Acknowledgments

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

I would like to express my deep gratitude, thanks and appreciations to my supervisor ***Dr. Majid H. Al-Gelawi*** *for his guidness, great support and advices the whole period of research. The knowledge I gained from him and both academic and non- academic matters have been invaluable and will definitely be beneficial to my future career.*

Thanks due to my family. I want them to know that I am very grateful for their unreseaved love and encouragement throughout my studies, to which has been a source of inspiration and moral support.

Also great thanks to Mr. As'ad Ghelani and his family for their great support to me throughout my study.

My gratitude to my colleagues and friends in the department of Biotechnology, college of science, Azad, Anas, Saif, Farah, Ahmad, Solaf, Sura, Mayassa, Muntaha, Riny, Yazen, Mr. Raed Bahar, Dr. Ali, Safa, Rana, Mustafa, Mutaz and Rawa. Also special thanks to my best friends Hassan Abdlhadi and Bassam Sameer. And a word of thank to staff of department of Biotechnology in Al-Nahrain University for their appreciable help specially Miss Oroba, Miss Shaima and Miss Tania, for their help and valuable advices.

Oday

وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم

التحسين الوراثي لانتاج الرامنولبيد من بكتريا *Pseudomonas aeruginosa*

رسالة
مقدمة الى كلية العلوم في جامعة النهرين
و هي جزء من متطلبات نيل درجة الماجستير علوم
في علوم التقانة الاحيائية

من قبل
عدي عدنان المقادسي
بكالوريوس تقانة احيائية-كلية العلوم-٢٠٠٠
جامعة النهرين

تشرين الثاني **2006**
شوال **١٤٢٧**

1-2 Literature Review

1-2-1 Definitions of Surface-Active Compounds:

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces (Greek, 1991).

These properties render surfactant capable of reducing surface and interfacial tension and forming micro emulsion where hydrocarbon can solubilize in water or where water can solubilize in hydrocarbon. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which make surfactant some of the most versatile process chemicals (Greek, 1990).

Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. The molecules reduce surface and interfacial tension in both aqueous solution and hydrocarbon mixtures, which make them potential candidates for enhancing oil recovery (Singer, 1985; Shennan and Levi, 1987; Sarkar *et al*., 1989) and emulsification process (Cairns *et al*., 1982).

Biosurfactants have several advantages over the chemical surfactants such as:

1- Lower toxicity and higher biodegradability (Zajic *et al*., 1977).
2- Better environmental compatibility (Georgiou *et al*., 1990).
3- Higher foaming (Razafindralambo *et al*., 1996).
4- High selectivity and specific activity at extreme temperature, pH and salinity (Velikonja and Kosaric 1993).
5- Ability to be synthesized from renewable feedstock.

For all these reasons, efforts have been directed toward biosurfactants, and to improve their production from microorganisms.

1-2-2 Biosurfactants Classification and Their Microbial Origin:

Unlike chemically synthesized surfactants which are classified according to the nature of their polar grouping, biosurfactant categorized mainly by their chemical composition and their microbial origin. In general their structure includes a hydrophilic moiety consisting of amino acid or peptide anions or cations; mono-, di, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, or saturated fatty acids. Although there are a number of reports on the synthesis of biosurfactants by hydrocarbon degrading microorganisms, some biosurfactants have been reported to be produced on water soluble compounds such as glucose, sucrose, glycerol, or ethanol (Cooper and Goldenberg, 1987; Palejwala and Desai, 1989; Guerra *et al*., 1991; Hommel *et al*., 1994). The biosurfactants-producing microbes are distributed among a wide variety of genera.

According to Desai *et al*., (1994), the major classes of biosurfactants include:

1- Glycolipids;
2- Lipopeptides and Lipoproteins;
3- Phospholipids and Fatty acids;
4- Polymeric surfactants; and
5- Particulate surfactants.

The microbial source of important types of microbial surfactants was shown in table (1-1).

Table (1-1) Major biosurfactants produced by microorganism (Makkar and Cameotra, 2002).

Biosurfactant	**Organisms**
Rhamnolipids	*Pseudomonas* sp., *P. aeuginosa*
Trehalolipids	*R. erythropolis, N. erythropolis* *Mycobacterium* sp.
Sophorolipids	*T. bombicola, T. apicola* *T. petrophilum*
Cellobiolipids	*U. zeae, U. maydis*
Peptide-Lipid	*B. licheniformis*
Serrawettin	*S. marcescens*
Viscosin	*P. fluorescens*
Surfactin	*B. subtilis*
Subtilisin	*B. subtilis*
Gramicidins	*B. brevis*
Polymyxin	*B. polymyxa*
Fatty acids	*C. lepus*
Neutral lipids	*N. erythropolis*
Phospholipids	*T. thiooxidant*
Emulsan	*A. calcoaceticus*
Biodispersan	*A. calcoaceticus*
Mannan-lipid protein	*C. tropicalis*
Liposan	*C. lipolytica*
Carbohydrate-protein-lipid	*P. fluorescens, D. polymorphis*
Protein PA	*P. aeuginosa*
Vesicles and Fimbriae	*A. calcoaceticus*
Whole cells	Variety of bacteria

1-2-2-1 Glycolipids:

Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best knowns are rhamnolipids, trehalolipids, and sophorolipids (Champion, 1995).

A- Rhamnolipids:

A model micelle-forming biosurfactant molecule is the rhamnolipid molecule produced by *Pseudomonas aeruginosa*. Production of rhamnose containing glycolipids was first described in *Pseudomonas aeruginosa* in 1949 by Jarvis and Johnson. Rhamnolipid is an anionic biosurfactant containing either one or two carboxylated sugar heads along with one or two lipid tails. For this reason, rhamnolipid is classified as a glycolipid. Rhamnolipid may exist in its micellar form as a sphere, a vesicle, or a lamella (bi-layered sheet) (Champion, 1995).

Rhamnolipid can be mono-headed (consisting of a six-carbon sugar) or multiheaded as well as mono-tailed (consisting of a seven-carbon alkyl) or multi-tailed. The chemical structure of one type of rhamnolipid, containing dual-head and dual-tail (rhamnolipid 1), is shown in figure (1-1 A). Rhamnolipids usual function is most probably to bring carbon sources into solution where they will be accessible by the bacterial cell. Rhamnolipids from *Pseudomonas* spp. have been demonstrated to lower the interfacial tension against *n*-hexadecane to 1 mN/m and the surface tension to 25 to 30 mN/m (Lang and Wagner, 1987; Parra *et al*., 1989).

B- Trehalolipids:

Several structural types of microbial trehalolipid biosurfactants have been reported (Li *et al*., 1984). Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids is associated with most species of *Mycobacterium*, *Nocardia*, and *Corynebacterium*. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation (Asselineau and Asselineau, 1978; Cooper *et al*., 1989). Trehalose dimycolate produced by *Rhodococcus erythropolis* (figure 1-1B) has been extensively studied (Desai and Banat, 1997). Trehalose lipids from *Rhodococcus erythropolis* and *Arthrobacter* sp. lowered the surface and interfacial tension in the culture broth to 25 to 40 and 1 to 5 mN/m, respectively (Rapp *et al*., 1979; Li *et al*., 1984).

C- Sophorolipids:

Sophorolipids, which are produced mainly by yeasts such as *Torulopsis bombicola*, consist of a dimeric carbohydrate sophorose linked to a long chain hydroxy fatty acid (figure 1-1C). These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides (Desai and Banat, 1997). It has been shown that *Candida bogoriensis* produce glycolipids in which sophorose is linked to docosanoic acid diacetate (Cutler and Light, 1979).

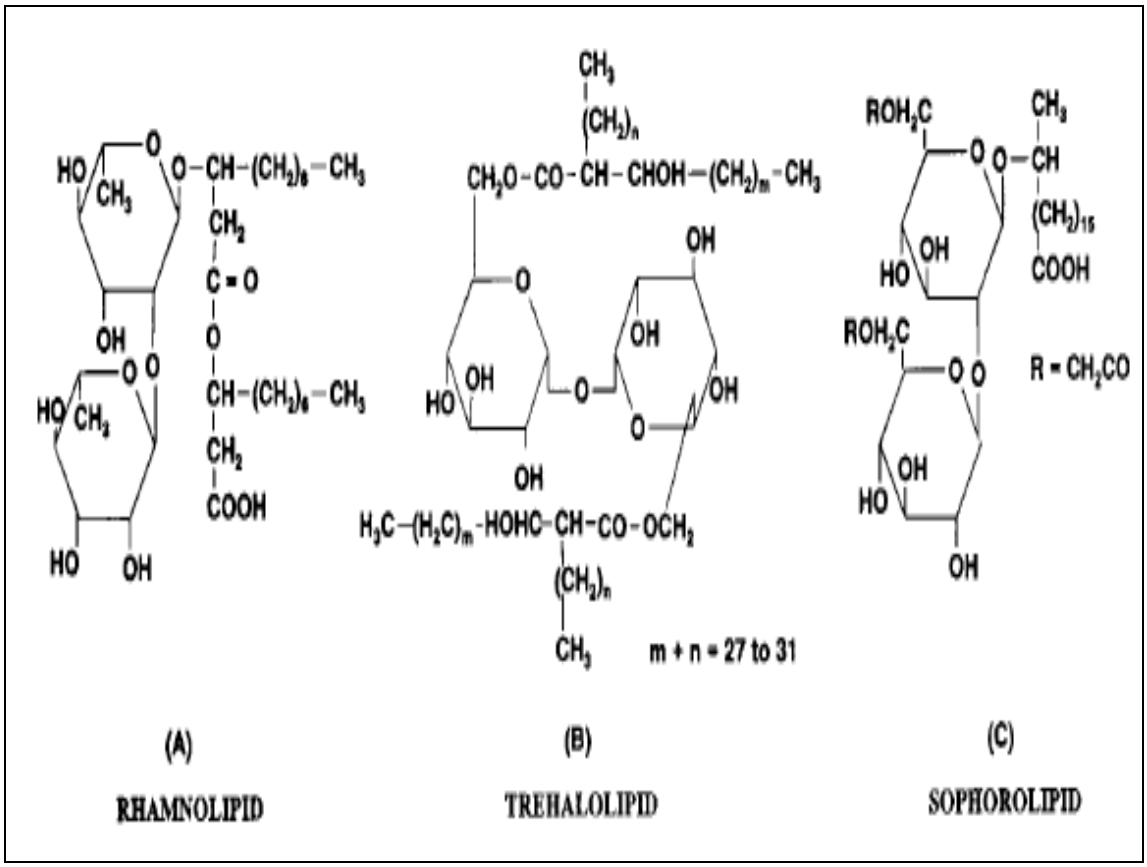

Figure (1-1). Structure of some common glycolipid biosurfactants (Champion, 1995).

(A) Rhamnolipid type 1 from *Pseudomonas aeruginosa* in which two rhamnose subunits are linked to two b-hydroxydecanoic acids in a side chain.

(B) Trehalose dimycolate from *Rhodococcus erythropolis*, in which disaccharide trehalose is linked to two long-chain α-branched β-hydroxy fatty acids.

(C) Sophorolipid from *Torulopsis bombicola* in which dimeric sophorose is linked to a long-chain (C18) hydroxy fatty acid.

1-2-2-2 Lipopeptides And Lipoproteins:

A large number of cyclic lipopeptides including decapeptide antibiotics and lipopeptide antibiotics possess remarkable surface-active properties. The cyclic lipopeptide surfactin (figure 1-2), produced by *Bacillus subtilis*, is one of the most powerful biosurfactants. It lowers the surface tension from 72 to 27.9 mN/m at concentrations as low as 0.5µg/mL (Arima *et al*., 1968). Viscosin, produced by the broccoli head rot pathogen *Pseudomonas fluorescens*, is also a potent lipopeptide biosurfactants. It can reduce the water surface tension from 72 to 27 mN/m at a concentration of 4µg/mL (Laycock *et al*., 1991).

Figure (1-2). Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis.* (Neu, 1990).

1-2-2-3 Phospholipids and Fatty acids:

Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on n-alkanes (Asselineau and Asselineau, 1978; Cooper *et al*., 1978; Cirigliano and Carman, 1985). Phosphatidylethanolamine (Figure 1-3) produced by *Rhodococcus erythropolis* grown on n-alkane causes a lowering of interfacial tension between water and hexadecane to less than 1mN/m and a CMC (critical micelle concentration) of 30mg/L (Kretschmer *et al*., 1982).

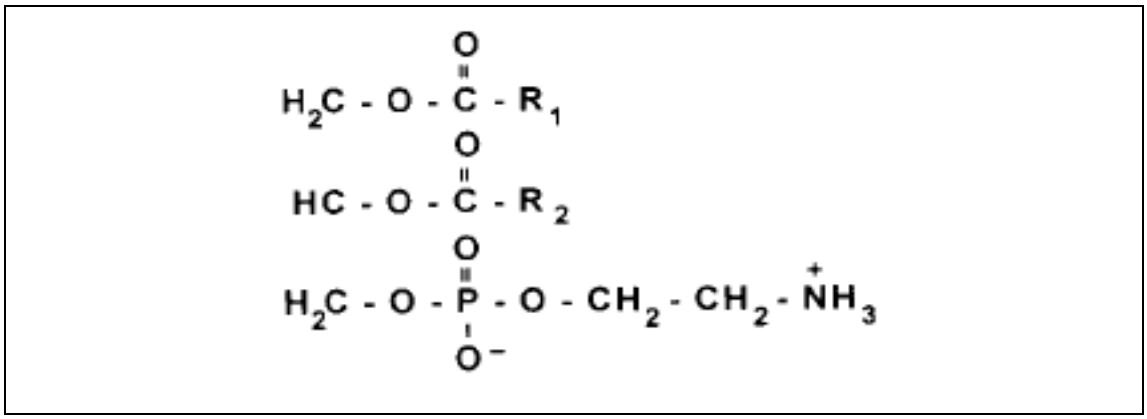

Figure (1-3). Structure of phosphotidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R1 and R2 are hydrocarbon chain of fatty acids (Cirigliano and Carman, 1985).

1-2-2-4 Polymeric Biosurfactants:

Polymeric biosurfactants usually have a high molecular weight. The best-studied polymeric biosurfactants are emulsan (Figure 1-4), liposan, mannoprotein, and other polysaccharide-protein complexes. Emulsan produced by *Acinetobacter calcoaceticus*, is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001% to 0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (Belsky *et al*., 1979; Zosim *et al*., 1982; Gutnick and Shabtai, 1987). Desai *et al*. (1988) demonstrated the production of bio-emulsifier by *Pseudomonas fluorescens* during growth on gasoline. This bio-emulsifier is composed of 50% carbohydrate, 19.6% protein and 10% lipid.

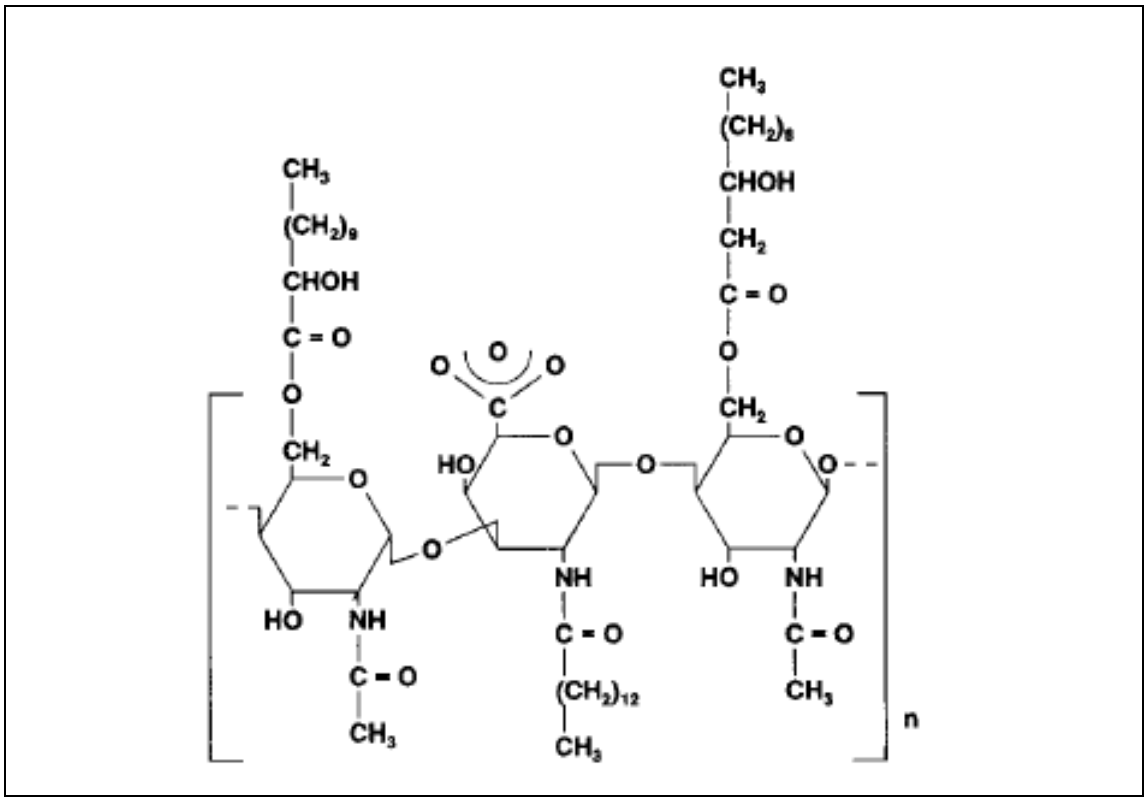

Figure (1-4). Structure of emulsan, produced by *Acinetobacter calcoaceticus* (Desai *et al*. 1988).

