H3R53**	80
P. aeruginosa **H3R54**	70
P. aeruginosa **H3R55**	70
P. aeruginosa **H3R56**	80
P. aeruginosa **H3R57**	60
P. aeruginosa **H3R58**	90
P. aeruginosa **H3R59**	70
P. aeruginosa **H3R60**	70

3.3.2 Chemical Mutagenesis:

3.3.2.1 Mutagenesis by MNNG

Chemical mutagenesis was achieved by treatment of *P. aeruginosa* H3 with 30µg/ml MNNG which has mutagenic effect on the bacterial cells concluded from the reduction in the total viable count $4.6x10^9$ CFU/ml in zero time to $1.98x10^9$ CFU/ml after the first incubation time (15 sec) with MNNG and the reduction in survival percent was 43%, while the next incubation periods caused 13.5%, 8.69% and 2.4% survival.

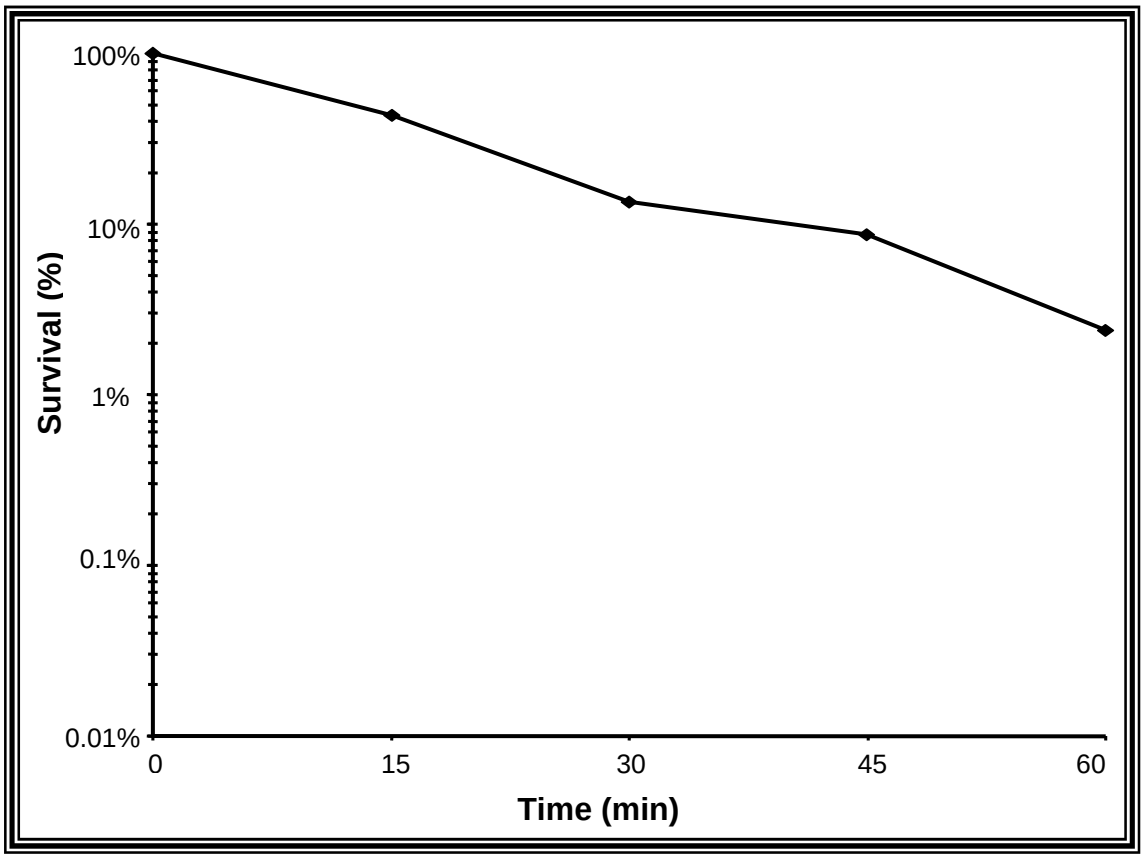

Figure (3-3) Survival of *P. aeruginosa* H3 After Incubation with MNNG (30µg/ml) for Different Time of Periods.

After mutagenesis with MNNG: nineteen mutant colonies (63.33%) gave with higher alginate production than the wild type (70 mg/L). The productivity of these mutants ranged between 80-160 for (H3R68, H3R73, H3R81, H3R78 mutant). Seven mutants (23.33%) with decreased alginate production. The alginate dry weight produced by these mutants ranged between 40 mg/L for (mutants H3R63 and H3R71) to 60 mg/L (mutants H3R62, H3R65, H3R69, H3R70 and H3R85),compared with the productivity of the wild type (70 mg/L). There were four mutants (13.33%) with the same alginate production (70 mg/L for the mutants H3R64, H3R74, H3R75 and H3R90) after MNNG treatment, table (3-4).

It is known that MNNG is effective mutagenic compound, it can generate mispairing lesion by adding alkyl (methyl group) to various position on nucleic acids, and hence missreplication of DNA, or missrepair of damaged DNA. Some of these lesions are potentially lethal as they can interfere with unwinding of the DNA during replication and transcription (Freifelder, 1987; Turner *et al.*, 2000). Mutation can be induced by an error prone DNA repair pathway (Stonesifer and Baltz, 1985; Abbas *et al.*, 2004).

Many studies dealing with the effect of MNNG on different bacteria (*Clostridium butricum*, *Micrococcus* sp. and *Gardnella vaginalis*) showed that these bacteria were sensitive to MNNG (carrasco and Soto, 1987; Al- Bakri and Umran, 1994; Al- Saady *et al.*, 2005).

Table (3-4) Ability of *P. aeruginosa* H3 in Alginate Production After Mutagenesis with MNNG.

Bacterial Isolate	Alginate concentration (mg/L)
P. aeruginosa **H3** wild type	70
P. aeruginosa **H3R61**	100
P. aeruginosa **H3R62**	60
P. aeruginosa **H3R63**	40
P. aeruginosa **H3R64**	70
P. aeruginosa **H3R65**	60
P. aeruginosa **H3R66**	130
P. aeruginosa **H3R67**	130
P. aeruginosa **H3R68**	80
P. aeruginosa **H3R69**	60
P. aeruginosa **H3R70**	60
P. aeruginosa **H3R71**	40
P. aeruginosa **H3R72**	90
P. aeruginosa **H3R73**	80
P. aeruginosa **H3R74**	70
P. aeruginosa **H3R75**	70
P. aeruginosa **H3R76**	90
P. aeruginosa **H3R77**	90
P. aeruginosa **H3R78**	160
P. aeruginosa **H3R79**	140
P. aeruginosa **H3R80**	150
P. aeruginosa **H3R81**	80
P. aeruginosa **H3R82**	140
P. aeruginosa **H3R83**	130
P. aeruginosa **H3R84**	130
P. aeruginosa **H3R85**	60
P. aeruginosa **H3R86**	120
P. aeruginosa **H3R87**	100
P. aeruginosa **H3R88**	110
P. aeruginosa **H3R89**	90
P. aeruginosa **H3R90**	70

3.3.4 Mutagenesis by Mitomycin C

Another type of chemical mutagen (Mitomycin C) was used to generate over producer mutants of alginate from *P. aeruginosa* H3. Results in figure (3-4) indicated that this mutagen has significant mutagenic and lethal effect on the bacterial cells of *P. aeruginosa* H3. This can be noticed from the reduction in the total viable count of bacterial cells from $5.2x10^8$ CFU/ml in the zero time to $2.55x10^8$ CFU/ml after the first incubation period (30 sec). The reduction in the survival percent after exposure to this dose was 49%, while the next incubation periods (30, 45 and 60 min.) caused severe reduction in the survival percent were 19.8%, 9.8% and 3.5%.

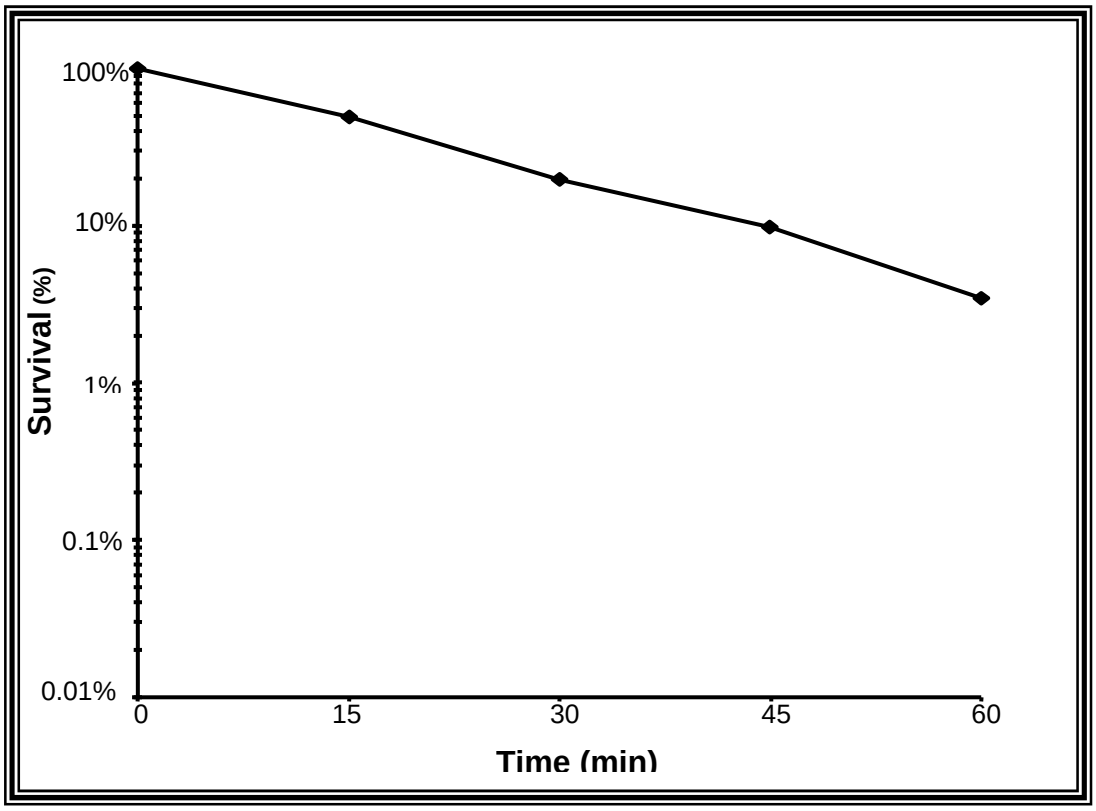

Figure (3-4) Survival of *P. aeruginosa* H3 After Incubation with Mitomycin C (10µg/ml) for Different Times.

After Mutagenesis with Mitomycin C, the illustrated results in table (3-5) appeared: nineteen mutants out of thirty (63.33%) with higher alginate production ranged between 80 mg/L for (mutants H3R95, H3R101, H3R104, H3R107, H3R111 and H3R113) to 160 mg/L for (H3R97 mutant), than the productivity of wild type (70 mg/L). Another six mutants (20%) showed decreasing in alginate production. The alginate dry weight produced by these mutants ranged between 30 mg/L for (mutant H3R91) to 60 mg/L (mutants H3R105, H3R106, H3R110 and H3R116). Five mutants (16.66%) have no changed in alginate production (70 mg/L for the mutants H3R96, H3R103, H3R115, H3R117 and H3R118).

The molecular basis of Mitomycin bioactivity derived mainly from its propensity to covalent interact with DNA sequences, causing lethal intra and interstrand cross-links as well as alkylation and formation of reactive oxygen species (Sheldon *et al.,* 1999).

Table (3-5) Ability of *P. aeruginosa* H3 in Alginate Production After Mutagenesis with Mitomycin C.

Bacterial Isolate	Alginate concentration (mg/L)
P. aeruginosa **H3** wild type	70
P. aeruginosa **H3R91**	30
P. aeruginosa **H3R92**	130
P. aeruginosa **H3R93**	140
P. aeruginosa **H3R94**	50
P. aeruginosa **H3R95**	80
P. aeruginosa **H3R96**	70
P. aeruginosa **H3R97**	160
P. aeruginosa **H3R98**	90
P. aeruginosa **H3R99**	120
P. aeruginosa **H3R100**	90
P. aeruginosa **H3R101**	80
P. aeruginosa **H3R102**	100
P. aeruginosa **H3R103**	70
P. aeruginosa **H3R104**	80
P. aeruginosa **H3R105**	60
P. aeruginosa **H3R106**	60
P. aeruginosa **H3R107**	80
P. aeruginosa **H3R108**	100
P. aeruginosa **H3R109**	90
P. aeruginosa **H3R110**	60
P. aeruginosa **H3R111**	80
P. aeruginosa **H3R112**	90
P. aeruginosa **H3R113**	80
P. aeruginosa **H3R114**	90
P. aeruginosa **H3R115**	70
P. aeruginosa **H3R116**	60
P. aeruginosa **H3R117**	70
P. aeruginosa **H3R118**	70
P. aeruginosa **H3R119**	100
P. aeruginosa **H3R120**	100

From the preceded results, UV radiation successfully enhanced alginate production from *P.aeruginosa* H3 since its productivity 2.43 fold higher than the alginate produced of the wild type.

Also, laser irradiation successfully developing the alginate productivity about 1.86 fold for the mutant H3R42 compared with productivity of the wild type. By comparing the highest alginate concentration 170mg/L for mutant (H3R1) that obtained after UV radiation and 130mg/L for mutant (H3R42) after Laser exposure, UV radiation was better than Laser in enhancing alginate productivity.

The chemical mutagens (MNNG and Mitomycin C) have the same efficiency in developing alginate production from *P. aeruginosa* H3 by producing 2.3 fold higher level for mutants (H3R78 and H3R97 respectively) which gave 160mg/L compared with the productivity of the wild type 70 mg/L. But still they are less efficient than the effect of UV radiation as mutagen, in the same time, these chemical mutagens have better effect than laser effect on *P. aeruginosa* H3.

Among the above results, table (3-6) shows that the physical mutagen (UV radiation) was the more efficient mutagen used to develop alginate productivity from *P. aeruginosa* H3 followed by MNNG, Mitomycin C and Laser respectively.

Table (3-6) Productivity of Alginate from the Over-Producer Mutants of *P. aeruginosa* H3 after Treatment with Physical and Chemical Mutagens.

Mutagen	Over-producer Mutant	Alginate (mg/L)
UV	**H3R1**	**170**
MNNG	H3R78	160
Mitomycin C	H3R97	160
Laser	H3R42	130

The mutation either occurred in the structural genes or in the regulatory genes that negatively regulate alginate production.

To explain the obtained results after mutagenesis with the physical mutagens and the chemical mutagens, the increment in alginate production by these mutants may be due to the effect of the mutagens which lead to inactivate the regulatory genes responsible for negative regulation of alginate production. the high productivity of alginate by *P. aeruginosa* mutants after exposure to different mutagens may be due to genetic mutations that inactivates negative regulatory genes (*muc* A, *muc* B, *muc* C and *muc* D) of the *alg* U leading to an increasing in alginate biosynthesis by direct action on *alg* D promoter or indirectly by up- regulation transcription of another regulatory gene *alg* R. Thus, genetic mutations in these regulatory genes are important in inactivation of *alg* D promoter and increase in alginate level, Gacesa, (1998); Martin *et al*.,(1993).

Reduction in alginate production by the mutants may be due to the genetic mutations induced in the structural genes responsible for alginate biosynthesis pathway (Bushell, 1983), or due to the mutations that may occur in the regulatory genes (*alg* U, *muc* A, *muc* B and *muc* C) which compromise the main switch controlling the conversion to the mucoidal growth (alginate production) (Gacesa, 1998).

Finally, unchangeable in alginate level expected due to genetic mutations in different genes of the chromosomal DNA of *P. aeruginosa* other than those responsible for production and regulation of alginate biosynthesis pathway, as it was mentioned by Martin *et al.* (1993); Gacesa (1998).

3.4 Analysis of Alginate Using FTIR Chromatography

Alginate produced by wild type and mutants of *P. aeruginosa* H3 after mutagenesis with physical and chemical mutagens was analyzed using FTIR chromatography to detect any structural variation in the chemical structure of alginate.

Results mentioned in figures (3-5), (3-6), (3-7), (3-8) and (3-9) showed the chemical structure of alginate produced by the wild type of *P. aeruginosa* H3 and the over-producer mutants (H3R1, H3R42, H3R78 and H3R97 that arose after mutagenesis with UV radiation, laser, MNNG and Mitomycin C respectively) using FTIR chromatography on a wide range of wave lengths (400-4000cm^{-1}).