1-2-2-5 Particulate Biosurfactants:

Extracellular membrane vesicles partition hydrocarbons to form a microemulsion, which plays an important role in alkan uptake by microbial cells. (Kappeli and Finnerty, 1979). The membrane vesicles contain about 5 times as much phospholipid and about 350 times as much polysaccharide as does the outer membrane of the same organism (Desai and Banat, 1997). Surface activity in most hydrocarbon-degrading and pathogenic bacteria is attributed to several cell surface components, which include structures such as M protein and lipoteichoic acid in the case of group A *Streptococci*, protein A in *Staphylococcus aureus*, layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* spp., gramicidins in *Bacillus brevis* spores, and thin fimbriae in *Aeromonas calcoaceticus* RAG-1 (Wilkinson and Galbraith, 1975; Fattom and Shilo, 1985).

1-2-3 Biosurfactants- Producing *Pseudomonas aeruginosa*

P. aeruginosa (family Pseudomonadacea), an aerobic, motile, gram-negative rod able to grow and survive in almost any environment, lives primarily in water, soil, and vegetation. However, despite abundant opportunities for spread, *P. aeruginosa* rarely causes community-acquired infections in immunocompetent patients.

P. aeruginosa produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination (figure 1-5). In vivo studies have shown that mutants defective in the production of exotoxin A, exoenzyme S, elastase, or alkaline protease are essential for maximum virulence of *P. aeruginosa;* however, the relative contribution of a given factor may vary with the type of infection (Nicas and Igliwski, 1985). Many

of these factors are controlled by regulatory systems involving cell-to-cell signaling.

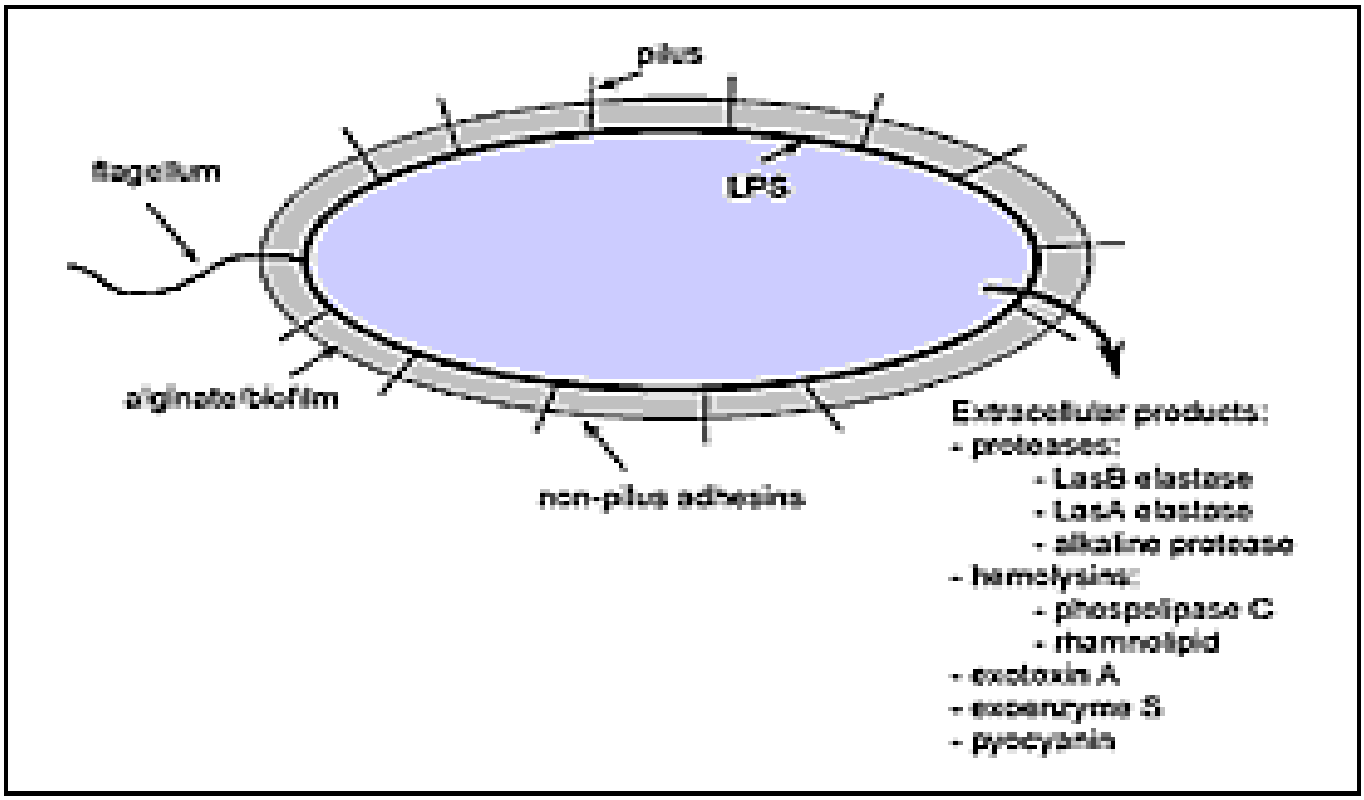

Figure (1-5). Virulence factors of *Pseudomonas aeruginosa* (Nicas and Igliwski, 1985).

The biologic effects of the most-studied extracellular virulence factors associated with acute *P. aeruginosa* infection were summarized as follows:

Exotoxin A: is produced by most *P. aeruginosa* strains that cause clinical infections. Like diphtheria toxin, *P. aeruginosa* exotoxin A catalyzes ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death (Nicas *et al*., 1985, a). Exotoxin A is responsible for local tissue damage, bacterial

invasion (Woods and Igliwski, 1983), and (possibly) immunosuppression (Vidal *et al*., 1993). Purified exotoxin A is highly lethal for mice, which supports its role as a major systemic virulence factor of *P. aeruginosa* (Woods and Igliwski, 1983).

Exoenzyme S: is also an ADP-ribosyl transferase, but unlike exotoxin A, it preferentially ribosylates GTP-binding proteins such as Ras (Iglewski *et al*., 1978). This exoproduct is responsible for direct tissue destruction in lung infection (Nicas *et al*., 1985, b) and may be important for bacterial dissemination.

Hemolysins: two hemolysins, phospholipase C and rhamnolipid, produced by *P. aeruginosa*, may act synergistically to break down lipids and lecithin. Both may contribute to tissue invasion by their cytotoxic effects. Rhamnolipid, a rhamnose-containing glycolipid biosurfactant, has a detergent like structure and is believed to solubilize the phospholipids of lung surfactant, making them more accessible to cleavage by phospholipase C (Liu, 1974). The resulting loss of lung surfactant may be responsible for the atelectasis associated with chronic and acute *P. aeruginosa* lung infection (Liu, 1979). Rhamnolipid also inhibits the mucociliary transport and ciliary function of human respiratory epithelium (Read *et al*., 1992). However, the relative role of rhamnolipid in acute or chronic infection is not known.

Proteases: are assumed to play a major role during acute *P. aeruginosa* infection. *P. aeruginosa* produces several proteases including LasB elastase, LasA elastase, and alkaline protease (Passador and Iglewski, 1995). The role of alkaline protease in tissue invasion and systemic infections is unclear; however, its role in corneal infections may be

substantial. The ability of *P. aeruginosa* to destroy the protein elastin is a major virulence determinant during acute infection. Elastin is a major part of human lung tissue and is responsible for lung expansion and contraction. Moreover, elastin is an important component of blood vessels, which rely on it for their resilience (Kernacki *et al*., 1995).

1-2-4 Genetics of Biosurfactants:

There are numerous reports on the isolation of mutants deficient in biosurfactant production with a concomitant loss in the ability to grow on water-insoluble substrates (Bar-Ness *et al*., 1988). The developments of a thin-layer chromatographic technique (Matsuyama *et al*., 1991), drop collapse test (Jain *et al*., 1991), hemolytic measurement test (Mulligan *et al*., 1984), technique for rhamnolipid detection on mineral agar (Siegmund and Wagner, 1991), and rapid screening method for hydrocarbon-degrading microorganisms (Hansen *et al*., 1993) have significantly accelerated the ability to rapidly isolate biosurfactant producing organisms. A clear understanding of biosurfactants genetics has emerged only through recent studies of *Pseudomonas* and *Bacillus* systems (Desai *et al*., 1994).

- **Genetics of rhamnolipid synthesis:**

Ochsner *et al*. (1994), b have extensively studied the genetics of rhamnolipid biosynthesis in *Pseudomonas aeruginosa*. The *rhlABR* gene cluster was found to be responsible for the synthesis of RhlR regulatory protein and a rhamnosyl transferase, both essential for rhamnolipid synthesis. The active rhamnosyl transferase complex is located in the cytoplasmic membrane, with the 32.5-kDa RhlA protein harboring a

putative signal sequence, while the 47-kDa RhlB protein is located in the periplasmic region and contains at least two putative membrane-spanning domains. The *rhlR* gene encodes a transcriptional activator, the 28-kDa RhlR protein belonging to the LuxR family, which positively regulates rhamnolipid biosynthesis. Ochsner and Reiser were identified another regulatory gene, *rhlI*, located down stream of the *rhlABR* gene cluster. Further, it was shown that the regulation of rhamnolipid production in *Pseudomonas aeruginosa* is mediated by the *rhlR-rhlI* system involving an autoinducer. Figure (1-6) shows the regulation of rhamnolipid synthesis, in which activation of RhlR regulatory protein on binding to its cognate autoinducers (factor 2 and/orVAI), the products of the autoinducer synthetase, and its regulatory role in genetic control of *rhlI* gene are proposed. The binding of activated RhlR protein upstream of the *rhlA* promoter enhances the transcription of the *rhlAB* operon encoding rhamnosyl transferase. The expression of the *rhlAB* genes in heterologous hosts has also been studied. In *Pseudomonas fluorescens* and *Pseudomonas putida*, these genes were expressed and produced rhamnolipids. However, in *Escherichia coli*, active rhamnosyl transferase was synthesized but rhamnolipids were not produced (Ochsner *et al*., 1995).

(Rh1G), is another *Pseudomonas aeruginosa* gene required for rhamnolipid production, which encodes the NADPH-dependent -ketoacyl-acyl carrier protein (ACP) reductase required for fatty acid synthesis. The insertional mutation of this rh1G gene produced no apparent effect on the growth rate and total lipid content of *P. aeruginosa* cells, but the production of rhamnolipids was completely abrogated.

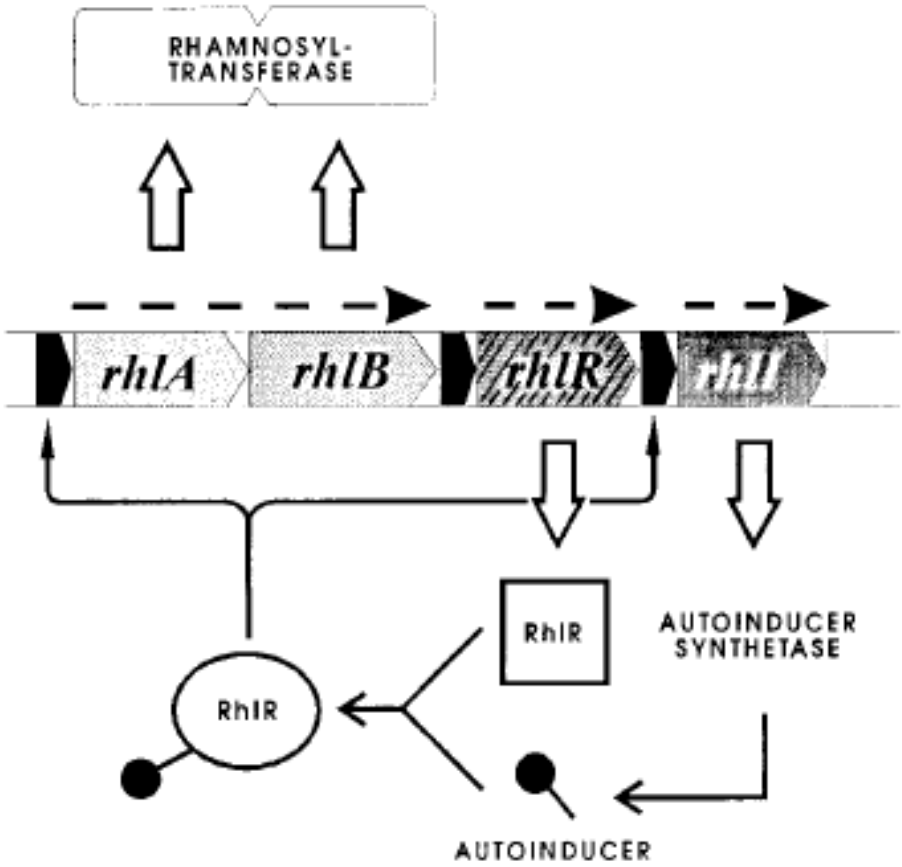

Figure (1-6). Genes involved in the synthesis and regulation of rhamnolipid in *Pseudomonas aeroginosa* (Ochsner *et al*. 1994).

These results suggest that the synthetic pathway for the fatty acid moiety of rhamnolipids is separate from the general fatty acid synthetic pathway, starting with a specific ketoacyl reduction step catalyzed by the RhlG protein (Jesus *et al*., 1998).

Another gene required for rhamnolipid biosynthesis is (rh1c), which catalyse the transfer of rhamnose from TDP-L-rhamnose to L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (monorhamnolipid) to produce dirhamnolipid (Rahim *et al.*, 2001).

1-2-5 Rhamnolipid Biosynthesis:

In general, the two more abundant rhamnolipids are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Lang and Wullbrandt, 1999; Maier and Soberon, 2000). According to the biosynthetic pathway proposed by Burger *et al.* (1963) , rhamnolipid synthesis proceeds by two sequential glycosyl transfer reactions, each catalysed by a different rhamnosyltransferase. The first rhamnosyltransferase, which catalyses the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkanoic acid (Figure 1-7), is encoded by the *rhlAB* operon (Ochsner *et al.*, 1995). Both genes, co-expressed from the same promoter, are essential for rhamnolipid synthesis but, whereas *rhlB* is known to encode the catalytic subunit of the rhamnosyltransferase, the function of *rhlA* is still unresolved. RhlA is probably an inner-membrane-bound protein (Rahim *et al.*, 2001), presumably involved in the synthesis or transport of rhamnosyltransferase precursor substrates or in the stabilization of the RhlB protein (Ochsner *et al.*, 1994, a). The second rhamnosyltransferase, encoded by *rhlC*, has been characterized and its expression shown to be co-ordinately regulated with *rhlAB*, and responsible for the transfer of the second rhamnose from TDP-L-rhamnose to L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (monorhamnolipid) to form L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (dirhamnolipid) (Rahim *et al.*, 2001).