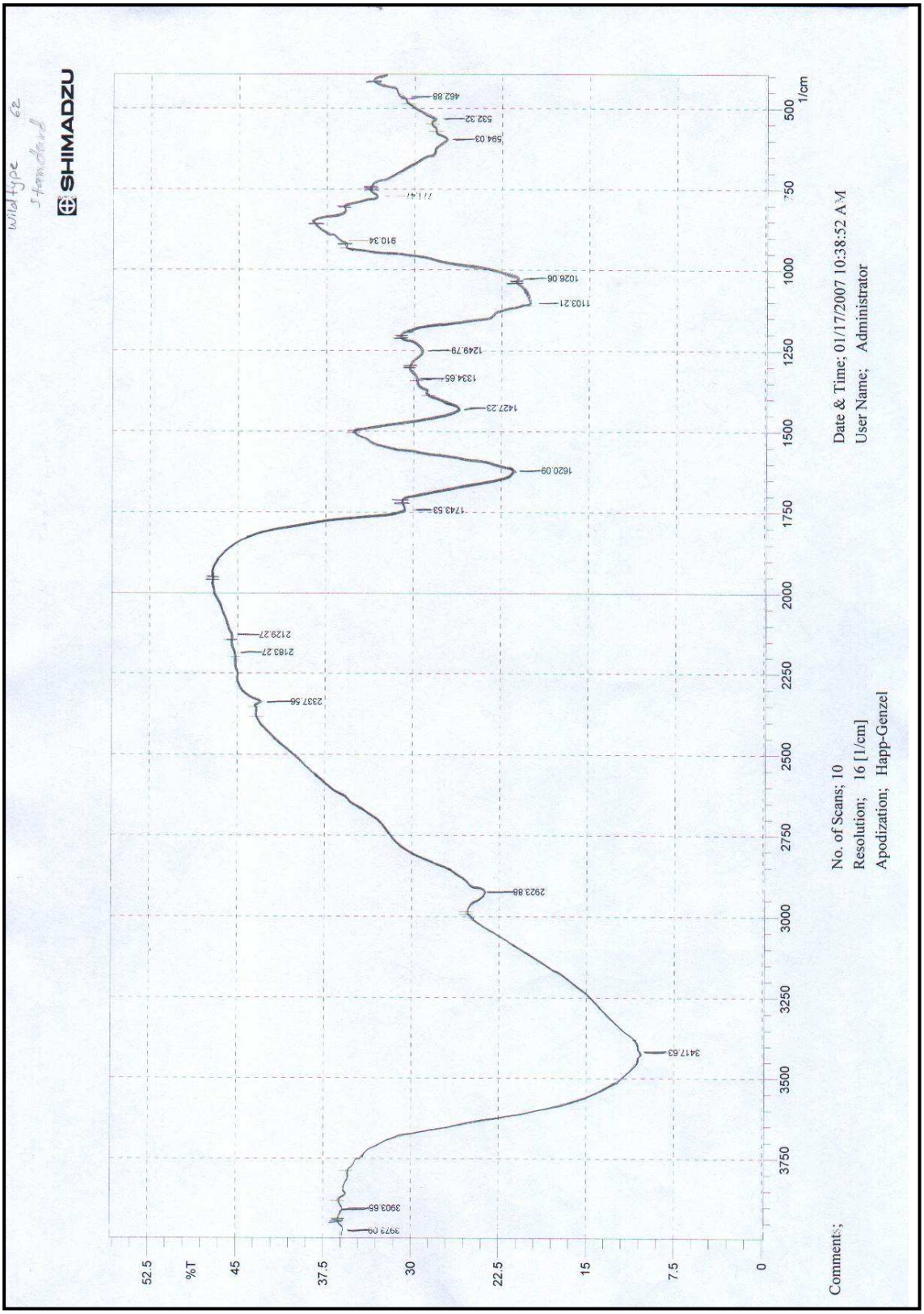

Figure (3-5) Structural Analysis of Alginate Produced by the Wild Type of *P. aeruginosa* H3 Using FTIR Chromatography.

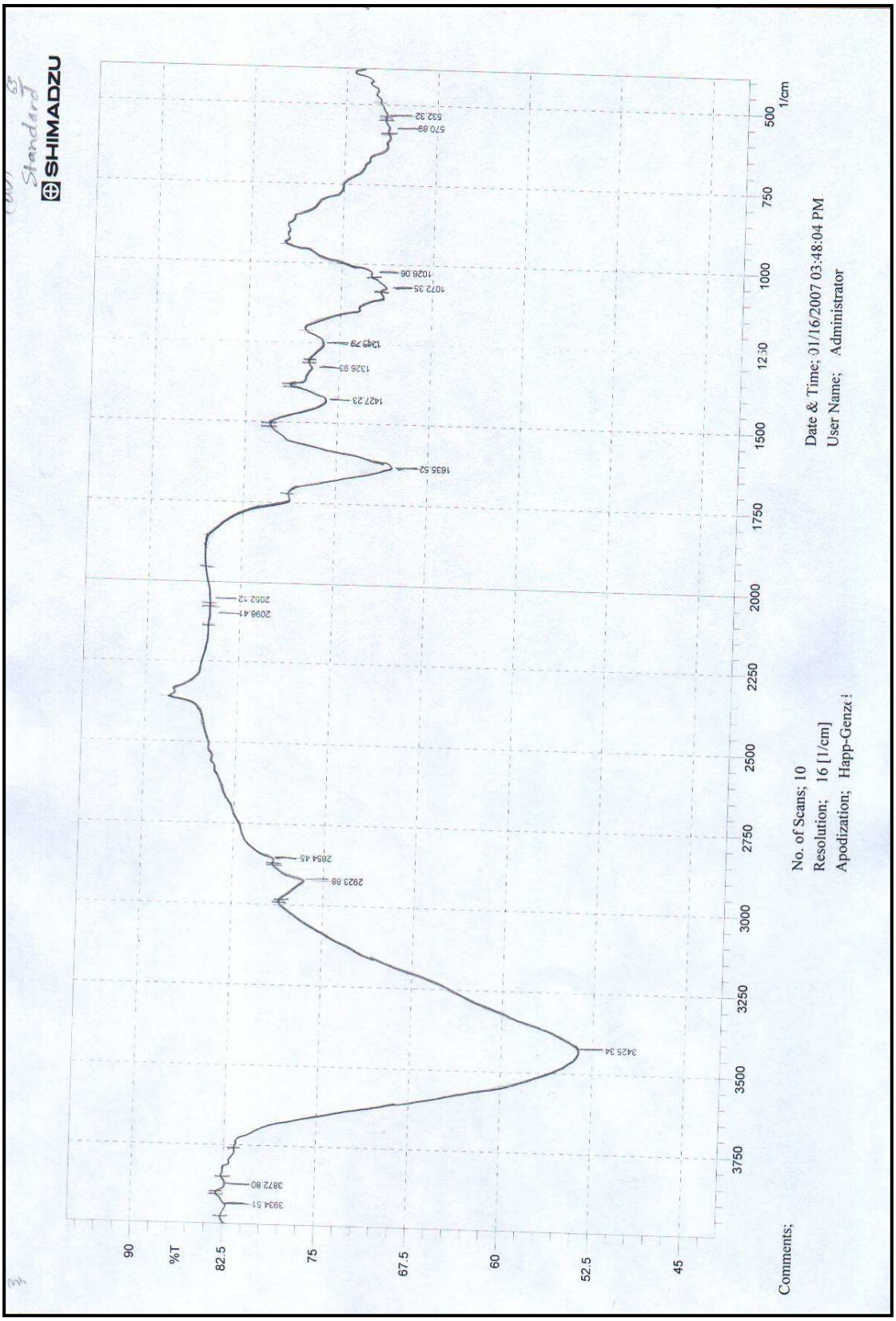

Figure (3-6) Structural Analysis of Alginate Produced by *P. aeruginosa* H3R1 Mutant Using FTIR Chromatography.

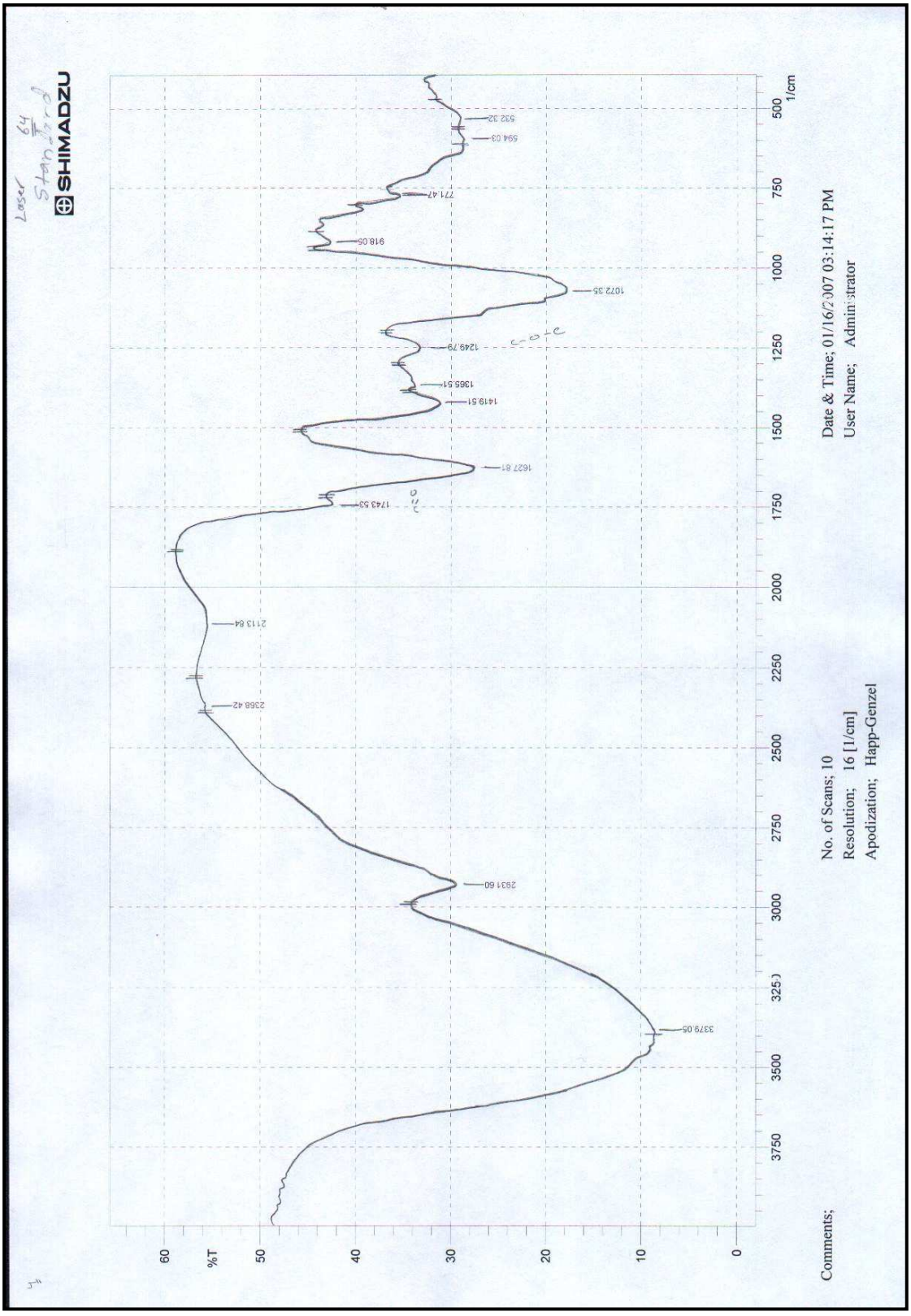

Figure (3-7) Structural Analysis of Alginate Produced by *P. aeruginosa* H3R42 Mutant Using FTIR Chromatography.

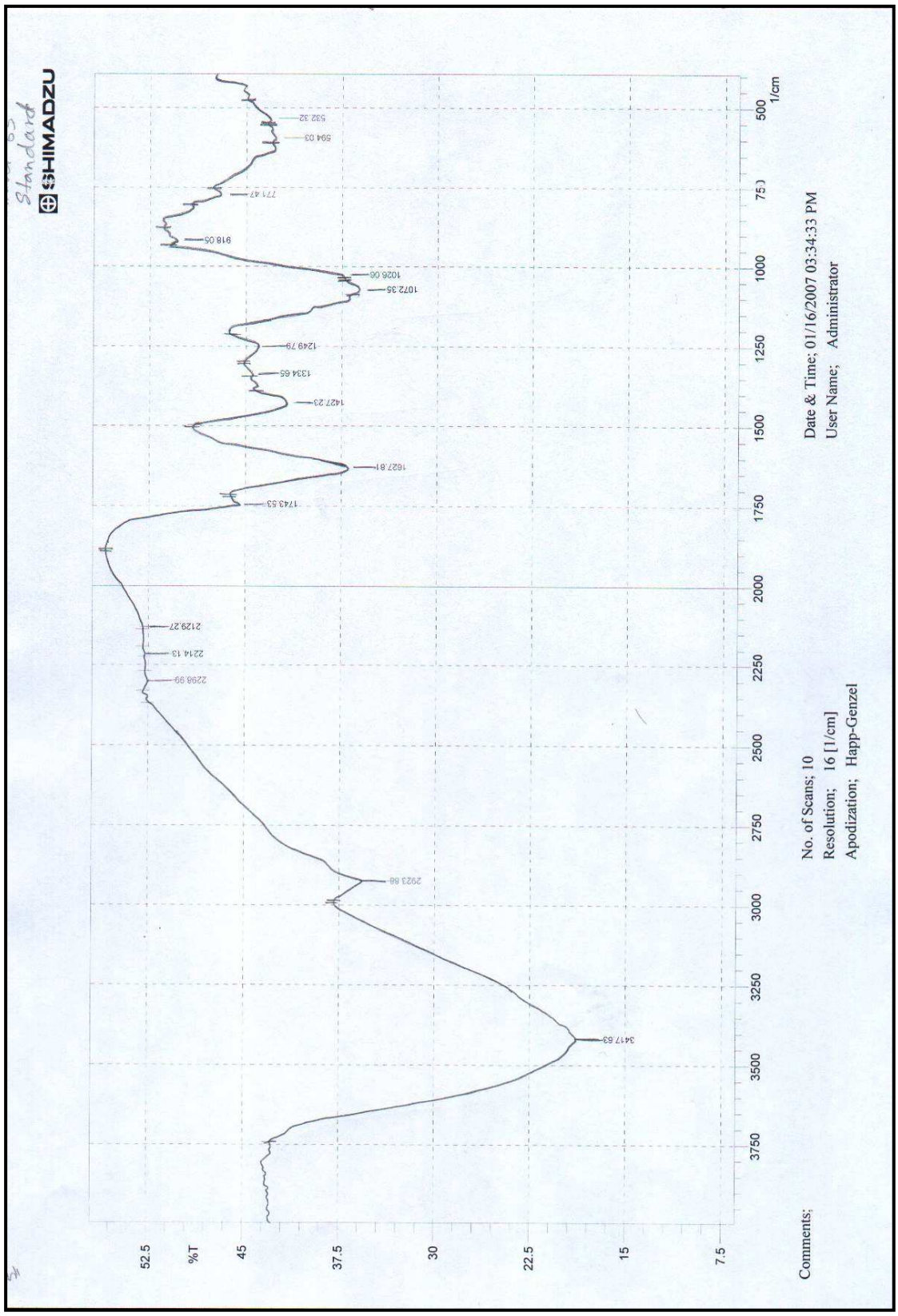

Figure (3-8) Structural Analysis of Alginate Produced by *P. aeruginosa* H3R78 Mutant Using FTIR Chromatography.