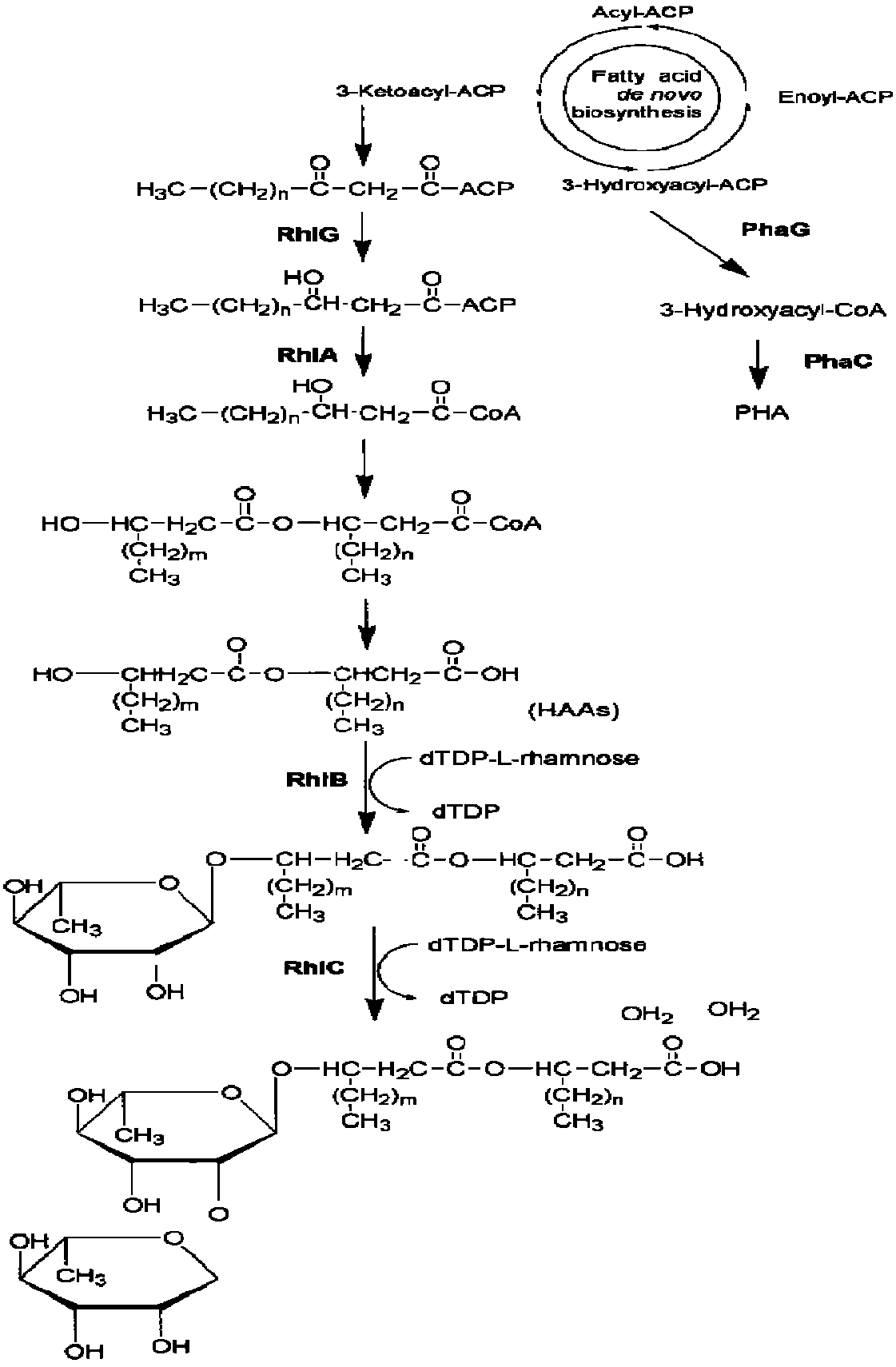

Figure (1-7). Biosynthesis pathway of rhamnolipid (Burger *et al*., 1963).

1-2-6 Cell to cell signaling system in *Pseudomonas aeruginosa* (Qourum sensing)

P. aeruginosa appears to control the production of many of its extracellular virulence factors by a mechanism that monitors bacterial cell density and allows communication between bacteria by cell-to-cell signaling. Bacteria are able to sense their environment, process information, and react appropriately; however, their ability to sense their own cell density, to communicate with each other, and to behave as a population instead of individual cells has only recently been understood (Fuqua *et al*., 1996; Gray, 1997). This phenomenon, called quorum sensing or cell-to-cell signaling, is a generic phenomenon described in many gram-negative (Greenberg, 1997) and gram-positive bacteria (Kleerebezem *et al*., 1997).

Cell-to-cell signaling systems of gram-negative bacteria are composed of a small molecule called an autoinducer, which is synthesized by a LuxI-type autoinducer synthetase and a LuxR-type transcriptional activator protein (R-protein) (Fuqua *et al*., 1996). The various autoinducers described in gram-negative bacteria are homoserine lactone-based molecules that differ between one another in length and substitutions on their acyl side chains. At low cell density, autoinducer is synthesized at basal levels and is thought to diffuse into the surrounding media, where it becomes diluted. With increasing cell density, the intracellular concentration of autoinducer increases until it reaches a threshold concentration. At this critical concentration, the autoinducer has been proposed to bind to a specific R-protein (Greenberg., 1997). The R-protein itself is not active without the corresponding autoinducer, and it is the R-protein/autoinducer complex that is proposed to bind to specific DNA sequences upstream of target genes enhancing their transcription

(Stevens and Greenberg, 1997). The resulting increase in expression of these genes can reach 1,000-fold. The autoinducer, therefore, allows the bacteria to communicate with each other (cell-to-cell signaling), to sense their own density (quorum-sensing), and together with a transcriptional activator to express specific genes as a population instead of individual cells.

- **The *rhl* Cell-to-Cell Signaling System of *P. aeruginosa***

P. aeruginosa contain a cell-to-cell signaling system, named the *rhl* system because of its ability to control the production of rhamnolipid. This system is composed of *rhlI*, the C4-HSL (*N*-butyrylhomoserine lactone) autoinducer synthase gene, and the *rhlR* gene encoding a transcriptional activator protein (Pearson *et al*., 1995). This system regulates the expression of the *rhlAB* operon that encodes a rhamnosyltransferase required for rhamnolipid production (Ochsner *et al*., 1994). The *rhl* system is also necessary for optimal production of LasB elastase, LasA protease, pyocyanin, cyanide, and alkaline protease (Pearson *et al*., 1997; Reimmann *et al*., 1997). Therefore, the *rhl* system, sometimes referred to as vsm (virulence secondary metabolites), regulates the expression of various extracellular virulence factors of *P. aeruginosa*. Interestingly, the *rhl* system also regulates the expression of *rpoS*, which encodes a stationary sigma factor (δ) involved in the regulation of various stress-response genes (Latifi *et al*., 1996).

1-2-7 Mutagenesis:

Mutation can be defined as a sudden change in the composition of a genome in a living cell caused by chemical or physical factors in the environment.

Mutation or heritable alteration in the genetic material may be gross at the level of the chromosome or point alteration, that is, mutations that affect only a very small part, often one nucleotide of the genome (Freifelder, 1987).

The first requirement for genetic studies is available of different mutants. So it should be determine the optimum conditions to obtain like these mutants by using different mutagens.

Many mutagens are available which can used to induce mutants in microorganisms and it can be divided generally to:

- ✓ Radiation: like UV light and ionizing radiation (x-ray and γ-ray).
- ✓ Base analogues: like 5-Bu, bromouracil (5-Bu) and 2-Ap.
- ✓ Base modifiers: like nitrus acid (N.A).
- ✓ Alkylating agents: like N-methy-N-nitro-nitrosoguanidin (MNNG).
- ✓ Intercalating agents: like ethidium bromide (E.B.) and acridine orange (A.O.)
- ✓ Cross- linking agents: like mitomycin C (Mt.C).

Mutagenesis of bacteria is induced by agents which either cause base misspairing (and hence misreplication of DNA) or misrepair of damaged DNA. Agents of the first type (such as MNNG and EMS), which yielded mispairing lesions, have been termed (direct mutagens) as they act largely independently of that complex system of induced cellular response to DNA damage that has been designated (SOS processing).

In contrast, those agents (such as UV radiation) whose mutagenasis is affected via misrepair of damaged DNA by SOS processing have been termed (indirect) mutagens (Al-Bakri and Umran, 1994).

Bacteria differ in their response to mutagenic agents; some bacteria is highly mutated by direct mutagens and poorly mutated by indirect mutagens and vice versa. Other bacteria are mutated by both types of

mutagens (direct and indirect). Mutagenesis can be divided to physical, chemical and molecular (transposon) mutagenesis.

1-2-7-1 Physical mutagenesis:

Exposure to ultraviolet radiation (UVR) occurs from both natural and artificial sources. The sun is the principal natural source. The known effects of UVR on man may be beneficial or detrimental, depending on a number of circumstances. Artificial UVR sources are widely used in industry and, because of the germicidal properties of certain portions of the UVR spectrum; they are also used in hospitals, biological laboratories, and schools. UVR is extensively used for therapeutic purposes, as in the prevention of vitamin D deficiency, the treatment of skin diseases, and for cosmetic purposes. Artificial UVR sources are available as consumer products (Internet 1).

UVR can be classified into UV-A, UV-B, and UV-C regions. Wavelengths in the UV-C region (200-280 nm) cause unpleasant, but usually not serious effects on the skin and eye. Although UV-C is very efficiently absorbed by nucleic acids, the overlying dead layers of skin absorb the radiation to such a degree that there is only mild erythema and, usually, no late sequelae, even after repeated exposures. Since solar UVR below 290 nm is effectively absorbed by stratospheric ozone, no such radiation reaches living organisms from natural sources (Miller *et al*., 1999).

UV-A generally causes indirect damage to DNA through the formation of chemical intermediates such as oxygen and hydroxyl radicals which interact with DNA to form strand breaks, alkali labile sites and DNA protein crosslinks. Conversely, adsorption of energy from UV-B induces direct damage to DNA. The two major lesions induced by UV-

B radiation are the cyclobutane pyrimidine dimer and the pyrimidine-pyrimidone (6-4) photoproduct. These photoproducts may be detected and quantified using specific radioimmunoassays (Peak and Peak, 1986).

Deoxyribonucleic acid (DNA) is one of the most important target molecules for photobiological effects. DNA can be represented as a double-stranded helix built up of purine and pyrimidine bases, held together by sugar and phosphate groups. If the features of the DNA macromolecule and the universality of the cell structure of living organisms in which DNA represents the genetic heritage are considered, it can be anticipated that any lesion inflicted on DNA, however slight, may have serious repercussions. A lesion in a cell genome is always serious, because, in general, the genome exists only in one copy in the cell concerned, whereas a lesion in a protein, even of the same magnitude, may remain undetected because there are many copies of the proteins. The latter is also true of ribonucleic acids (RNA) (Internet 1).

The effect of UVR is above all destructive. The most common changes produced in DNA are damage to the bases and to the polynucleotide chains. Damage to the bases may be unimolecular or bimolecular. Since pyrimidine bases are ten times more sensitive to UVR than purine bases, the only unimolecular reaction discussed will be the formation of pyrimidine hydrates. Bimolecular reactions are very numerous. They may occur between two bases, or between a base and another molecule. The most important effect is the formation of dimer compounds, particularly thymine dimers. The dimer brings about a twisting of the secondary helical structure of DNA and causes local denaturation. New biochemical methods have made it possible to detect dimers *in vivo* in all types of irradiated cells studied. The number of dimers has been shown to be proportional to the dose of UVR and to

vary with wavelength with a peak at 280 nm. While the production of dimers has been shown to be directly linked to the harmful effects of UVR on biological material, it is not the only serious lesion produced in DNA by UVR (figure 1-8) (Dale, 1998).

The distortion produced in the DNA-molecule prevents it from carrying out its functions, i.e., transcription and replication may be blocked. These lesions can be recognized by repair enzymes or may act as a signal for other biological processes to intervene. They may result in cell death, genetic recombination, mutagenesis, or even carcinogenesis (Mount, 1996).

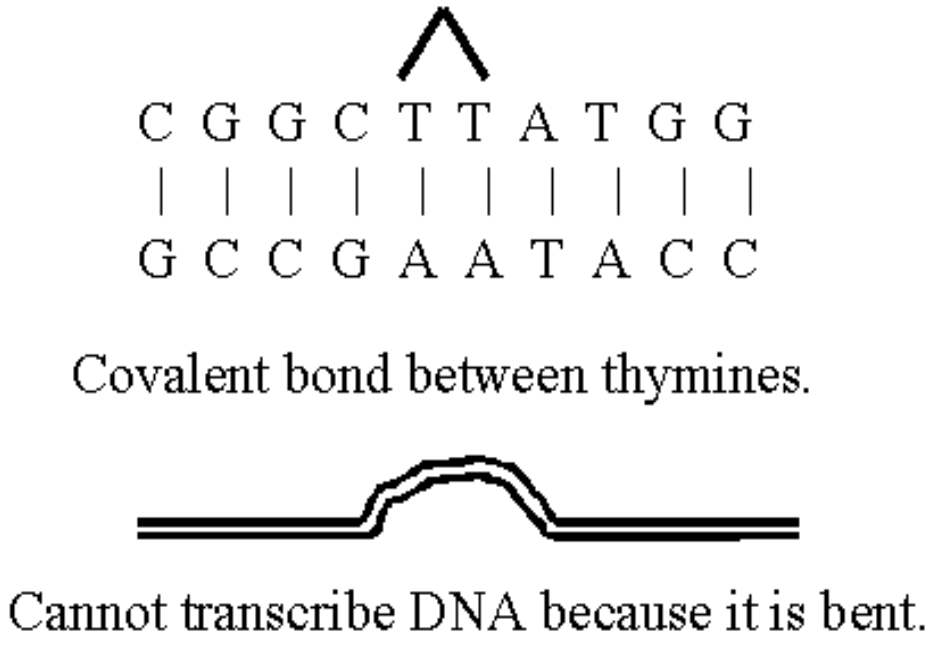

Figure (1-8). Formation of thymine dimmer by UVR (Dale, 1998).

1-2-7-2 Chemical Mutagenesis:

Chemical mutagens are defined as those compounds that increase the frequency of some type of mutations. They vary in their potency since this term reflects their ability to enter the cells, their reactivity with DNA, their general toxicity and the likelihood that the type of chemical change they introduce into the DNA will be corrected by a repair system (Snyder and Champness, 1997). Among the most widely used mutagenic reagents with microorganisms are the alkylating agents include ethylmethane sulfonate, methylmethane sulfonate and N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Alkylating agent cause transversions rather than transition (Crow, 1993).

- **N-methyl-N-nitro-N-nitrosoguanidine(MNNG).**

The first chemical determination and use of MNNG was in 1947. MNNG (figure 1-9) is stable at pH=5 but at low acidity it hydrolysis to diazomethane (Snyder and Champness, 1997). MNNG act against the DNA within cells, rather than against isolated DNA. MNNG act by introducing alkyle groups onto the nucleotides at various positions, especially the O_6 position of guanine. This change may be directly mutagenic or may lead to depurination (the loss of purine base from the nucleotide) (Turner *et al*., 1996). Depurination may be remedied by the action of the DNA repair system, but in the event of a replication fork reaching the depurination site before repair has occurred replication will cease.

The action of an error-prone repair system known 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).

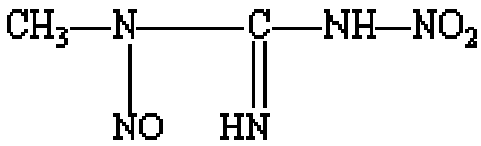

Figure (1-9). Chemical structure of MNNG (Botstein and Jone, 1969)

Results and Discussion

3.1 Identification of Bacterial Isolates:

Four bacterial isolates (RB19, RB27, RB29, and RB31) belonging to the species *Pseudomonas aeruginosa* were isolated and identified in previous study (Nasir *et al*., 2002).

In this study the morphological, physiological and biochemical tests were performed to ensure the identification of the bacteria mentioned above, and also to identify the species of other two bacterial isolates belonging to the genus *Pseudomonas* (RB55, and RB67) (Nasir, Personal communication).

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

Results (table 3-1) showed that all isolates gave a positive results for oxidase, catalase, pyoverdin, gelatinase, urease, growth at 42ºC, growth on King A, and growth on citrimide, while they gave negative results for growth on King B, and growth at 4ºC.

In addition to the above tests, biochemical identification was also done using API 20E system (figure 3-1), to confirm the previous results.

Results in table (3-1) and figure (3-1) showed that *Pseudomonas* RB19, RB27, RB29, RB31, RB55, and RB67 were all belonging to the species *Pseudomonas aeruginosa*.

Results of morphological, physiological and biochemical tests for *Psedomonas* spp. were in agreement with Holt *et al*., (1994).

Table (3.1) Morphological, physiological and Biochemical characteristics of the locally isolated *Pseudomonas* Spp.

Isolates / Tests	RB19	RB27	RB29	RB31	RB55	RB67
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Pyoverdin	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+
Urease	+	+	+	+	+	+
Growth at 4°C	-	-	-	-	-	-
Growth at 42°C	+	+	+	+	+	+
Growth on King A	+Green	+Green	+Green	+Green	+Green	+Green
Growth on King B	Growth without pigment	Growth without pigment	Growth without pigment	Growth without pigment	Growth without pigment	Growth without pigment
Growth on citrimide	+	+	+	+	+	+
Gram's stain	-ve	-ve	-ve	-ve	-ve	-ve

+: Positive results
-: Negative results

Figure (3.1) Identification of *Pseudomonas* spp. using API 20E kit.