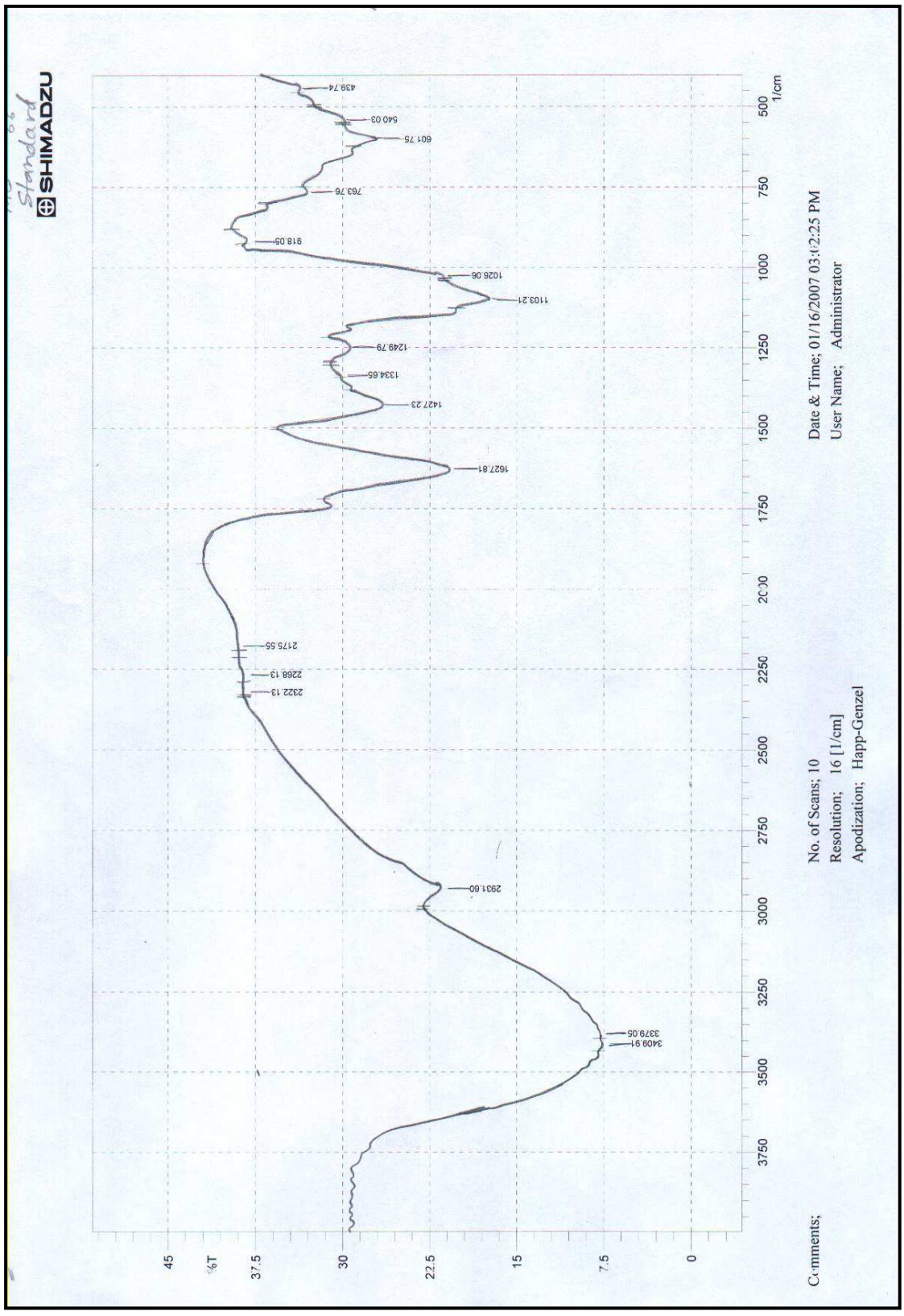

Figure (3-9) Structural Analysis of Alginate Produced by *P. aeruginosa* H3R97 Mutant Using FTIR Chromatography.

According to these results, there are no significant differences between the chemical structure of alginate by over –producer mutants and the alginate produced by the wild type. On the other hands, results indicated in table (3-7) showed that the absorbances of the active groups of alginate structure occurred nearly the same wave lengths (for hydroxyl group, carbonyl group and ether group). From the mentioned results, O-H broad band of alginate from the wild type (H3) and mutants (H3R1, H3R42, H3R78 and H3R97) were approximately the same. But for the C=O and C-O-C band, no differences were noticed between the values.

From these results it can be concluded that there were no differences in the chemical structure of alginate produced by these over producer mutants after treatment with different mutagens in comparison with alginate produced by the wild type *P. aeruginosa* H3. This may be due to the effect of different mutagens on other chromosomal genes rather than those coding for different enzymes dealing with alginate biosynthesis pathway.

Table (3-7) Absorbance of the Active Groups of Alginate Produced by the Wild Type of *P. aeruginosa* H3 and Over-Producing Mutants.

Bacterial Isolate	Mutagen	Functional Group	Wave Length (cm^{-1})
P. aeruginosa H3 (wild type)	—	O-H	3417.63
		C=O	1743.53
		C-O-C	1249.79
P. aeruginosa H3R1 mutant	UV radiation	O-H	3425.34
		C=O	1743.53
		C-O-C	1249.79
P. aeruginosa H3R42 mutant	Laser	O-H	3379.05
		C=O	1743.53
		C-O-C	1249.79
P. aeruginosa H3R78 mutant	MNNG	O-H	3417.63
		C=O	1743.53
		C-O-C	1249.79
P. aeruginosa H3R97 mutant	Mitomycin C	O-H	3409.91
		C=O	1743.53
		C-O-C	1249.79

4.1 Conclusions:-

1. Physical and chemical mutagens may lead to alginate over producer mutants for *P. aeruginosa* H3.
2. Physical mutagens (UV radiation and Laser) are better than the mutagens (MNNG and Mitomycin C) in increasing the alginate productivity of *P. aeruginosa* H3.
3. UV radiation is the most effective mutagen compared with laser, MNNG and Mitomycin C by producing alginate about 2.43 fold higher than the wild type.
4. The chemical structure of alginate produced by over-producer mutants after mutagenesis with physical mutagens (UV radiation and Laser) and chemical mutagens (MNNG and Mitomycin C) did not altered from the wild type.

4.2 Recommendations:-

1. Improving the ability of *P. aeruginosa* H3 in alginate production using transposon mutagenesis.
2. Studying the optimum conditions of alginate production by H3R1 over-producing mutant using liquid fermentation.
3. Large scale production of alginate by H3R1 over-producing mutant under the optimum conditions.
4. Attempt to clone alginate genes in suitable host other than *P. aeruginosa*.

List of Abbreviations

Abbreviation	Meaning
Ca-alginate	Calcium-alginate
CPD	Cyclobutane Pyrimidine Dimer
D.W.	Distilled Water
EMS	Ethyl Methane Sulfonate
FTIR	Fourier Transform- Infrared
GDP-Mannuronic acid	Guanosin di-phosphate Mannuronic acid
IR	Infrared
J/m^2	Joule/ meter square
LB	Luria Bertani
MMC	Mitomycin C
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
rpm	Round per minute
UV	Ultraviolet
UVR	Ultraviolet Radiation

Acknowledgements

First of all Praise to ***Allah*** *the lord of the universe, peace be upon* ***Mohammed*** *the messenger of* ***Allah*** *and upon his* ***Relatives****.*

First and foremost my grateful thanks must go to my esteem supervisor ***Dr. Hameed M. Al- Dulaimi*** *for his invaluable guidance and great support throughout my study and preparation.*

I am grateful to ***Miss. Shaimaa Hussain*** *for her love, Kindness, help and encouragement throughout the study.*

With pleasure I would like to thank my friends for their kind support: Rasha, Hadeel, Zainab, Mayassa, Safa, Sura, Sahar, Rafah, Rawaa, Raghda, Marwa, Linda, Oday, Hassan, Saddam, Jameel, Hussam and to whom that I didn't mention.

My special thank to my close friends Mayasa, Saja, Dahlia.

At last I would like to thank my family who support and understand me with great love.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تأثير بعض المطفرات الفيزيائية والكيميائية في إنتاج بكتيريا *Pseudomonas aeruginosa* H3 للألجنيت

رسالة
مقدمه إلى كلية العلوم/ جامعة النهرين
وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانه الأحيائية

من قبل

رفـل سامي ذيـاب

(بكالوريوس تقانة أحيائية ٢٠٠٤)

ربيع الثاني- ١٤٢٨

أيار- 2007

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Effect of Some Physical and Chemical Mutagens on *Pseudomonas aeruginosa* H3 Alginate Production

A thesis
Submitted to the College of Science of Al-Nahrain University
in partial Fulfillment of the Requirements for the Degree of
Master of Science in Biotechnology

By

Rafal Sami Thiab

B. Sc. Biotechnology (2004)

May- 2007

Rabi'a al- Thani -1428

List of Contents

Chapter Two: Materials and Methods

Chapter Three: Results and Discussion

Chapter Four: Conclusions and Recommendations

List of Figures

List of Tables

Summary

This study was aimed to alter the ability of *Pseudomonas aeruginosa* H3 in alginate production by using some physical and chemical mutagens. Physical mutagenesis were UV radiation and laser mutagens. Chemical mutagenes were N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and Mitomycin C.

Physical mutagenesis was performed by subjection the cell suspension of *P. aeruginosa* H3 to different doses of UV radiation (2, 4, 6, 8 and 10 J/m^2) and laser for (30, 60, 90 and 120 sec.), then, cell suspension was spread on LB agar plates and incubated at 37 ˚C for 24hrs. After that, random selection of thirty colonies that arose from the cells subjection to the effect of different doses of UV radiation and laser were made (90% of the suspended cells were killed and were tested for their alginate production). Results showed that the mutagenesis by UV radiation caused an increase in the ability of *P. aeruginosa* H3 in alginate production. Over-producer mutant, H3R1, was obtained from this treatment. The productivity of this mutant was 170mg/L in comparison with the productivity of wild type (70mg/L). On the other hand, results also showed that mutagenesis using laser radiation caused an increase in the ability of *P. aeruginosa* H3 in alginate production. Over-producer mutant, H3R42, was obtained from this treatment characterized by its high ability in alginate production was 130mg/L in comparison with the productivity of wild type (70mg/L).