3.2 Screening of Biosurfactant Producer Isolates:

Four bacterial isolates belonging to *Pseudomonas aeruginosa* (RB19, RB27, RB29, and RB31) were known for their ability to produce rhamnolipid (Saifour *et al*., 2004).

These isolates in addition to *P. aeruginosa* RB55 and *P. aeruginosa* RB67 were grown on minimal salt medium that contain sun flower oil as a sole source of carbon and energy, and tested for their ability to produce biosurfactant compounds by measuring the surface tension for culture supernatant.

Results in table (3-2) showed that all isolates were able to produce surface-active compounds as compared with the control. *P. aeruginosa* RB67 gave the best results in its ability to produce surface active compounds by minimizing the surface tension to 30 mN/m, while other isolates showed a divergence in their ability to produce surface active compounds. For this reason *P. aeruginosa* RB 67 was selected for further study.

The growth of these isolates under such condition require the production of surface active compounds in order to emulsify the sun flower oil and increase its bioavailability for utilization by bacteria. Other studies showed that insoluble hydrocarbons promote the bacteria to produce surface active compounds which capable of reducing the surface tension at the interface between liquids, solids, and gases thereby, allowing them to mix readily as emulsion in water (Banat *et al*., 1991; Zouboulis *et al*., 2003).

It can conclude that the biosurfactant produced by *P. aeruginosa* RB67 is rhamnolipid, hence it was referred that rhamnolipid have been identified predominantly from *pseudomonas aeruginosa* (Beal and Betts, 2000).

Table (3.2) Surface tension of cell free supernatants of *P. aeruginosa* grown in minimal salt medium containing 2% sun flower oil with shaking (180 rpm), at 30ºC for 72 hrs.

Isolates	**Symbols**	**Surface Tension mN/m**
Control-	_	58
Control+	_	68
P. aeruginosa	RB 27	32
P. aeruginosa	RB 29	40
P. aeruginosa	RB 30	33
P. aeruginosa	RB 31	32
P. aeruginosa	RB 55	41
P. aeruginosa	RB 67	30
Methanol	_	23
Benzene	_	28.9

Control-: Medium without carbon source.
Control+: Medium without bacteria.

3.3 Mutagenesis of *P. aeruginosa* RB67:

P. aeruginosa RB67 was mutaginized in an attempt to increase its ability to produce rhamnolipid. The lethal and mutagenic effect of UV light and MNNG were studied on this bacterium.

3.3.1 UV survival curve and Mutagenesis:

The survival curve of *P. aeruginosa* RB67 after exposure to different doses of UV light is shown in figure (3-2). Results indicated that *P. aeruginosa* RB67 was UV sensitive; it was lost its viability exponentially. The survival curve showed increase of lethal percentage exponentially with the increase of UV doses and the highest lethal percentage or the less survival percentage was (4.7%) when the bacterium exposed to $20J/m^2$ of UV irradiation.

Results in figure (3-2) indicated that the survival percentage of *P. aeruginosa* RB67 was 10.4% (89.6% killing) when it was exposed to $15J/m^2$ of UV light. So this treatment was selected for mutants isolation because many studies mentioned that treatment allowing survival of approximately 10% of cell population generate more mutants (Queener and Lively, 1986; Abbas *et al*., 2004).

It is known that mutation in bacteria can be induced by agent such as UV irradiation whose mutagenesis is affected via missrepair of damaged DNA by SOS repair system and have been termed indirect mutagens (Al-Bakri and Umran, 1994).

Physical mutagenesis by UV light was used successfully with *Xanthomonas campestris* and *Gardnerella vaginalis*, and many mutants were

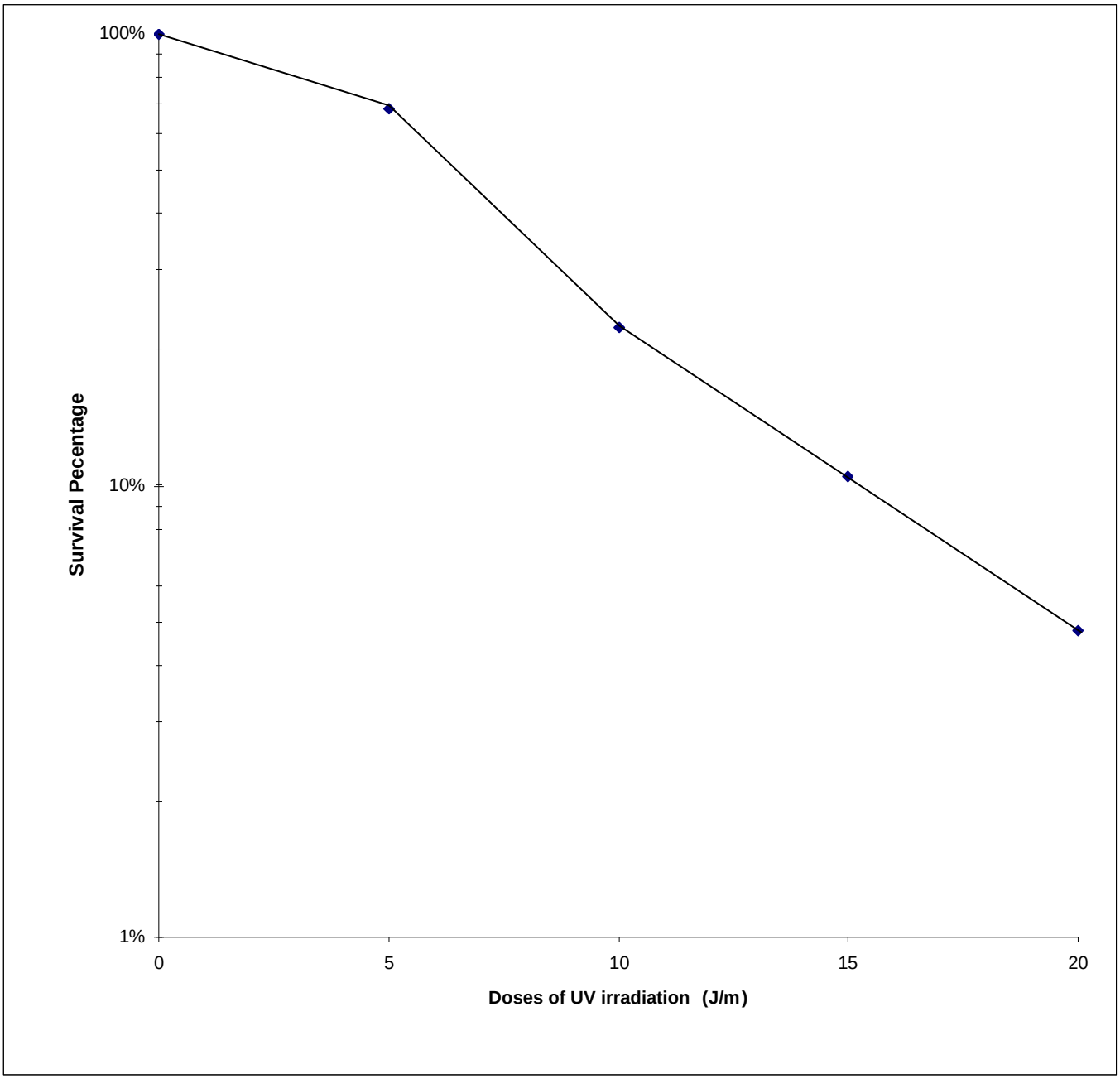

Figure (3.2) Effect of UV irradiation on a suspension of *P. aeruginosa* RB67 in phosphate buffer (pH=7).

obtained from these bacteria, indicating that UV light is an efficient mutagen (Al- Delaimi and Al-Gelawi, Unpublished Data; Al- Saady *et al*., 2005).

Baho, (2006) indicated that *P. aeruginosa* is sensitive to the lethal and mutagenic effect of UV light.

3.3.2 MNNG Survival Curve and Mutagenesis:

The survival curve of *P. aeruginosa* RB67 after treatment with different concentration of MNNG is shown in figure (3-3).

It was found that this bacterium was sensitive to MNNG. The survival curve showed increase of the lethal percentage exponentially with the increase of MNNG concentration, and the highest lethal percentage or the less survival percentage was (11.2%) when the bacterium treated with 40µg/ml of MNNG.

This treatment (40µg/ml) was led to a survival percentage of 11.2% (88.8% killing) therefore; it was selected for mutants' isolation.

It is known that MNNG is an effective mutagenic compound, it can generate mispairing lesion by adding alkyl (e.g. 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 the unwinding of the DNA during replication and transcription (Freifelder, 1987; Turner *et al*., 1996). Also it can induce mutation 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 butyricum Micrococcus* sp. and *Gardnerella vaginalis*) showed that these bacteria were sensitive to MNNG (Carrasco and Soto, 1987; Al-Bakri and Umran, 1994; Al- Saady *et al*., 2005).

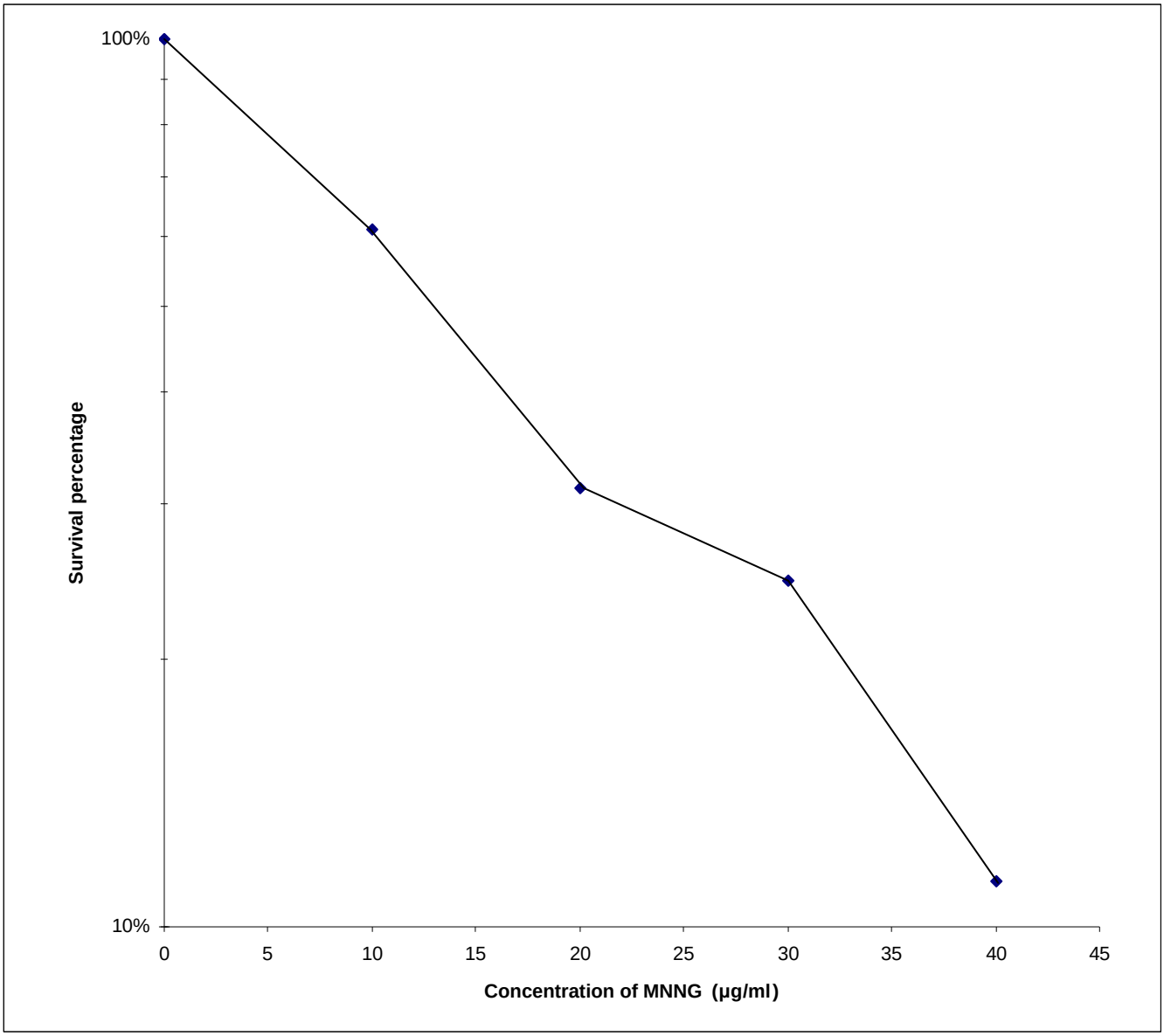

Figure (3.3) Effect of MNNG on a suspension of *P. aeruginosa* RB67 in phosphate buffer (pH=7).

3.4 Selection of hyperproducer mutants:

In order to isolate rhamnolipid overproducer mutants from *P. aeruginosa* RB67, mutagenized culture from the selected treatments (3-3) (bacteria exposed to 15J/m^2 of UV light and/or treated with 40μg/ml of MNNG) were replica plated on blood agar plates to detect the haemolytic zone, which is used as an indicator for rhamnolipid production. Results indicated that all colonies showed approximately the same haemolytic zone. However the extent of hemolytic zone formation on blood agar plate is not solely dictated by the concentration of rhamnolipid and may be affected by divalent ions and other hemolysins produced by the microbe under investigation (Mulligan *et al*., 1989). For this reason, an alternative method for the detection of extracellular rhamnolipid was employed for screening of rhamnolipid biosurfactant produced by *P. aeruginosa* RB67 mutants. In which fifty colonies from each UV and MNNG treatment were replica plated on CTAB-methylene blue agar (Sigmund- Wagner medium) plates. This test was developed based on the property that the concentration of anionic rhamnolipid in aqueous solution can be determined by the formation of insoluble ion pairs with various cationic substances (ex. CTAB). The formation of insoluble ion pairs precipitate in the agar plate containing methylene blue exhibited dark blue color against light blue background. The diameter of the dark blue region has been shown to be semiquantitavely proportional to the concentration of rhamnolipid biosurfactant (Siegmund and Wagner, 1991).

Twelve colonies from each treatment (UV and MNNG) were selected (termed UOM1 to UOM12 and MOM1 to MOM12, respectively) which gave the largest dark blue halo against light blue background and used for measuring rhamnose concentration.

These colonies were screened for their ability to produce rhamnolipid by measuring the partially purified rhamnose concentration according to Patel and Desai, (1997) (2.2.6.2) and depending on the standard curve of glucose (figure 2.1) (Dupois *et al*., 1956).

Results (Table 3-3) showed that the rhamnose concentration produced by *P. aeruginosa* RB67 (wild type) was 70 μg/ml, while the concentration of rhamnose after exposure to UV light was varied among these twelve mutants, in which some of them were closed to that of the wild type, while other mutants showed decrease in rhamnose concentration. On other hand mutants UOM11 and UOM12 gave the highest concentration of rhamnose (85 μg/ml and 89 μg/ml, respectively).

While results in table (3-4) showed that the rhamnose concentration produced after treatment with MNNG was also varied between the twelve mutants, in which some of them showed a decrease in rhamnose concentration while others showed no differences in rhamnose content, however, MOM11 and MOM12 showed a significant increase in rhamnose content (82 μg/ml and 94 μg/ml, respectively).

UV light and MNNG were used successfully to mutagenize and to get different mutants from different bacteria.

Al- Delaimi and Al-Gelawi, (Unpublished Data) and Baho, (2006), used UV light to mutagenize *Xanthomonas campestris* and *P. aeruginosa*, respectively and they successfully get hyperproducer mutants for protease and lectins, respectively. On the other hand, MNNG was used successfully by Al-Gelawi, (1999) to isolate salt sensitive mutants from *Micrococcus* sp. strain G1. It was used also by Abbas *et al*., (2004) to mutagenize *P. aeruginosa*, and he was successfully isolated rhamnolipid hyperproducer mutants.

Table (3.3) Rhamnose concentration produced from *P. aeruginosa* RB67 after exposure to UV light (15 J/m²).

Isolates	**Symbols**	**Rhamnose Concentration (µg/ml)**
P. aeruginosa wild type	RB67	70
P. aeruginosa mutant	UOM1	52
P. aeruginosa mutant	UOM2	54
P. aeruginosa mutant	UOM3	61
P. aeruginosa mutant	UOM4	61
P. aeruginosa mutant	UOM5	64
P. aeruginosa mutant	UOM6	68
P. aeruginosa mutant	UOM7	69
P. aeruginosa mutant	UOM8	78
P. aeruginosa mutant	UOM9	81
P. aeruginosa mutant	UOM10	82
P. aeruginosa mutant	UOM11	85
P. aeruginosa mutant	UOM12	89

Table (3.4) Rhamnose concentration produced after treatment with MNNG (40µg/ml).