Chemical mutagenesis was achieved by incubation *P. aeruginosa* H3 with 30µg/ml of MNNG and 10µg/ml of mitomycin C separately for 60 minutes, then cell suspension was spread on LB agar plates and incubated at 37 ˚C for 24 hrs. After that, random selection of thirty colonies that arose from the cells incubation with the chemical mutagens for the period that caused killing of 90% of the cells was made,

and the alginate production of *P. aeruginosa* H3 was examined. Results showed that mutagenesis by MNNG caused an increase of *P. aeruginosa* H3 alginate production. Over-producer mutant H3R78 was obtained, the productivity of this mutant was 160mg/L. Results also showed that mutagenesis by mitomycin C caused an increase of *P. aeruginosa* H3 alginate production. Over-producer mutant H3R97, was obtained, the productivity of this mutant was 160mg/L compared with the productivity of the wild type.

In order to analyze the chemical structure of alginate produced by *P. aeruginosa* H3 (wild type) and the over producer mutants, Fourier Transform-Infrared was performed. Results showed that there are no differences in the chemical structure of alginate produced by the wild type and over producer mutants.

Dedication

To the Gorgeous and Best Family that One Can Ever Get

Rafal

بسم الله الرحمن الرحيم

وَلله غَيْبُ السَّمَاوَاتِ وَالأَرْضِ وَإِلَيْهِ يُرْجَعُ الأَمْرُ كُلُّهُ فَاعْبُدْهُ وَتَوَكَّلْ عَلَيْهِ وَمَا رَبُّكَ بِغَافِلٍ عَمَّا تَعْمَلُونَ ۝

صدق الله العظيم

سورة هود
الآية{١٢٣ }

الـخـــــــلاصـــــــة

تهدف هذه الدراسة إلى استخدام المطفرات الفيزيائية والكيميائية لتطوير قابلية بكتيريا *P. aeruginosa* H3 في إنتاج الألجينيت بأستخدام بعض المطفرات الفيزيائية والكيميائية. أجريت عملية التطفير الفيزيائي باستخدام الأشعة فوق البنفسجية والليزر وعملية التطفير الكيميائي باستخدام مادة MNNG و MitomycinC.

تمت عملية التطفير الفيزيائي بتعريض عالق خلايا بكتيريا *P. aeruginosa* H3 إلى جرعات مختلفة من الأشعة فوق البنفسجية (٢، ٤، ٦، ٨و ١٠ جول/م^{٢}) وأشعة الليزر (٣٠، ٦٠، ٩٠ و ١٢٠ ثانية) ثم نشر العالق على أطباق LB المتصلب بمادة الأكار وحضنت بدرجة ٣٧ °م لمدة ٢٤ ساعة، تم بعدها الانتقاء العشوائي لثلاثين من المستعمرات الناشئة عن الخلايا المتعرضة لتأثير الجرعات من الأشعة فوق البنفسجية وأشعة الليزر التي أدت إلى قتل ٩٠% من خلايا العالق، ثم غربلة قابليتها على إنتاج الألجينيت. وقد أشارت النتائج إلى إن التطفير بالأشعة فوق البنفسجية أدى إلى زيادة قابلية بكتيريا *P. aeruginosa* H3 في إنتاج الألجينيت، حيث تم الحصول على طافرة بكتيرية (H3R1) تميزت بكفاءتها العالية في إنتاج الألجينيت، إذ بلغت إنتاجيتها ١٧٠ ملغم/ لتر مقارنة بإنتاجية النوع البري (٧٠ ملغم/لتر). من ناحية أخرى أشارت النتائج إلى إن التطفير بأشعة الليزر أدى إلى زيادة قابلية البكتيريا *P. aeruginosa* H3 في إنتاج الألجينيت، حيث تم الحصول على طافرة بكتيرية (H3R42) تميزت بكفاءتها العالية في إنتاج الألجينيت، إذ بلغت إنتاجيتها ١٣٠ملغم/لتر مقارنة بإنتاجية النوع البري (٧٠ ملغم/لتر).

أجريت عملية التطفير الكيميائي لبكتريا *P. aeruginosa* H3 بحضن هذه البكتريا مع مادة MNNG بتركيز ٣٠ مايكرو غرام/مل و مادة Mitomycin C بتركيز ١٠ مايكرو غرام/ مل كل على انفراد ولمدة ٦٠ دقيقة، نشر خلالها عالق الخلايا على أطباق LB المتصلب بمادة الأكار وحضنت بدرجة ٣٧ °م لمدة ٢٤ ساعة ، تم بعدها الانتقاء العشوائي لثلاثين من المستعمرات الناشئة عن لخلايا المتعرضة للتطفير بكلا المطفرين الكيميائيين واللذان أديا إلى قتل ٩٠% من خلايا العالق، ثم غربلة قابليتها على إنتاج الألجينيت. وقد أشارت النتائج إلى التطفير بمادة MNNG أدى إلى زيادة قابلية بكتريا *P. aeruginosa* H3 في إنتاج الألجينيت حيث تم الحصول على طافرة بكتيرية (H3R78) تميزت بكفاءتها العالية في إنتاج الألجينيت، إذ بلغت إنتاجيتها ١٦٠ ملغم/لتر مقارنة بإنتاجية النوع البري (٧٠ملغم/لتر).

كما أشارت النتائج أيضا إلى إن التطفير بمادة Mitomycin C أدى إلى زيادة قابلية بكتريا*P. aeruginosa* H3 في إنتاج الألجينيت حيث تم الحصول على طافرة بكتيرية (H3R97) تميزت بكفاءتها العالية في إنتاج الألجينيت، إذ بلغت إنتاجيتها ١٦٠ ملغم/لتر مقارنة بإنتاجية النوع البري.

تم استخدام تقنية (FTIR) Fourier Transform- Infrared من اجل تحليل التركيب الكيميائي للألجينيت المنتج من بكتيريا *P. aeruginosa* H3 (النوع البري) والطافرات الأكثر إنتاجا. وقد أشارت النتائج إلى عدم وجود اختلافات بالتركيب الكيميائي للألجينيت بين النوع البري والطافرات.

Supervisor Certification

I, certify that this thesis was prepared under my supervision in Al-Nahrain University/ College of Science/ Department of Biotechnology as a partial requirement for the degree of Master of Science in Biotechnology.

Signature:

Supervisor: Dr. Hameed M. Al-Dulaimi

Scientific Degree: Assistant Professor

Date:

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature:

Name: Dr. Nabeel Al-Ani

Scientific Degree: Assistant Professor

Title: Head of Biotechnology Department

Date:

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:
Name:
Scientific Degree:
(Chairman)

Signature:
Name:
Scientific Degree:
(Member)

Signature:
Name:
Scientific Degree:
(Member)

Signature:
Name: Dr. Hameed M. Al-Dulaimi
Scientific Degree:
(Member/ Supervisor)

I hereby certify upon the decision of the examining committee.

Signature:
Name: Dr. Laith Abdul Aziz
Scientific Degree:
Title: Dean of the College of Science
Date:

Aarons, S. J.; Sutherland, I. W.; Chakrabarty, A. M. and Gallagher, M. P. (1997). A novel gene, *algK*, from the alginate biosynthetic cluster of *Pseudomonas aeruginosa*. *Microbiolo.* 143: 641-652.

Al-Bakri, G. H. and Umran, M.A. (1994). Mutagenesis of novel halotolerant bacteria (Micrococcus sp.)using ultraviolet light and N-methyl N'-nitro N-nitrosoguanidine. Iraq. J. Microbial 6: 55-65.

Abbas, T.; Fatemeh, K. and Muhnaz, M. A. (2004). Improved production of rhamnolipids by a *Pseudomonas aeruginosa* mutant. Iran. Biomed. J. 8(1): 25-31.

Albrecht, M. T. and Schiller, N. L. (2005). Alginate lyase (AlgL) activity is required for alginate biosynthesis in *Pseudomonas aeruginosa*. Bacteriol. 187: 3869–3872.

Al-Emarra, F. J.M. (1990). Laser As New Technique. 1st ed. pp. 68.

Al-Janabi, H. A. H. (2006). Production and characterization of alginate produced by the locally isolated *Pseudomonas aeruginosa*. M.Sc. Thesis in Biotechnology, College of Science/ Al- Nahrain University. Iraq.

Al-Khafaji, A. S. (2002). A study of the photosensitization technique by low power He: Ne laser on *Pseudomonas aeruginosa* infection burn wound. M.Sc. Thesis in Biotechnology, College of Science / Baghdad University. Iraq.