Isolates	**Symbols**	**Rhamnose Concentration (µg/ml)**
P. aeruginosa wild type	RB67	70
P. aeruginosa mutant	MOM1	38
P. aeruginosa mutant	MOM2	45
P. aeruginosa mutant	MOM3	48
P. aeruginosa mutant	MOM4	57
P. aeruginosa mutant	MOM5	61
P. aeruginosa mutant	MOM6	65
P. aeruginosa mutant	MOM7	65
P. aeruginosa mutant	MOM8	73
P. aeruginosa mutant	MOM9	74
P. aeruginosa mutant	MOM10	78
P. aeruginosa mutant	MOM11	82
P. aeruginosa mutant	MOM12	94

Results indicated that both UV light and MNNG caused random mutation in the rhamnolipid gene(s).

The mutation may be occurred in the regulatory gene site that regulate the production of rhamnolipid positively or in the structural gene that code for rhamnolipid production and hence this may led to decrease the production of rhamnolipid from these mutants.

Also, a mutation in the structural gene (Rh1G) that responsible for the formation of fatty acid moiety of rhamnolipid will inhibit rhamnolipid production, while the growth rate and total lipid content of *P. aeruginosa* cells showed no apparent effect (Jesus *et al*., 1998).

While the increase in the rhamnolipid production from other mutants may be attributed to many reasons, first the mutation may occur in promoter region that render it to be more similar with that of the consensus sequence, and as a result the promoter strength may increase, resulting in increase the efficiency of transcription.

It was referred that promoter strength is directly proportional to the degree of similarity with the consensus sequence (Freifelder, 1987).

Second, a mutation may occur in the structural gene (Rh1C) that codes for dirhamnolipid synthesis, this mutation may prevent the formation of dirhamnolipid, so abundance of rhamnose will accumulate in the cell leading to the synthesis of large amount of monorhamnolipid (Rahim *et al*., 2001).

3.5 Characterization of Rhamnolipid Using FTIR:

The partially purified rhamnolipid was subjected to the Fourier transformer infrared spectroscopy in order to get the chemical structure of this compound. Figure (3.5 a,b,c).

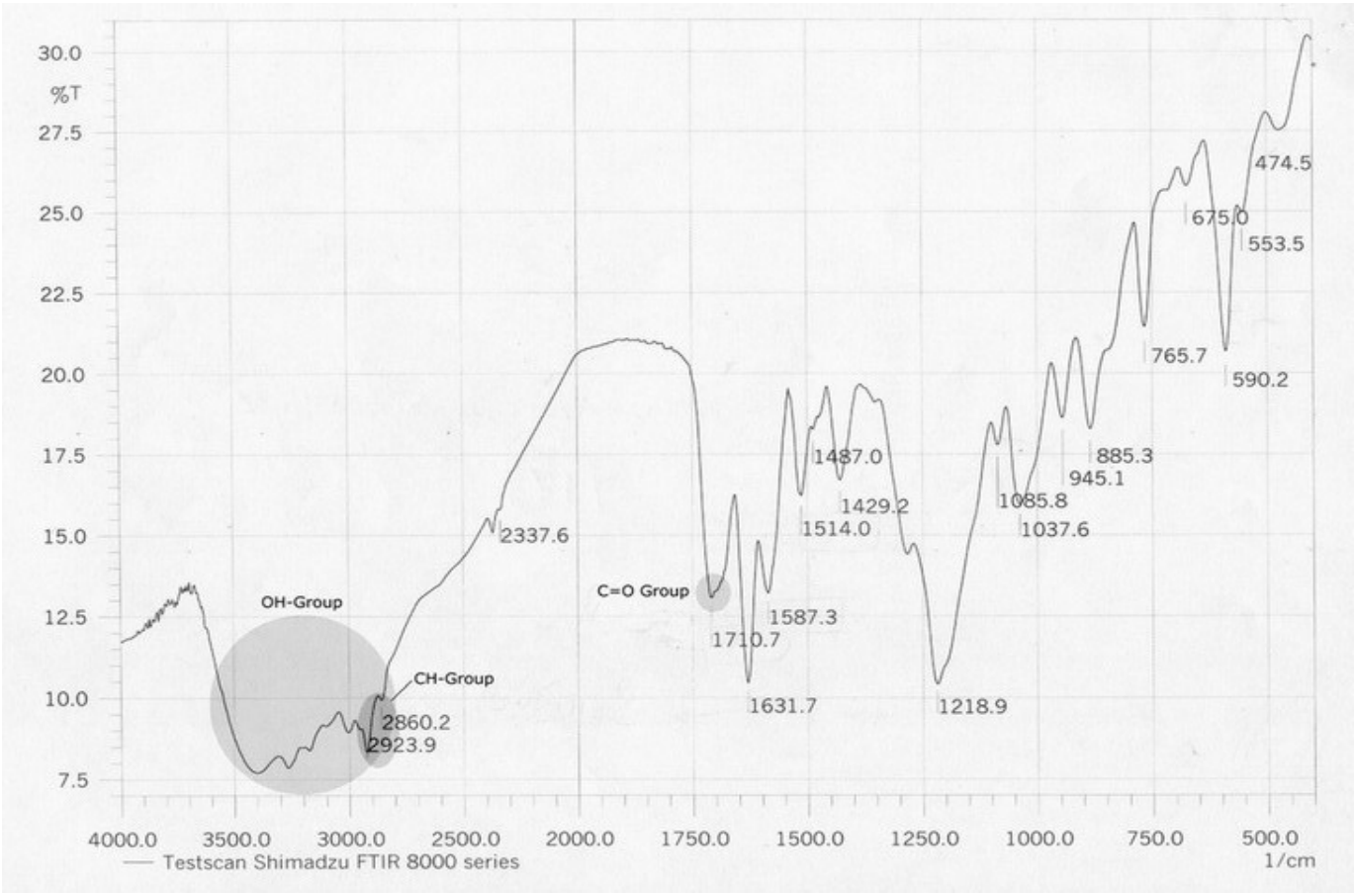

Figure (3-5, a) FTIR Spectroscopy of Partially Purified Rhamnolipid Produced from *P. aeruginosa* RB67 (wild type).

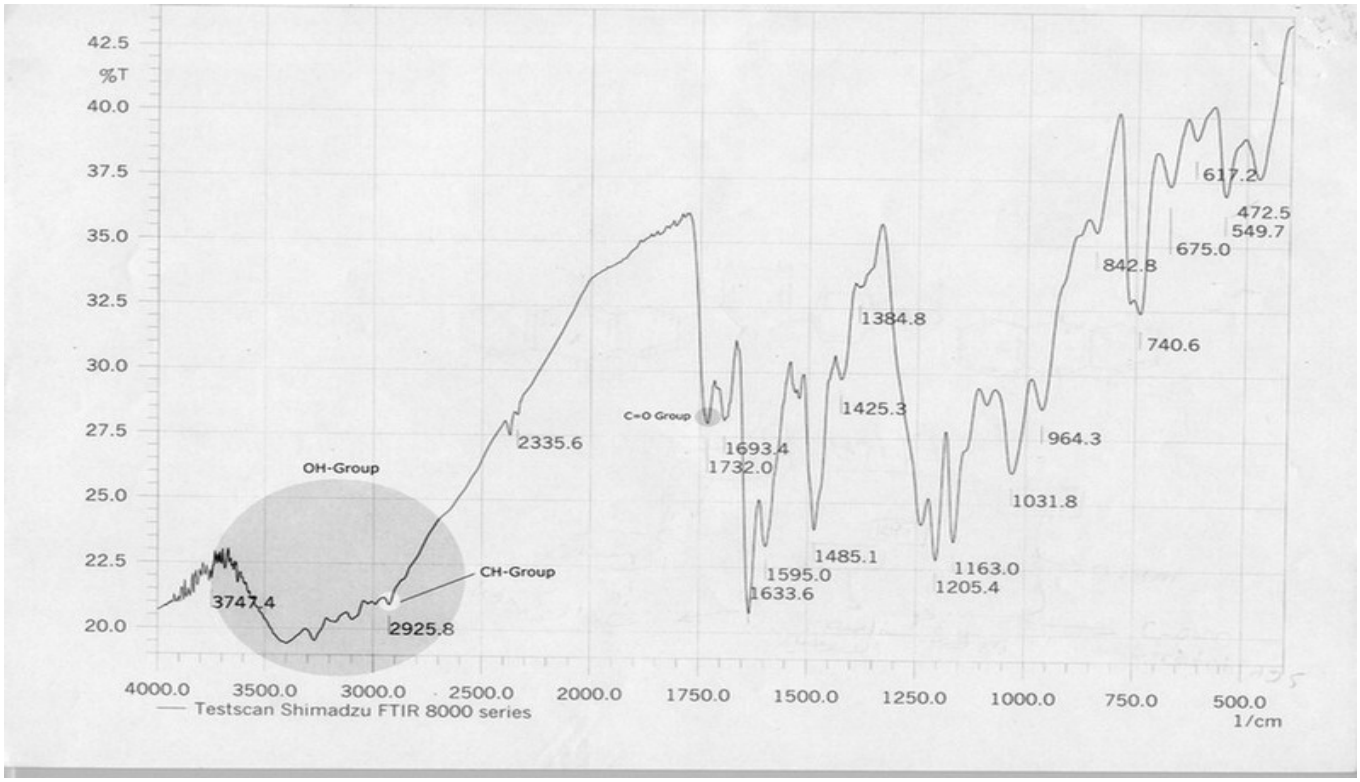

Figure (3-5, b) FTIR Spectroscopy of Partially Purified Rhamnolipid Produced from UOM12 mutants

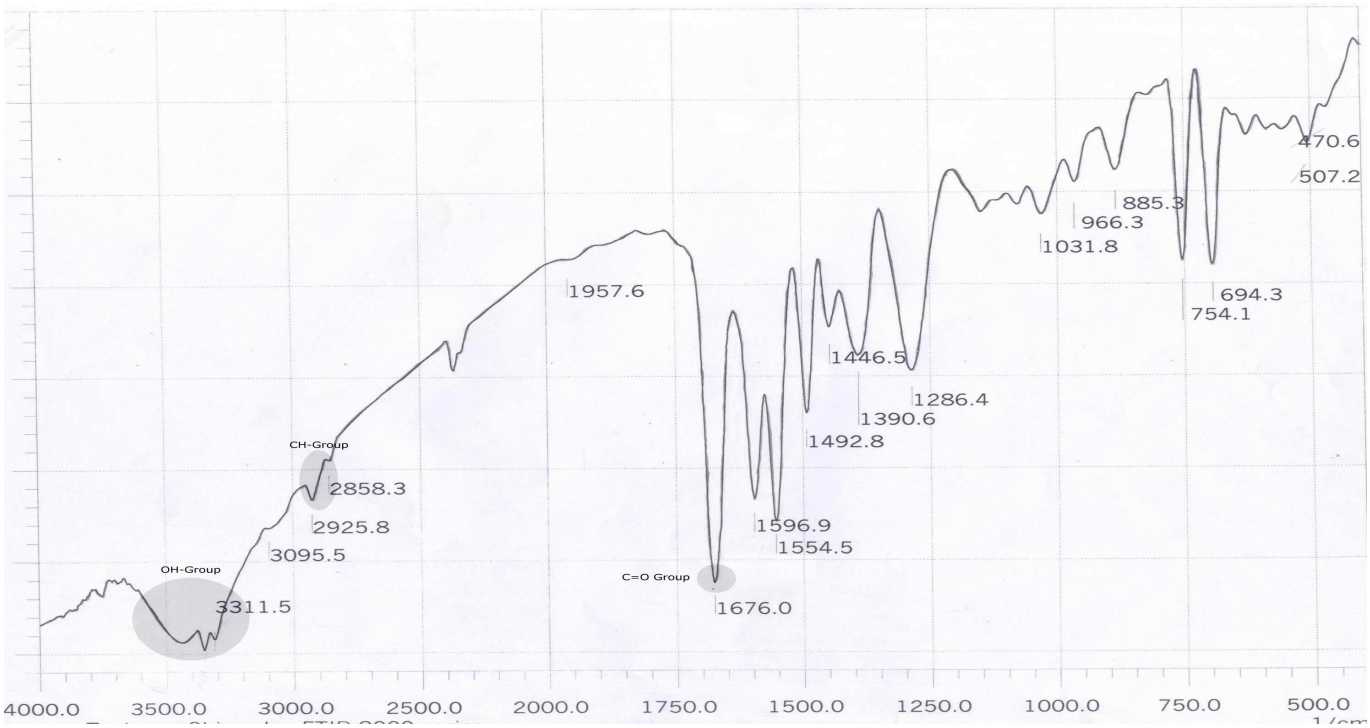

Figure (3-5, c) FTIR Spectroscopy of Partially Purified Rhamnolipid Produced from MOM12 mutants

The IR spectrum of the rhamnolipid produced from *P. aeruginosa* RB67 (wild type, UOM12, and MOM12) were shown in figure (3-5 a, b, and c).

Results from these figures were used to compare between the rhamnolipid produced from these three isolates. The value of the bands were as follows:

1- for the wild type RB67:

A- A broad band at 3550-2880 /cm resulting from O-H group.

B- A band at 2923.9-2860.2 /cm resulting from C-H aliphatic group.

C- A band at 1710 /cm resulting from C=O group.

2- For the UOM12:

A- A broad band at 3600-2700 /cm resulting from O-H group.

B- A band at 2925.8 /cm resulting from C-H aliphatic group.

C- A band at 1732 /cm resulting from C=O group.

3- For the MOM12:

A- A broad band at 3600-3100 /cm resulting from O-H group.

B- A band at 2925.8-2858.3 /cm resulting from C-H aliphatic group.

C- A band at 1676.0 /cm resulting from C=O group.

These results showed that the OH band of the rhamnolipid produced from these three bacterial isolates were approximately the same.

The same values can be seen with C-H group and C=O group between these three isolates.

The above values showed also small differences in the band domain between these three isolates, and this may be attributed to the presence of impurities in the partially purified rhamnolipd compounds, which may lead to a slight divergence in the band domain.

By comparison between the rhamnolipid produced from *P. aeruginosa* RB67 (wild type), UOM12, and MOM12 one can conclude that there is a quantitative increase in rhamnolipid production, but no apparent qualitative changes were observed.

2.1 Materials:

2.1.1 Equipments and Apparatus:

The following Equipments and Apparatus were used throughout this study:

Equipment	Company (origin)
Autoclave	Tomy (Japan)
Light microscope	Olympus (Japan)
Cool centrifuge	Harrier (U.K.)
Distillator	GallenKamp (England)
Oven	GallenKamp sanyo (U.K.)
Hot plate with magnetic stirrer	GallenKamp (England)
Shaker incubator	GFL (Germany)
pH-meter	Metter-GmpH Toledo (U.K.)
Sensitive balance	Sartorius (Germany)
Spectrophotometer	Aurora instrument Ltd.(U.K.)
Vortex mixer	Stuart scientific (U.K.)
Shaker water bath	Kotterman (France)
Micropipettes	Brand (West Germany)
UV-transiluminator	Ultraviolet products (USA)
Laminar air flow	Memmert (West Germany)
Tensiometer	Kruss
Rotary evaporator	Buchi (Switzerland)
Fourier transform infrared (F.T.IR)	SHIMADZU (8300) (Japan)
Millipore filter paper unit	Millipore corp (USA)

2.1.2 Chemicals:

The following chemicals were used throughout this study:

Material	Company(Origin)
Peptone	BDH (England)
Glycerol	Riedel-Dehaeny- (Germany)
K_2SO_4	BDH (England)
Citramide	Riedel-Dehaeny- (Germany)
Agar	Difco (USA)
KH_2PO_4	BDH (England)
K_2HPO_4	BDH (England)
NH_4NO_3	Riedel-Dehaeny- (Germany)
$CaCl_2.2H_2O$	Riedel-Dehaeny- (Germany)
$FeCl_3.6H_2O$	Riedel-Dehaeny- (Germany)
Chloroform	Riedel-Dehaeny- (Germany)
Methanol	Riedel-Dehaeny- (Germany)
Sodium bicarbonate	BDH (England)
NaOH	Merk (Germany)
HCl	BDH (England)
Phenol	Riedel-Dehaeny- (Germany)
H_2SO_4	AnalaR (England)
NaH_2PO_4	BDH (England)
Na_2HPO_4	BDH (England)
N-methyl-N-nitro-N-nitrosoguanidine solution (MNNG)	BDH (England)

Material	Company (Origin)
CTAB	Merk (Germany)
Methylene blue	BDH (England)
Glucose	Oxoid (England)
$MgSO_4.7H_2O$	BDH (England)
Ethanol	Riedel-Dehaeny- (Germany)
Iodine	BDH (England)
Saphranin	BDH (England)
Crystal violet	BDH (England)
Sun flower oil	Margarine (Iran)
Benzyne	BDH (England)
Phenol red	Difco (USA)
NaCl	Merk (Germany)
Gelatin	Biolife (Italy)
$MgCl_2$	BDH (England)

2.1.3 Media:

2.1.3.1 Ready Made Culture Media:

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

- Brain heart infusion agar (Difco, USA).
- Brain heart infusion broth (Difco, USA).
- Nutrient agar (Oxoid, England).
- Nutrient broth (Oxoid, England).

2.1.3.2 Laboratory Prepared Culture Media:

1. Cetrimide agar (Stolp and Gadkari, 1984):

This medium composed of:

Peptone	20g
$MgCl_2$	4.5g
K_2SO_4	10g
Cetrimide	0.3g
Agar	15g

dissolved in 850 ml of distilled water, adjusted pH to 7, sterilized by autoclaving then the volume was completed to 1000 ml.

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

Prepared by adding 12% w/v gelatin to nutrient broth, then sterilized by autoclaving at 121°C for 15 min.

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

This medium composed of:

Phenol red	0.012g
NaCl	5g
KH_2PO_4	2g
Peptone	1g
Glucose	1g
Agar	15g

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

4. Mineral Salt Medium: Bushnell-Hass (Patel and Deasi, 1997):

This medium was used to study the ability of bacteria to produce surfactants, which composed of:

KH_2PO_4	1g
K_2HPO_4	1g
NH_4NO_3	1g
$CaCl_2.2H_2O$	0.02g
$FeCl_3.6H_2O$	0.05g
$MgSO_4.7H_2O$	0.2g

dissolved in 850 ml of distilled water, adjusted pH to 7, sterilized by autoclaving then the volume was completed to 1000 ml.

5. Siegmund- Wagner (SW) Medium (Siegmund and Wagner, 1991):

This medium was used for the detection of anionic extracellular rhamnolipid produced by *Pseudomonas aeruginosa*, which composed of:

CTAB	0.2g
Methylene blue	0.005g
Agar	15g

dissolved in 850 ml of distilled water, adjusted pH to 7, sterilized by autoclaving then the volume was completed to 1000 ml.

6. King A Medium (Starr *et al*., 1981).

It is composed of:

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

dissolved in 850 ml of distilled water, adjusted pH to 7, sterilized by autoclaving then the volume was completed to 1000 ml.

7. King B Medium (Starr *et al*., 1981).

It is composed of:

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

dissolved in 850 ml of distilled water, adjusted pH to 7, sterilized by autoclaving then the volume was completed to 1000 ml.

8. Blood Agar (Harely and Prescotte, 1996):

Blood agar was used for semiquantitative detection of rhamnolipid hyperproducer mutants. It was prepared by dissolving 37g of blood agar base in distilled water, pH was adjusted to 7.3 and complete volume to 950ml with distilled water sterilized by autoclaving. After cooling (45°C), 50ml of sterile, defibrinated sheep blood was added and mixed well.

2.1.4 Api 20E Kit (Api Bio Merieux, Lyon, France):

Api 20E Kit consist of:

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

(B) Api 20E reagents:

- Oxidase reagent (1% tetra-methyl-p-phenyle-diamine)

- Kovac's reagent (p-dimethyl aminobenzaldehyde at 4% in HCl isoamyl alcohol).
- Voges-Proskauer reagent:
  - Vp1 (40% potassium hydroxide).
  - Vp2 (6% alpha-nephthol).
- Ferric chloride 3.4%.

2.1.5 Bacterial Isolates:

Bacterial isolates used in this study are listed below:

Bacteria	Phenotype	Source
Pseudomonas spp. RB55, RB67	Wild type	Nasir, R.B., Biotechnology Department, Baghdad University.
Pseudomonas aeruginosa RB19, RB27, RB29, RB31	Wild type	Biotechnology Department, Al nahrain University.

2.1.6 Reagents:

- **Catalase Reagent (Atlas *et al*., 1995):**

This reagent composed of 3% hydrogen peroxide.

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

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

2.1.7 Stains:

- **Gram' Stain (Atlas *et al*., 1995):**

It's composed of four reagents;

1 A primary stain-crystal violet.
2 A mordant-Gram's iodine solution.
3 A decolorizing agent- an organic solvent (alcohol).
4 A secondary stain or counter stain- safranin.

2.1.8 Solutions and Buffers:

- **Glucose Solution:**

The stock solution of glucose (100 µg/ml) was prepared by dissolving 0.01g of glucose in 100ml-distilled water.

- **Phenol solution (5%):**

It was prepared by dissolving 5g of phenol in 90ml distilled water and the volume was completed to 100ml with distilled water.

- **N-methyl-N-nitro-N-nitrosoguanidine (MNNG) solution:**

It was prepared as stock solution of 1mg/ml in 0.1N citrate buffer pH (5.5).

- **Citrate Buffer (0.1 N, pH = 5.5):**

Citric acid 10.5g and NaOH 4.4g were dissolved in distilled water, and then completed the volume to 500ml, pH was adjusted to 5.5.

- **Phosphate buffer (pH=7.0):**

Component	g/L
Na_2HPO_4	9.52
NaH_2PO_4	6.00

Sterilized by autoclaving at 121°C for 15 min.

2.2 Methods:

2.2.1 Maintenance of Bacterial Isolates:

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

- **Short term storage:**

Isolates of bacteria were maintained for period of few weeks on the surface of nutrient agar plates. The plates were tightly wrapped in parafilm and stored at 4°C.

- **Medium term storage:**

Isolates of bacteria were maintained in stab culture for period of 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:**

Bacteria 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 an exponential growth of bacteria in a screw-capped bottle with final volume 10 ml and stored at -20°C.

2.2.2 Identification of Bacterial Isolates:

2.2.2.1 Morphological, Physiological and Biochemical Tests:

A. Gram's stain (Atlas *et al*., 1995):

A single colony was transferred by a loop to a clean slide. The smear was stained with crystal violet, treated with iodine, decolorized with 95% alcohol, and counterstained with safranine, then examined by a microscope.

B. Growth on cetrimide agar (Greenwood *et al*., 1997):

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

C. **Catalase production test (Atlas *et al*., 1995):**

This test was peformed by adding drops of hydrogen peroxide (3% H_2O_2) on a single colony grown on brain heart infusion agar plate. Appearance of bubbles was regarded as positive result.

D. Oxidase production test (Atlas *et al*., 1995):

Filter paper was saturated with the substrate (tetramethyl-p-phenylene-diamine-dihydrochloride), colony of bacterial isolate to be tested was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to deep blue indicates a positive result.

E. Fluorescein pigment production test (Todar, 2004).

Bacterial isolates grown on cetrimide agar plates were examined under the source of UV light. Yellow-green fluorescent pigment indicates the presence of pyoverdin and then a positive result.

F. Gelatinase test (Atlas *et al*., 1995).

A nutrient broth-gelatin tube was inoculated with the bacterial isolate and incubated at 37°C for 24 hour, after that the tubes were placed in a refrigerator for 30 minutes. Liquefaction of the media indicates a positive result.

G. Urease test (Atlas *et al*., 1995).

The surface of the Christensin urea agar slants was inoculated with the bacterial isolates and incubates at 37°C for 24 hour. After incubation, the appearance of red-violet color indicates a positive test, while the appearance of a yellow-orange color indicates a negative test.

H. Growth at 4°C (Palleroni, 1985).

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

I. Growth at 42°C (Palleroni, 1985).

Bacterial isolates were grown at 42°C, by inoculating 50 ml of nutrient broth with 50µl of freshly prepared cultures of local isolates of *Pseudomonas* spp. separately. Appearance of growth (O.D. 600nm) indicates a positive result.

J. Growth on King A (Cruickshank *et al*., 1975).

Inoculate the bacteria on the plate by streaking and incubate at 37°C for 24 hrs. This test was performed to study the production of the characteristic pigment, pyocyanin.

K. Growth on King B (Cruickshank *et al*., 1975).

Inoculate the bacteria on the plate by streaking and incubate at 37°C for 24 hrs. then the plates were exposed to UV. This test was performed to study the production of the characteristic pigment, flourescein.

2.2.2.2 Identification of Bacterial Isolates Using API 20 E System:

Local isolates that have the same features and characteristics of *P. aeruginosa* on nutrient agar plates subsequently identified using biochemical tests were further characterized using API 20 E system as a standardized

characterization system for Enterobacteriaceae and other non-fastidious Gram-negative rods. The system consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension, which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents.

Biochemical tests included in this system are:

1. ONPG: Beta-galactosidase test.
2. ADH: Arginine dihydrolase test.
3. LDE: Lysine decarboxylase test.
4. ODC: Ornithine decarboxylase test.
5. CIT: Citrate utilization test.
6. H_2S: Hydrogen sulphide test.
7. URE: Urease test.
8. TDA: Tryptophane deaminase test.
9. IND: Indole test.
10. VP: Voges proskauer test.
11. GEL: Gelatin liquifaction test.
12. GLU: Glucose fermentation test.
13. MAN: Manitol fermentation test.
14. INO: Inositol fermentation test.
15. SOR: Sorbitol fermentation test.
16. RHA: Rhamnose fermentation test.
17. SAC: Sucrose fermentation test.
18. MEL: Melibiose fermentation test.
19. AMY: Amygdalin fermentation test.

20. ARA: Arabinose fermentation test.

A. Preparation of the galleries

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

B. Preparation of the inoculum

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

C. Inoculation of the gallereis

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

D. Reading the galleries

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

1. One drop of VP reagent to VP microtube and wait for 10 min then the result was recorded immediately.

2. One drop of 3.4% ferric chloride to TDA microtube then the result was recorded immediately.
3. One drop of Kovac's reagent to the IND microtube.
4. One drop of oxidase reagent to either H_2S or ONPG microtube.

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

2.2.3 Screening of Bacterial Isolates for Rhamnolipid Production in Liquid Media:

A fifty ml of mineral salt (Bushnell-Hass) medium were dispensed into 250ml Erlenmeyer flasks. Then 2% of sunflower oil was added as a sole carbon source. Following sterilization by autoclaving, flasks were inoculated with 1% of *Pseudomonas aeruginosa* fresh (18hrs, 30°C) culture, and then incubated in shaker incubator (180 rpm, 72 hrs, 30°C). After incubation the supernatant was obtained by centrifugation at 4000 rpm. for 10 min. Then surface tension test was performed by using tensiometer device in which the ring of the device was emerged inside the supernatant and then remove it and read the value (mN/m) at the time of ring separation from the supernatant.

2.2.4 UV Survival curve and mutagenesis of *Pseudomonas aeruginosa* RB67.

Culture of *Pseudomonas aeruginosa* RB67 grown in brain heart infusion broth at 18 hrs., was pelleted from 5ml by centrifugation at 4000 rpm for 10 min., washed twice and resuspended in the same volume of phosphate buffer (pH=7.0). The UV source was UV- transiluminator-Cross Linker [FLX-20-M, Vibler Lourmat, France]. The tray for irradiation approximately 15x25 cm exposes samples on Petri dishes, multi-well plates, membranes, etc, to direct irradiation from four of 15 watts, 254 nm bulbs.

A UV photoelectric cell detects actual intensity. The dose rate of UV irradiation was 2.5 $J/m^2/s$.

The experiment in this study was performed under red light. The distance between UV source and irradiated suspension was 11cm.

Five ml samples of the bacterial suspension in phosphate buffer were irradiated in sterile Petri dish for the following doses: (0, 5, 10, 15, and 20 J/m^2). Then 0.1 ml samples from appropriate dilution of each treatment were spreaded on brain heart infusion agar plates, and then incubated at 37°C for 24 hrs. to determine the total viable count (survival percentage).

According to the UV survival curve, the treatment that led to a survival percentage of approximately 10% as compared with the control was suspected to have the higher mutation frequency. From this treatment a number of colonies were picked up and tested for rhamnolipid production.

2.2.5 MNNG Survival curve and mutagenesis of *Pseudomonas aeruginosa* RB67

Culture of *Pseudomonas aeruginosa* RB67 grown in brain heart infusion broth at 18 hrs. was pelleted from 10 ml by centrifugation at 4000 rpm for 10 min., washed twice and resuspended in the same volume of

phosphate buffer (pH=7.0), and dispensed into 250 ml Erlenmeyer flasks. MNNG dissolved in citrate buffer (pH 5.5) was added to the bacterial suspension at different concentration (0, 10, 20, 30, 40, 50 µg/ml).

The mixture was incubated at 37°C with quite shaking for 20 min. Then 0.1 ml samples from appropriate dilutions of each treatment were spreaded on brain heart infusion agar plates, and then incubated at 37°C for 24 hrs. to determine the total viable count (survival percentage).

According to the MNNG survival curve, the treatment that led to a survival percentage of approximately 10% as compared with the control was suspected to have the higher mutation frequency. From this treatment a number of colonies were picked up and tested for its ability in rhamnolipid production.

2.2.6 Detection of *Pseudomonas aeruginosa* rhamnolipid hyperproducer mutants.

2.2.6.1 Semiquantitative detection of hyperproducer mutants:

Bacterial colonies obtained from each mutagenized culture (the selected treatment of UV and MNNG) were replica plated on blood agar plates (2.1.3.2.8) and incubated at 30°C for 72 hrs. Rhamnolipid hyperproducer mutants on blood agar plates were identified following the formation of a definite clear zone (β-haemolysis) around each colony. Colonies formed largest hemolytic zone were suspected as rhamnolipid hyperproducer, which were selected to measure rhamnolipid.

Fifty colonies from each selected treatment were replica plated on cetyltrimethyl-ammonium bromide (CTAB)- methylene blue agar plates (SW medium). Rhamnolipid producing colonies on SW agar plates were identified according to the formation of dark blue halos around the colonies on a light blue-plate background. Colonies formed largest halos were suspected as rhamnolipid hyperproducer, which were candidated to measure rhamnolipid.

2.2.6.2 Rhamnose concentration measurement:

Rhamnolipid amount was determined according to rhamnose concentration measurement (Saifour *et al*., 2004).

- **Preparation of glucose standard curve:**

A glucose stock solution (100 µg/ml) was prepared by dissolving 0.01g of glucose in 100ml of distilled water (Table 2-1). In which 1ml of 5% phenol was added to 1ml of the glucose stock solution and mixed gently, then 5ml of concentrated H_2SO_4 was added to the mixture with gentle mixing then cool for 15 min.

The absorbance was measured at 490 nm, the rhamnose concentration (µg/mL) was determined from the standard curve of glucose (Dupois *et al*., 1956).

- **Determination of rhamnolipid:**

In order to determine rhamnolipid produced by *P. aeruginosa* RB67 (wild type) and selected mutants, they were grown on minimal salt medium contained 2% sunflower oil (2.1.3.2.4) and incubated in shaker incubator

(180 rpm) at 30°C for 72hrs. After incubation, the culture was centrifuged, then pH of 10 ml of the supernatant was adjusted to 2, and then left for 24 hrs. at 4°C. After that it was extracted with chloroform/methanol (2:1 V/V) and the solvent was evaporated using rotary evaporator. After evaporation the precipitate was dissolved in 2ml of 0.1M sodium bicarbonate (Patel and Desai, 1997). The resultant was used to
measure rhamnose concentration according to Dupois *et al*., (1956) and as mentioned above.

Table (2-1) preparation the standard curve of glucose.