Al-Saady, R. E.; Al-Gelawi, M. H. and Al-Ani, Z. N. (2005).Mutagenesis of locally isolate Gardnella vaginalis using nitrosoguanodine and ultraviolet light. J. Al- Nahrain University. 8: (1).

Atlas, R.M.; Parks, L.C. and Brown, E.A. (1995). Laboratory Manual of Experimental Microbiology. Mosby-Year Book, Inc., Wesline Industrial Drive, St. Louis, Missouri.

Bagewadikar, R. S. and Bhattacharya, R. K. (1982). Inhibition of *in-vitro* amino acid incorporation by the carcinogen N-methyl N'-nitro N-nitrosoguanidine. J. Bioscience. 4(2): 183-190.

Bhawalker, D. D. and Kukreja, L. M. (1992). Lasers in Medicine. Indian Institution of Technology, New Delhi. pp: 1-15.

Bowring, S. N. and Morris, J. G. (1985). Mutagenesis of *Clostridium acetobutylicum.* J. Appl. Bacteriology. 58: 577-584.

Bushell, M. E. (1983). Progress in Industrial Microbiology. Microbial Polysaccharides. Elsevier, UK. 1st ed. pp: 45-83.

Carmichael, J. and Wheeler, L. (1997). Molecular biology of *Pseudomonas aeruginosa* infections in Cyctic fibrosis. J. Biochem. 1: 1-23.

Cerada- Olmedo, E. and Hanawalt, P. C. (1967). Macromolecular action of nitrosoquanidine in *E.coli*. Bio. Chem. E.T. Biophys. Acta. 143: 450-464.

Cheba, B. O. (2000). A study on the effect of He: Ne and nitrogen lasers radiation on the viability of *E.coli* and its genomic content. M.Sc. Thesis in Biotechnology, College of Science/ Baghdad University. Iraq

Chopra, S. and Chawla, H. M. (1992). Lasers in Chemical and Biological Sciences. Wiley Eastern Limited, New Delhi.1: 2-182.

Chu, L.; May, T.B.; Chakrabarty, A.M. and Misra, T.K. (1991). Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *pseudomonas aeruginosa* strains. Gene. 107: 1-10.

Coyne, M. J.; Russell, K. S.; Coyle, C. L. and Goldberg, J. B. (1994). The *Pseudomonas aeruginosa algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. J. Bacteriol. 176: 3500-3507.

Cruickshank, R.; Duguid, J. P.; Marmior, B. P. and Swain, R. H. A. (1975). Medical Microbiology. Churchill Livingstone, London. 12th ed., Vol. 17.

Dale, J.W. (1998). Molecular Genetics of Bacteria. John Wiley & Sons, UK. 3rd ed.

Deretic, V. and Konyecsni, W. M. (1990). A prokaryotic regulatory factor with a histon H1-like carboxyl-terminal domain: clonal variation of repeats within *alg P,* a gene involved in regulation of mucoidy in *Pseudomonas aeruginosa*. J. Bacteriol.172: 5544-5554.

Deretic, V.; Gill, J. F. and Chakrabarty, A. M. (1987). *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. 15: 4567–4581.

De Simone, N. A.; Christian, C. and Dore, D. (1999). Bacteriocidal effect of 0.95 mW helium-neon and 5 m Windium-gallium-aluminum- phosphate laser irradiation at exposure times 30, 60 and 120 seconds on photosensitize *Staphylococcus aureus* and *Pseudomonas aeruginosa* in vitro. Physical ther.79 (9).

Draget, K.I.; Onsøyen, E.; Fjaereide, T.; Simensen, M. K. and Smidsrød, O. (1997). Use of G-block polysaccharides. Carbohydr. Polym. 29: 209-215.

Eisenstark, A. (1989). Bacterial genes involved in response to near ultraviolet radiation. Adv. Genet. 26: 99–147.

Ertesvag, H. and Valla, S. (1998). Biosynthesis and applications of alginates. Polym. Degrad. Stabil. 59: 85-91.

Espevik, T. and Skjak-Braek, G. (1996). Application of alginate in biotechnology and biomedicine. Carbohydr. Eur. 14: 19-25.

Franklin, M. J.; Chitnis, C. E.; Gacesa, P.; Sonesson, A.; Wite, D. C. and Ohman, D. E. (1994). *Pseudomonas aeruginosa* AlgG is a polymer level alginate C-5 mannuronan epimerase. J. Bacteriol. 176: 1821-1830.

Franklin, M. J. and Ohman, D. E. (1993). Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J. Bacteriol. 175: 5057–5065.

Franklin, M. J. and Ohman, D. E. (1996). Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. J. Bacteriol. 178: 2186–2195.

Freifelder, D. (1987). Molecular Biology. Jones and Bartlett, Boston. USA. 2nd ed.

Gacesa, P. (1998). Bacterial alginate biosynthesis- recent progress and future prospects. Microbiol. 144: 1133–1143.

Goodenough, U. (1984). Genetics. Saunders College publishing, Philadelphia. USA. 3rd ed.

Govan, J. R. W. and Deretic, V. (1996). Microbial pathogenesis in cyctic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepaci* .J. Microbiol. Rev. 60 (3): 539-574.

Greenwood, D.; Slack, R. and Peatherer, J. (1997). Medical Microbiology. 5th ed. Churchill Livingstone, London

Grinberg, T.; Pirog, T. P.; Malashenko, Y. R. and Vlasov, S. A. (1995). Ethapolan: A new microbial exopolysaccharide for oil industry. J. Energy and Fuels. 9: 1086-1089.

Harely, P. J. and Prescott, M. L. (1996). Laboratory Exercises in Microbiology. ASM press, Washington.

Hawkey, P. and Lewis, D. (1998). Medical Bacteriology. Oxford University Press. UK.

Henderson, C. I. (1993). Recent advances in the usage of mitomycin. J. Oncol. 50:1–84.

Iglewski, BH (1996). *Pseudomonas*. In: Baron's Medical Microbiology. Churchill Livingstone, London. 4th ed.

Jain, S.; Franklin, M. J.; Ertesvag, H.; Valla. S.; and Ohman, D. E. (2003). The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. J. Mol. Microbiol. 47: 1123-1133.

Jain, S.; and Ohman, D. E. (1998). Deletion of *algk* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J. Bacteriol. 180: 634-641.

Jarman, T. R.; Deavin. I.; Slocombe, S. and Righelato, R. C. (1978). Investigation of the effect of environmental conditions on the rate of exopolysaccharides synthesis in *Azotobacter vinelandii*. J. Gen. Microbiol. 107: 59-64.

Jawetz, M. D.; Melnick, J. K. and Adelberg, E. A. (1998). *Pseudomonades.* In: Medical Microbiology Review. Appleton and Lange, USA. 21st ed. 231-233.

Kandela, S. A. (1988). Laser Physics and Technical Applications. Ministry of Higher Education. Baghdad University. 1st ed.

Kvam, B. (1987). Conformational conditions and ionic interactions of charged polysaccharides. Application of NMR techniques and Poisson- Boltzman equation, Ph.D. Thesis, Norwegian Institution of technology, Trondheim.

Kidambi, S. P.; Sundin, G. W.; Palmer, D. A.; Chakrabarty, A. M. and Bender, C. L. (1996). Copper as a signal for alginate synthesis in *Pseudomonas syringae* pv. *syringae*. Appl. Environ. Microbiol. 61: 2172-2179.

Kim, J. J. and Sundin, G. W. (2001). Construction and analysis of photolyase mutants of *Pseudomonas aeruginosa* and *Pseudomonas syringae*: Contribution of photoreactivation, nucleotide excision repair, and mutagenic DNA repair to cell survival and mutability following exposure to UV-B radiation. Appl. Environ. Microbiol. 67(4): 1405-1411.

Lebrun, L.; Junter, G. A.; Jouenne, T. and Mignot, L. (1994). Exopolysaccharide production by free and immobilized microbial cultures. Enz. Microbiol. Technol. 16: 1048-1054.

Maharaj, R.; May, T. B.; Wang, S. K. and Chakrabarty, A. M. (1993). Sequence of the *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. Gene 136: 267-269.

Maki, H. (2002). Origins of spontaneous mutations: specificity and directionality of base-substitution, frame shift, and sequence-substitution mutagenesis. Ann. Rev. of Genet. 36: 279-303.

Maniatis, T.; Fritch, E. F. and Sambrock, J. (1982). Molecular Cloning: A Laboratory Manual. Cold spring Harbor Laboratory, New York.

Martin, D. W.; Schurr, M. J.; Mudd, M. H.; Govan, J. R.; Holloway, B. W. and Deretic, V. (1993). Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. 90:8377-8381.