Tube no.	**Stock solution volume (ml)**	**Added distilled water volume (ml)**	**Total volume (ml)**	**Glucose concentration (µg/ml)**
1*	0.00	1.00	1.00	0.00
2	0.08	0.92	1.00	8
3	0.24	0.76	1.00	24
4	0.40	0.60	1.00	40
5	0.56	0.44	1.00	56
6	0.80	0.20	1.00	80
7	1.00	0.00	1.00	100

Tube (1*) represents the blank solution

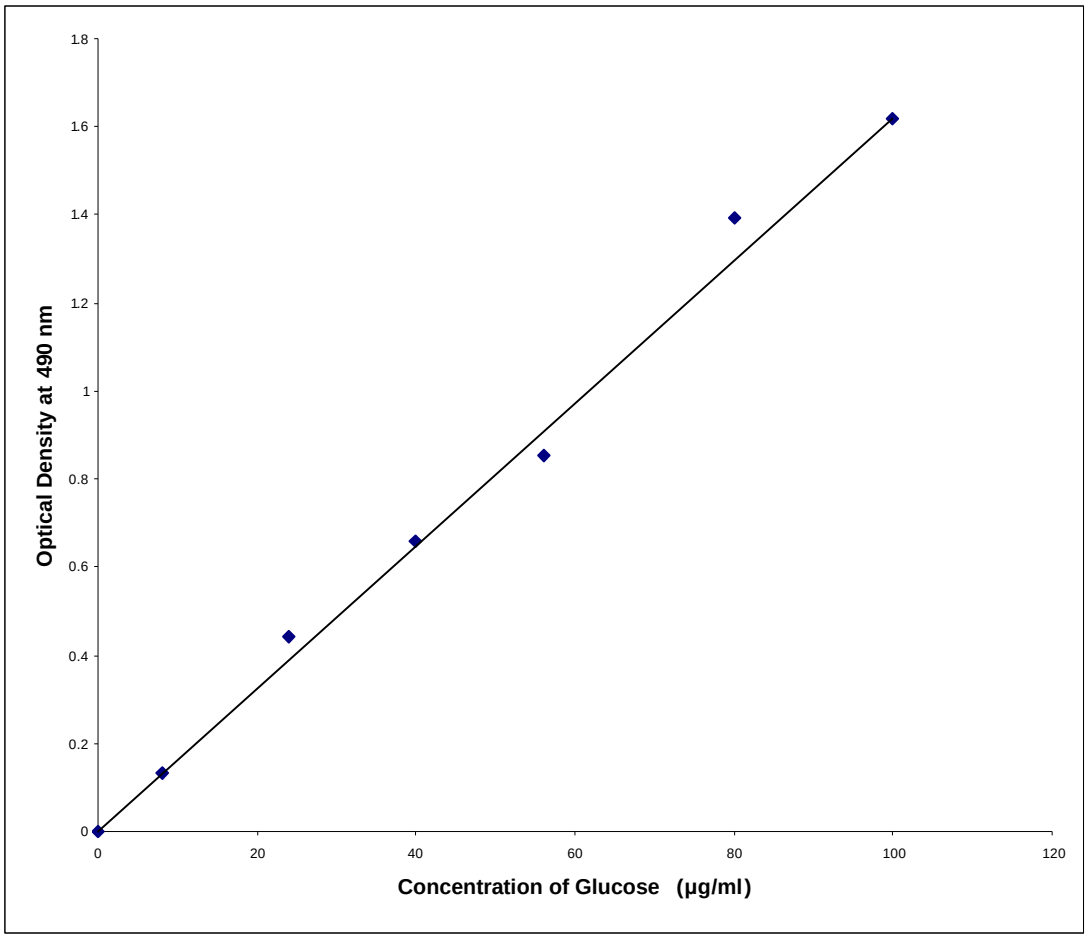

Figure (2.1) The standard curve of glucose according to Dupois *et al*., (1956) method. (Phenol-Sulfuric acid).

2.2.7 Characterization of Rhamnolipid using FTIR Analysis:

The partially purified rhamnolipid obtained from *P. aeruginosa* RB67 (wild type, UV mutant (UOM12), and MNNG mutant (MOM12)), were subjected to FT- infrared (FTIR) for the characterization of the compound. The FTIR spectrum which is advanced type of infrared (IR) spectrometry, will give the functional chemical groups that are found in the compounds in order to compare between them and identify if there is a qualitative differences between them or not.

Supervisor Certification

I certify that this was prepared under my supervision in Al-Nahrain University-College of Science as a partial fulfillment of degree of master of Science in Biotechnology.

Signature:
Supervisor:
Date:

In review of the available recommendation I forward this thesis for debate by examining committee.

Signature:

Name: Dr. Nabeel AL-Ani

Chairman 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:
Date:
(Chairman)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member/Supervisor)

I hereby certify upon the decision of the examining committee

Signature:

Name: **Dr.Laith Abdul Al-Aziz Al-Ani**.

Scientific Degree: Assistant Professor

Title: Dean of College of Science

Date:

4.1 Conclusions:

1. It was shown that all bacterial isolates, were able to produce biosurfactants, and the best one was *P. aeruginosa* RB67.
2. *P.aeruginosa* RB67 was able to produce rhamnolipid biosurfactant.
3. *P. aeruginosa* RB67 was sensitive to the lethal and mutagenic effect of UV light and MNNG.
4. Both mutagens (UV and MNNG), were efficient agents in inducing rhamnolipid overproducer mutants from *P. aeruginosa* RB67.
5. It was found that there is no qualitative differences between genuine rhamnolipid (wild type) and that produced by mutants.

4.2 Recommendations:

1. Attempts to develop efficient isolates in their ability to produce rhamnolipid via genetic engineering techniques.
2. More detailed studies for the possibility to take up rhamnolipid in an industerial importance.
3. Examine the biological activity of rhamnolipid produced by *P. aeruginosa* RB67 as, antimicrobial, antiviral, and antitumor agents.
4. Study the Quorum sensing system and its role in regulation of rhamnolipid production by *Pseudomonas* spp.

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

Genetic Improvement of Rhamnolipid Production from *Pseudomonas aeruginosa*

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

By

Oday Adnan Al- Makadci

B. Sc., Biotechnology, College of Science, 2000

Al-Nahrain University

November **2006**

Shawal **1427**

1-1 Introduction:

Surface-active agents are substances that have the ability to stabilize dispersions of one liquid in another, e.g. oil-in-water emulsions. The reason that surfactants concentrate at interface is that they are amphipathic, i.e. they contain both hydrophobic and polar groups (Rosenberg and Ron, 1998).

Surfactants can be divided into low-molecular-weight molecules that lower surface and interfacial tensions efficiently, and high-molecular-weight polymers that bind tightly to surfaces (Rosenberg and Ron, 1999; 2000).

Surfactants provide remarkable benefits in many processes that involve interaction of surfaces include emulsification, chemical or dye adsorption on fibers, adhesion, vaporization, melting, heat transfer, catalysis, foaming and defoaming. Specific function of surface active agents include removing soil (scouring), wetting, rewetting, softening, retarding dyeing rate, fixing dyes, stabilizing dispersions, coagulating suspended solids (Warren, 1998).

Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. The molecules reduce surface and interfacial tension in both aqueous solution and hydrocarbon mixtures, which make them potential candidates for enhancing oil recovery (Sarkar *et al*., 1989) and emulsification process. They also used in food industries, hydrometallurgy, medicine, biotechnology and environmental protection in the separation of hydrocarbons and gases, and in the concentration and separation of amino acids, metal ions and other mixtures and suspensions (Kaminski and Kwapinski, 2000; Stubenrauch, 2001).

Rhamnolipid, a rhamnose containing glycolipidis is one of the most important biosurfactant produced by *Pseudomonas aeruginosa* (Champion, 1995). It is considered to be one of the virulence-associated exoprducts of *P. aeruginosa* (Olvera *et al*., 1999). Also rhamnolipid possess antimicrobial activity against many Gram-positive and Gram-negative bacteria (ex. *Bacillus subtilis*) (Borjana *et al*., 2002). In addition to the above, rhamnolipid biosurfactant has been shown to be capable of binding to heavy metals (e.g. it can bind to cadmium, lead and zinc and removing them from soil) (Sandrin *et al*., 2000).

In order to improve rhamnolipid production from *P. aeruginosa*, genetic manipulation using physical (UV light) and chemical (MNNG) mutagenes were performed. Abbas *et al*., (2004) successfully improved rhamnolipid production using chemical mutagen (MNNG).

Aims of the Study:

According to the great importance of rhamnolipid, and because of the limited studies on it, this study was aimed to:

1. Screening the ability of *Pseudomonas* spp., which were isolated previously, to produce biosurfactants.

2. Study the lethal and mutagenic effect of UV and MNNG, in an attempt to enhance rhamnolipid production by *P. aeruginosa*.

3. Characterization of the rhamnolipid produced by *P. aeruginosa* (wild type) and mutants, in order to identify if there is a qualitative differences or not.

References

A

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.

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

Al-Delaimi, H. M. and Al-Gelawi, M. H. Effect of molecular and physical mutagens on the ability of *Xanthomonas campestris* H6 in protease production. (Unpublished Data).

Al- Gelawi, M. H. (1999). Isolation of salt sensitive mutants from *Micrococcus* sp. strain G1. Iraqi J. Sci., Vol: **80** No. (1).

Al- Saady, R. E.; Al-Gelawi, M. H. and Al-Ani, Z. N. (2005). Mutagenesis of locally isolate *Gardnerella vaginalis* using nitrosoguanidine and ultraviolet light. J. Al- Nahrain University. Volume: **8** No. (1).

Arima, K. A.; Kakinuma and Tamura, G. (1968). Surfaction, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Biophys. Res. Commun. **31:** 488-494.

Asselineau, C. and Asselineau**,** J. **(**1978). Trehalose containing glycolipids. Prog. Chem. Fats Lipids **16:** 59–99.

Atlas, R. M.; Parks, L. C. and Brown, E. A. (1995). Laboratory manual of experimental microbiology. Moby-Year Book, Inc., Wesline Industerial Drive, St. Louis, Missouri.

B

Baho, S. A. (2006). Genetic study of the locally isolated *Pseudomonas aeruginosa* and its ability in lectin production. M. Sc. Thesis, Al- Nahrain university, College of science, Biotechnology department.

Banat, I. M.; Samarah, N.; Murad, M.; Horne, R. and Benerjee, S. (1991). Biosurfactant production and use in oil tank clean-up. World J. Microbiol. Biotechnol. **7**: 80-84.

Bar-Ness, R.; Avrahamy, N.; Matsuyama, T. and Rosenberg**,** M. **(**1988). Increased cell surface hydrophobicity of a *Serratia marcescens* NS 38 mutant lacking wetting activity. J. Bacteriol. **170:** 4361–4364.

Beal, R. and Betts, W. B. (2000). Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. J. Appl. Microbiol. **89**: 158-168.

Belsky, I.; Gutnick, D. L. and Rosenberg, E. **(**1979). Emulsifier of *Arthrobacter RAG-1*: determination of emulsifier bound fatty acids. FEBS Lett. **101:** 175–178.

Borjana, K. T.; George, R. I. and Nelly, E. C. (2002). Biosurfactant production by new *Pseudomonas putida* strain. Acad. Sci. Microbiol. **57c**: 356-360.

Botstien, D. and Jones, E. W. (1969). Non random mutagenesis of the *E. coli* genome by nitrosoguanidine. J. Bacteriol. **98**: 847-848.

Burger, M. M.; Glaser, L. and Burton, R. M. (1963). The enzymatic synthesis of a rhamnose-containing glycolipid by extracts of *Pseudomonas aeruginosa*. J. Biol. Chem. **238**: 2595–2602.

C

Cairns, W. L.; Cooper, D. G.; Zajic, J. E.; Wood, J. M. and Kosaric, N. **(**1982). Characterisation of *Nocardia amaree* as a potent biological coalescing agent of water-oil emulsions. Appl. Environ. Microbiol. **43:** 362–366.

Carrasco, A. and Soto, C. (1987). Mutagenesis of *Clostridium butyricum.* J. Appl. Bact. **63**: 539-543.

Champion, J. T. (1995). Electron microscopy of rhamnolipid (biosurfactant) Morphology: Effect of pH, cadmium and octadecane. J. Coll. Interf. Sci. **170(2):** 569-574.

Cirigliano, M. C. and Carman, G. M. **(**1985). Purification and characterization of liposan, a bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol. **50:** 846–850.

Colline, C. H. and Lyne, P. M. (1987). Microbiological methods, 3rd ed. Butte worth and Co. Publisher Ltd., London.

Cooper, D. G.; Liss, S.; Longay, N. R. and Zajic, J. E. **(**1989). Surface activities of *Mycobacterium* and *Pseudomonas*. J. Ferment. Technol. **59:** 97–101.

Cooper, D. G. and Goldenberg, B. G. **(**1987). Surface active agents from two *Bacillus* species. Appl. Environ. Microbiol. **53:** 224–229.

Cooper, D. G.; Zajic, J. E. and Gerson, D. F. (1978). Production of surface active lipids by *Corynebacterium lepus*. Appl. Environ. Microbiol. **37:** 4–10.

Crow, J. F. (1993). Environmental and molecular mutagenesis. **21**: 122-129.

Cruickshank, R.; Marmiom, B. P.; Duguid. (1975). Medical microbiology, 12th ed., Vol. 2. Churchill Livingstone, London.

Cutler, A. J. and Light, R. J. (1979). Regulation of hydroxydocosanoic and sophoroside production in *Candida bogoriensis* by the level of glucose and yeast extract in the growth medium. J. Biol. Chem. **254:** 1944–1950.

D

Dale, J. W. (1998). Molecular genetics of bacteria. 3rd ed., University of Surrey, UK.

Desai, J. D. and Banat, I. M. (1997). Microbial production of surfactants and their commertial potential. Microbiol. Mol. Biol. Rev. **61:** 47-64.

Desai, A. J.; Patel, R. M. and Desai, J. D. **(**1994). Advances in production of biosurfactants and their commercial applications. J. Sci. Ind. Res. **53:** 619– 629.

Desai, A. J.; Patel, K. M. and Desai, J. D. **(**1988). Emulsifier production by *Pseudomonas fluorescens* during the growth on hydrocarbons. Curr. Sci. **57:** 500–501.

Dupois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A. and Smith, F. (1956). Colorimetric method for determination of sugar and related substances. Anal. Chem. **28**: 350-356.

F

Fattom, A. and Shilo, M. (1985). Production of emulcyan by *Phormidium* J-1: its activity and function. FEMS Microbiol. Ecol. **31:** 3–9.

Friefelder, D. (1987). Molecular biology. 2nd ed. Yones and Bttett. Boston. USA.

Fuqua, C.; Winans, S. C. and Greenberg, E. P. (1996). Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50**: 727-51.

G

Georgiou, G.; Lin, S. C. and Sharma, M. M. **(**1990). Surface active compounds from microorganisms. Biotechnology. **10:** 60–65.

Gray, K. M. (1997). Intercellular communication and group behaviour in bacteria. Trends Microbiol. **5**: 184-8.

Greek, B. F. (1990). Detergent industry ponders products for new decade. Chem. Eng. News. **68:** 37–38.

Greek, B. F. **(**1991). Sales of detergents growing despite recession. Chem. Eng. News. **69:** 25–52.

Greenberg, E. P. (1997). Quorum sensing in gram-negative bacteria. ASM News. **63**: 371-7.

Greenwood, D.; Slack, R. and Peatherer, J. (1997). Medical microbiology (5^{th} ed.) Churchill livingstone.

Guerra-Santos, L. H.; Kappeli, O. and Flechter, A. **(**1991). Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol. Biotechnol. **24:** 443–448.

Gutnick, D. L. and Shabtai, Y. (1987). Exopolysaccharide bioemulsifiers. Pp. 211–246. *In* N. Kosaric, W. L. Cairns, and N. C. C. Gray (ed.), Biosurfactants and biotechnology. Marcel Dekker, Inc., New York, N.Y.

H

Hansen, K. G.; Desai, J. D. and Desai, A. J. (1993). A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnol. Tech. **7:** 745–748.

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

Holt, J. G.; Krieg, N. R.; Sneath, H. A.; Staley, J. T. (1994). Bergey's Manual of Determinative Bacteriology. 9th ed. Williams and Wilkins, USA.

Hommel, R. K.; Weber, L. A.; Weiss, U.; Himelreich, O. Rilke and Kleber, H. P. **(**1994). Production of sophorose lipid by *Candida (Torulopsis) apicol*a grown on glucose. J. Biotechnol. **33:** 147–155.

I

Iglewski, B. H.; Sadoff, J.; Bjorn, M. J. and Maxwell, E. S. (1978). *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Natl. Acad. Sci. U S A. **75**: 3211-5.

J

Jain, D. K.; Thompson, D. L.; Lee, C. H. and Trevors, J. T. **(**1991). A drop-collapsing test for screening surfactant producing microorganisms. J. Microbiol. Methods **13:** 271–279.

Jarvis, F. G. and Johnson, M. J. **(**1949). A glycolipid produced by *Pseudomonas aeruginosa*. J. Am. Chem. Soc. **71:** 4124–4126.

Jesus, C. G.; Alma, D. C.; Rebeca, N.; Raina, M.; Ragheb, A. and Gloria, S. (1998). The *pseudomonas aeruginosa* rh1G gene encodes an NADPH-dependent β-ketoacyl reductase, which is specifically involved in rhamnolipid synthesis. J. Bact. Pp. 4442-4451.

K

Kaminski, W. and Kwapinski, W. (2000). Pol. J. Environ. Stud. Pp. 37-43.

Kappeli, O. and Finnerty, W. R. **(**1979). Partition of alkane by an extracellular vesicle derived from hexadecane-grown *Acinetobacter*. J. Bacteriol. **140:** 707–712.

Kernacki, K. A.; Hobden, J. A.; Hazlett, L. D.; Fridman, R. and Berk, R. S. (1995). In vivo bacterial protease production during *Pseudomonas aeruginosa* corneal infection. Invest. Ophthalmol. Vis. Sci. **36**: 1371-8.

Kleerebezem, M.; Quadri, L. E.; Kuipers, O. P. and Vos V. M. (1997). Quorum sensing by peptide pheromones and two-component signal-

transduction systems in gram-positive bacteria. Mol. Microbiol. **24**: 895-904.

Kretschmer, A.; Bock, H. and Wagner, F. **(**1982). Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grown on *n*-alkane. Appl. Environ. Microbiol. **44:** 864– 870.

L

Lang, S. and Wullbrandt, D. (1999). Rhamnose lipids – biosynthesis, microbial production and application potential. Appl. Microbiol. Biotechnol. **51**: 22–32.

Lang, S. and Wagner, F. **(**1987). Structure and properties of biosurfactants, Pp. 21–47. *In* Kosaric, N.; Cairns, W. L. and Gray, N. C. C. (ed.), Biosurfactants and biotechnology. Marcel Dekker, Inc., New York, N.Y.

Latifi, A.; Foglino, M.; Tanaka, K.; Williams, P. and Lazdunski, A. (1996). A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. **21**: 1137-46.

Laycock, M. V.; Hildebrand, P. D.; Thibault, P.; Walter, J. A. and Wright, J. L. (1991). Viscosin, a lipopeptide biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas flourescens*. J. Agric. Food. Chem. **39**: 483-489

Li, Z. Y.; Lang, S.; Wagner, F.; Witte, L. and Wray, V. **(**1984). Formation and identification of interfacial-active glycolipids from resting microbial cells of *Arthrobacter* sp. and potential use in tertiary oil recovery. Appl. Environ. Microbiol. **48:** 610–617.

Liu, P. V. (1974). Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**: 94-9.

Liu, P. V. (1979). Toxins of *Pseudomonas aeruginosa.* In: Doggett RG, editor. *Pseudomonas aeruginosa.* Clinical manifestations of infection and current therapy. New York: Academic Press. Pp. 63-88.

M

Maier, R. M. and Soberon, C. G. (2000). *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl. Microbiol. Biotechnol. **54**: 625–633.

Makkar, R. S. and Cameotra, S. S. (2002). An update on the use of unconventional substrates for biosurfactant production and their new applications. Appl. Microbial. Biotechnol. **58**: 428-434.

Matsuyama, T.; Sogawa, M. and Yano, I. (1991). Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. Appl. Environ. Microbiol. **53:** 1186–1188.

Miller, R. V.; Jeffrey, W.; Mitchell, D. and Elasri, M. (1999). Bacterial responses to ultraviolet light. American society for microbiology, **65**: 535-542.

Mount, D. W. (1996). Programming transcription nature. J. general microbial. **383**: 763-769.

Mulligan, C. N.; Chow, T. Y. K. and Gibbs, B. F. (1989). Enhanced biosurfactant production by a mutant *Bacillus subtilis* strain. Appl. Microbiol. Biotechnol. **31**: 486-489.

Mulligan, C. N.; Cooper, D. G. and Neufeld, R. J. **(**1984). Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferment. Technol. **62:** 311–314.

N

Nasir, R. B.; Ali, N. A. and Al-Gelawi, M. H. (2002). Isolation and identification of crude oil and hydrocarbon utilizing bacteria. Iraq. J. Sci. Vol: **43B** No (1).

Neu, T. R. and Poralla, K. **(**1990**).** Emulsifying agent from bacteria isolated during screening for cells with hydrophobic surfaces. Appl. Microbiol. Biotechnol. **2:** 521–525.

A) Nicas, T. I.; Bradley, J.; Lochner, J. E. and Iglewski, B. H. (1985). The role of exoenzyme S in infections with *Pseudomonas aeruginosa*. J. Infect. Dis. **152**: 716-21.

B) Nicas, T. I.; Frank, D. W.; Stenzel, P.; Lile, J. D. and Iglewski, B. H. (1985). Role of exoenzyme S in chronic *Pseudomonas aeruginosa* lung infections. Eur. J. Clin. Microbiol. **4**: 175-9.

Nicas, T. I. and Iglewski, B. H. (1985). The contribution of exoproducts to virulence of *Psedomonas aeruginosa*. Can. J. Microbiol. **31**: 387-92.

O

Ochsner, U. A. and Reiser, J. **(**1995). Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:** 6424–6428.

Ochsner, U. A.; Reiser, J.; Fiechter, A. and Witholt, B. **(**1995). Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterogeneous host. Appl. Environ. Microbiol. **61:** 3503–3506.

A) Ochsner, U. A.; Fiechter, A. and Reiser, J. **(**1994). Isolation, characterization and expression in *Escherichia coli* of the *Pseudomonas aeruginosa rhl*AB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J. Biol. Chem. **269:** 19787–19795.

B) Ochsner, U. A.; Koch, A. K.; Fiechter, A. and Reiser, J. **(**1994). Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:** 2044–2054.

Olvera, C.; Goldberg, J. B.; Sanchez, R. and Soberon-Chavez, G. (1999). The *Pseudomonas aeruginosa* algC gene product participates in rhamnolipid biosynthesis. FEMS Microbiol. Lett. **179**: 85-90.

P

Palejwala, S. and Desai, J. D. **(**1989). Production of extracellular emusifier by a Gram-negative bacterium. Biotechnol. Lett. **11:** 115–118.

Palleroni, N. J. (1985). Pseudomonadaceae. Sized from Berjy's Manual of systematic microbiology. Vol (1). Williams and Wilkins. Baltemore. London.

Parra, J. L.; Guinea, J.; Manresa, M. A.; Robert, M.; Mercade, M. E.; Comelles, F. and Bosch, M. P. **(**1989). Chemical characterization and physicochemical behaviour of biosurfactants. J. Am. Oil Chem. Soc. **66:** 141–145.

Passador, L. and Iglewski, B. H. (1995). Quorum sensing and virulence gene regulation in *Pseudomonas aeruginosa*. 2nd ed. Washington: American Society for Microbiology. Pp. 65-78.

Patel, R. N. and Desai, A. J. (1997). Surface active properties of rhamnolipid from *Pseudomonas aeruginosa* GS3. J. Basic Microbiol. **32**: 518-520.

Peak, M. J. and Peak, J. G. (1986). DNA to protein cross- links and backbone breaks caused by far and near ultraviolet and repair; Implication for carcinogenesis and risk asessment, Pp. 193-202. M. G. Simie; L. Grossman and A. C. Upton (eds). New York: Plenum press.

Pearson, J. P.; Passador, L.; Iglewski, B. H. and Greenberg, E. P. (1995). A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U S A. **92**: 1490-4.

Pearson, J. P.; Pesci, E. C. and Iglewski, B. H. (1997). Role of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in the control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179**: 5756-67.

Q

Queener, S. W. and Lively, D. H. (1986). Screening and selection for strain improvement. In: Manual of Industerial Microbiology and Biotechnology (Demain, A. L. and Solomon, M. A. eds.), American Society for Microbiology, Washington, D. C. Pp. 155-169.

R

Rahim, R.; Ochsner, U. A.; Olvera, C.; Graninger, M.; Messner, P.; Lam, J. S. and Soberon-Chavez, G. (2001). Cloning and functional characterization of the *Pseudomonas aeruginosa* rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol. Microbiol. **40**: 708–718.

Rapp, P.; Bock, H.; Wray, V. and Wagner, F. (1979). Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on n-alkanes. J. Gen. Microbiol. **115:** 491–503.

Razafindralambo, H.; Paquot, M.; Baniel, A.; Popineau, Y.; Hbid, C.; Jacques, P. and Thonart, P. **(**1996). Foaming properties of surfactin, a lipopeptide biosurfactant from *Bacillus subtilis*. J. Am. Oil Chem. Soc. **73:** 149–151.

Read, R. C.; Roberts, P. and Munro, N. (1992). Effect of *Pseudomonas aeruginosa* rhamnolipids on mucociliary transport and ciliary beating. J. Appl. Physiol. **72**: 2271-7.

Reimmann, C.; Beyeler, M.; Latifi, A.; Winteler, H.; Foglino, M.; Lazdunski, A. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol. Microbiol. **24**: 309-19.

Rosenberg, E. and Ron, E. Z. (2000). Biosurfactants. In the prokaryotes. Dworkin, M. (ed.). New York: Springer-Verlag.

Rosenberg, E. and Ron, E. Z. (1999). High- and low-molecular-mass microbial surfactants. Appl. Microbiol. Biotechnol. **52**: 154-162.

Rosenberg, E. and Ron, E. Z. (1998). Surface active polymer from the genus *Acinobacter*. In biopolymer from renewable resources. Kaplan, D. L. (ed.). New York: Springer-Verlag, Pp. 281-291.

S

Saifour, M.; Aziz, G. M. and Al-Gelawi, M. H. (2004). Determination of cultural conditions for the production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa* RB28. Iraqi J. Biotech. **2(2):** 35-49.

Sandrin, T. R.; Chech, A. M. and Mair, R. M. (2000). A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. Appl. Environ. Microbiol. **66**: 4585-4588.

Sarkar, A. K.; Goursand, J. C.; Sharma, M. M. and Georgiou, G. **(**1989). A critical evaluation of MEOR process. In Situ **13:** 207–238.

Shennan, J. L. and Levi, J. D. **(**1987). *In situ* microbial enhanced oil recovery, Pp. 163–181. *In* N. Kosaric, W. L. Cairns, and Gray, N. C. C. (ed.), Biosurfactants and biotechnology. Marcel Dekker, Inc., New York, N.Y.

Siegmund, I. and Wagner, F. **(**1991). New method for detecting rhamnolipids exerted by *Pseudomonas* species grown on mineral agar. Biotechnol. Tech. **5:** 265–268.

Singer, M. E. **(**1985). Microbial biosurfactants. Microbes Oil Recovery **1:** 19– 38.

Snyder, L. and Champness, W. (1997). DNA repair and mutagenesis. In molecular genetics of bacteria, Pp. 237-264. Asm press. Washington, D.C.

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.

Stevens, A. M. and Greenberg, E. P. (1997). Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. J. Bacteriol. **179**: 557-62.

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

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

Stubenrauch, C. (2001). Curr. Opin. Colloid Interface Sci. Pp. 160-170.

T

Todar, Kennech. (2004). University of Wisconsin-Madison.

Turner, P. C.; Mclennan, A. G.; Bates, A. D. and White, M. R. H. (1996). Mutagenesis by UV radiation. Molecular biotechnology. Pp. 89-102.

V

Velikonja, J. and Kosaric, N. **(**1993). Biosurfactant in food applications, Pp. 419–446. *In* N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker Inc., New York, N.Y.

Vidal, D. R.; Garrone, P. and Banchereau, J. (1993). Immunosuppressive effects *Pseudomonas aeruginosa* exotoxin A on human B-lymphocytes. Toxicon. **31**: 27-34.

W

Warren, S. P. (1998). Surfactants- A Primer. Technical Editor. Pp. 51-54.

Wilkinson, S. G. and Galbraith, L. **(**1975). Studies on lipopolysaccharides from *Pseudomonas aeruginosa*. Eur. J. Biochem. **52:** 331–343.

Woods, D. E. and Iglewski, B. H. (1983). Toxins of *Pseudomonas aeruginosa*: new perspectives. Rev. Infect. Dis. 5 Suppl **4**: S715-22.

Z

Zajic, J. E.; Gignard, H. and Gerson, D. F. **(**1977). Properties and biodegradation of a bioemulsifier from *Corynebacterium hydrocarboclastus*. Biotechnol. Bioeng. **19:** 1303–1320.

Zosim, Z.; Gutnick, D. L. and Rosenberg, E. **(**1982). Properties of hydrocarbon- in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. Biotechnol. Bioeng. **24:** 281–292.

Zouboulis, A. I.; Matis, K. A.; Lazaridis, N. K. and Golyshin, P. N. (2003). The use of biosurfactants in flotation: application for the removal of metal ions. Minerals Engineering. **16**: 1231-1236.

World Wide Web References

Internet (1):
http://WWW.inch.org/documents/ech/ech/ech014.htm#partNumber:1.

Summary

Six bacterial isolates (four of them belonging to *Pseudomonas aeruginosa* and the other two were belonged to *Pseudomonas* spp.) isolated previously, were identified and/or ensured their identification. Results showed that these isolates were belonged to *P. aeruginosa* according to morphological, physiological, and biochemical characteristics, which was confirmed by using API 20E system.

All these isolates were screened for their ability to produce rhamnolipid biosurfactant depending on surface tension measurment (mN/m), which referred that all these isolates were capable of producing rhamnolipid, and the best one was *P. aeruginosa* RB67.

In order to get rhamnolipid hyperproducer mutants, mutagenesis of *P. aeruginosa* RB67 using UV light and MNNG were performed. The lethal and mutagenic effect of UV light and MNNG were studied on this bacterium. Results showed that this bacterium was sensitive to UV and MNNG. Fifty colonies from each treatment (UV and MNNG) were selected and screened for their ability to produce rhamnolipid semiquantitavely by replica plated on blood agar. Results indicated that all colonies showed approximately the same hemolytic zone. So, these colonies were replica plated on CTAB-methylene blue agar (Sigmend-Wagner medium) as an alternative method. Depending on this method twelve colonies from each treatment (UV and MNNG) were selected (based on the formation of largest dark blue halo against light blue background) and used for measuring rhamnose concentration.

Results showed that these mutants were varied in their ability to produce rhamnolipid. Two mutants from each treatment showed an increase in rhamnolipid production and the highest rhamnose

concentration (94 μg/ml) was gained by the mutant (MOM12) compared with rhamnose concentration (70 μg/ml) produced from *P. aeruginosa* RB 67 (wild type).

In order to qualify the rhamnolipid produced from *P. aeruginosa* RB67 (wild type), UOM12, and MOM12, Fourier transformer infrared (FTIR) spectroscopy was performed.

Results showed that there are no apparent qualitative differences in rhamnolipid produced from these three isolates.

الخلاصة

تم تشخيص وتاكيد تشخيص ستة عزلات بكتيرية (اربعة منها تعود للنوع *Pseudomonas aeruginosa* وعزلتين تعود للجنس *Pseudomonas* spp.) معزولة سابقاً. واظهرت النتائج ان هذه العزلات تعود للنوع *Pseudomonas aeruginosa* اعتمادا على الصفات المظهرية والفسلجية والبايوكيمياوية والتي اكدت باستعمال نظام API 20E.

اختبرت قابلية جميع هذه العزلات على انتاج الرامنولبيد اعتمادا على قياس الشد السطحي (ملي نيوتن/م) والذي اشار الى ان جميع هذه العزلات كانت قادرة على انتاج الرامنولبيد وان افضل عزلة هي *P. aeruginosa* RB67.

ولاجل الحصول على طافرات ذات انتاجية عالية من الرامنولبيد، طفرت بكتريا *P. aeruginosa* RB67 باستخدام الاشعة فوق البنفسجية ومادة MNNG، ودرس التاثير القاتل والمطفر لهذين العاملين على هذه البكتريا. اظهرت النتائج ان هذه البكتريا كانت حساسة لكل من الاشعة فوق البنفسجية ومادة MNNG. انتخبت خمسين مستعمرة من كل معاملة (الاشعة فوق البنفسجية ومادة MNNG) وغربلت لقابليتها على الانتاج الشبه كمي للرامنولبيد وذلك بتكرارها على وسط اكار الدم. اوضحت النتائج ان جميع المستعمرات اظهرت تقريبا مناطق التحلل نفسها. لذا فقد كررت هذه المستعمرات على وسط CTAB-methylene blue agar (Sigmend-Wagner medium) كطريقة بديلة. اعتمادا على هذه الطريقة ١٢ مستعمرة من كل معاملة (الاشعة فوق البنفسجية ومادة MNNG) انتخبت (بالاعتماد على تكوين اكبر هالة زرقاء داكنة على خلفية زرقاء باهتة) واستعملت لقياس تركيز الرامنوز. اظهرت النتائج ان هذه الطافرات كانت متغايرة في قابليتها على انتاج الرامنولبيد. واظهرت طافرتين لكل معاملة زيادة في انتاج الرامنولبيد وان اعلى تركيز للرامنوز (٩٤ µg/ml) تم الحصول عليه بفعل الطافرة (MOM12) مقارنة بتركيز الرامنوز (٧٠ µg/ml) المنتج من قبل العزلة البرية *P. aeruginosa* RB67.

من اجل التقييم النوعي للرامنولبيد المنتج من قبل *P. aeruginosa* RB67 (wild type, UOM12, and MOM12)، استخدمت تقنية FTIR. اظهرت النتائج عدم وجود تغيرات نوعية في الرامنولبيد المنتج من قبل هذه العزلات.

Chapter One

Introduction and Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Chapter Four

Conclusions and Recommendations

References