Mathee, K.; Kharazmi, A. and Hoiby, N. (2002). Role of Exopolysaccharide in Biofilm Matrix Formation: The Alginate Paradigm. Mol. Ecol. Biofilms. 1: 1-34.

McBeth, D. L. (1988). Effect of degradative plasmid CAM-OCT on responses of *Pseudomonas* bacteria to UV light. J. Appl. Bacteriol. 171(2): 975-982.

McHugh, D.J. (1987). Production properties and uses of alginates in: Production and Utilization of Products from Commercial Seaweeds, FAO Fisheries Technical Paper. 288: 58-115.

Mercado, C. M. and Tomasz, M. (1972). Inhibitory effects of mitomycin-related compounds lacking the C1-C2 aziridine ring. Antimicrob. Chemother. 1(1): 73-77.

Merza, R. A. (1988). Laser in Medicine. Aldewani, Baghdad. Iraq. 1st ed. 12-19.

Miller, R.V.; Jefrrey, W.; Mitchell, D. and Elsari, M. (1999). Bacterial responses to ultraviolet light. American Society for Microbiology. 65: 535-542.

Moe, S. T.; Draget, K. I.; Skjak Braek, G. and Smidsrod, O. (1995). Alginates, In: Food Polysaccharide and their Applications. Marcel Dekker, New York 9: 245-286.

Nyvall, P.; Corre, E.; Boisset, C.; Barbeyron, T.; Rousvoal, S.; Scornet, D.; Kloereg, B. and Boyen, C. (2003). Characterization of mannuronan C-5 epimerase genes from the brown algae *Laminaria digitata*. Plant Physiol. 133: 726-735.

Onsøyen, E. (1996). Commercial applications of alginates, Carbohydr. Eur. 14: 26-31.

Palleroni, N. J. (1985). Pseudomonadaceae. Cited from Bergy's Manual of Systematic Bacteriology. Vol. (1). Williams and Wilkins, USA.

Paustian, T. D. and Kurtz, R. S. (1994). Transposon Mutagenesis of *Rhodobacter sphaeroides*. Department of Biology. Yale University. 15th ed. 15: 45-61.

Qui, X.; Sundin, G. W.; Chai, B.; Tiedje, J. M. (2004). Survival of *Shewanella oneidensis* MR-1 after UV radiation exposure. Appl. Environ. Microbiol. 70 (11): 6435-6443.

Rehm, B. H. A.; Boheim, G.; Tommassen, J. and Winkler, U. K. (1994). Over expression of AlgE in *Escherichia coli* subcellular localization, purification and ion channel properties. Bacteriol. 176: 5639-5647.

Rehm, B. H. A. and Valla, S. (1997). Bacterial alginates: biosynthesis and applications. Appl. Microbiol. Biotechnol. 48:281-288.

Reich, E.; Shatkin, A. J. and Tatum, E. L. (1961). Bacteriocidal action of mitomycin C. Biochim. Biophys. Acta. 53:132-149.

Remminghorst, U. and Rehm, B. H. A. (2006). In Vitro Alginate Polymerization and the Functional Role of Alg8 in Alginate Production by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 72 (1): 289-305.

Roberts, G. (2003). Chemical mutagens. Review. Edited by Timothy Paustian, University of Wisconins-Madison.

Robles-Price, A.; Wong, T. Y.; Sletta, H.; Valla, S.; and Schiller, N. L. (2004). AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. Bacteriol. 186: 7369-7377.

Schiller, N. L.; Monday, S. R.; Boyd, C. M.; Keen, N. T. and Ohman, D. E. (1993). Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): cloning, sequencing, and expression in *Escherichia coli*. Bacteriol. 175: 4780–4789.

Sekiguehi, M. and Takagi, Y. (1960). Effect of mitomycin C on the synthesis of bacterial and viral deoxyribonucleic acid. Biochim. Biophys. Acta. 41:434-443.

Setty, R.S. and Sreekrish, V. (2004). Biotechnology including: Cell Biology, Genetics and Microbiology. New Age International (P) Ltd., New Delhi. 1st ed. Pp. 212-222.

Shankar, S.; Schlictman, R. W. and Chakrabarty, A. M. (1995). Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. Adv. Enzymol. Relat. Areas Mol. Biol. 70:221–255.

Sheldon, P. J.; Johnson D. J; August, P. R.; Liu, H.W. and Sherman, D.H. (1999). Characterization of a Mitomycin-binding drug resistance Mechanism from the producing organism. Bacteriol. 179 (5): 1796-1804.

Shinabarger, D.; Berry, A.; May, T. B.; Rothmel, R.; Fialho, A.; and Chakrabarty, A. M. (1991). Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase- a bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. Biol. Chem. 266: 2080-2088.

Simpson, J. A.; Smith, S. E. and Dean, R. T. (1989). Scavenging by alginate of free radicals released by macrophage. Free radical Biol. Med. 6: 347-353.

Smidsrød, O. and Skjåk-Bræk, G. (1990). Alginate as immobilization matrix for cells. Trends biotechnol. 8: 71-78.

Soon-Shiong, P.; Feldman, E.; Nelson, R.; Heintz, R.; Yao, Q.X.; Yao, Z. W.; Zheng, N.; Merideth, G.; Skjåk-Bræk, G.; Espevik, T.; Smidsrød, O. and Sandford, P. A. (1993). Long -term reversal of diabetes by the injection of immunoprotected islets. proc. Natl. Acad. Sci. 90: 5843-5847.

Soon-Shiong, P.; Heintz, R. E.; Merideth, N.; Yao, Q.X.; Yao, Z. W.; Zheng, T. L.; Murphy, M.; Moloney, M. K.; Schmehl, M.; Harris, M.; Mendz, R. and Sandford, P. A. (1994). Insulin Independence in a type 1 diabetic patient after encapsulated islets transplantation. Lancet. 343: 950-951.

Soon-Shiong, P.; Otterlei, M.; Skjåk-Bræk, G.; Smidsrød, O.; Heintz, R. E.; Lanza, R. P. and Espevik, T. (1991). An immunologic basis for the fibrotic reaction to implanted microcapsules. Transplant Proc. 23: 758-759.

Starr, M. P.; Teuper, H. G.; Balaws, A. (1981). The Prokaryotes: A Handbook of Habitat, Isolation and Identification of Bacteria. McGrow-Hill, New York, USA.

Stean, W. M. (1998). Laser Materials Processing. 2nd ed.

Stokke, B. T; Smidsrød, O.; Zanetti, F.; Strand, W. and Skjåk-Bræk, G. (1993). Distribution of urinate residues in alginate chains in relation to gelling properties: Enrichment of -D- mannuronic acid and depletion of -L-guluronic acid in the sol fraction. Carbohyd. Polym. 21:39-46.

Stolp, H. and Gadkari, D. (1984). Non pathogenic members of genus *Pseudomonas*. In the prokaryotes. Pp: 721-74.

Stonesifer, J. and Baltz, R. H. (1985). Mutagenic DNA repair in Streptomyces. Proc. Natl. Acad. Sci. USA. 82: 1180-1183.

Sutherland, I. W. (1997). Microbial Exopolysaccharides-Structural Subtleties and their Consequences. Appl. Chem. 69:1911-1917.

Sutherland, I. W. (2003). Biotechnology of Exopolysaccharides. 1st Cambridge: Cambridge University Press. pp. 1-151.

Suzuki, H. and Kilgore, W.W. (1966). Effects of Mitomycin C on macromolecular synthesis in *E. coli*. Bacteriol. 93(1): 675-682.

Szybalski, W. and Iyer, V. N. (1964). Crosslinking of DNA by enzymatically or chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics. Federat Proc. 23:946-957.

Todar, K. (1997). Bacteriology 330 Lecture Topics: *Pseudomonas aeruginosa*. Annual Reports of Wisconsin University.

Toft, K.; Grasdalen, H. and Smidsrød, O. (1986). Synergistic gelation of alginates and pectins. Symp. Ser. 310: 117-132.

Tomasz, M.; Chawla, A. K. and Lipman, R. (1988). Mechanism of monofunctional and bifunctional alkylation of DNA by mitomycin C. Biochem. 27: 3182–3187.

Tombs, M. P. and Harding, S. E. (2003). An Introduction to Polysaccharide Biotechnology. Taylor and Francis, London. 1st ed. Pp. 123-170.

Van Geel, I. (2004). Synthesis of exopolysaccharides by *Lactococcus reuteri and Lactobacilli*. Center for carbohydrate engineering. Pp. 1-27.

Voskanyan, K. Sh. (1990). 633 nm light induces mutations. Study Biophysica. 139 (1): 43-46.