1 - LITERATURE REVIEW

1.1 Genus *Pseudomonas*

Pseudomonads are motile by one or several polar flagella (rarely non motile), Gram-negative, aerobic that utilize glucose oxidatively, characterized by straight or slight curved rod but not helical ,pili forming , catalase and oxidase are positive , growth occurs from 4°C or lower to 43°C and were chemo-organotrophic (Holt, *et al.*, 1994a).

Members of this genus are classified into five groups based on ribosomal RNA homology. These bacteria are clinically important because they are resist to most antibiotics and they are capable of surviving in conditions that few other organisms can tolerate. They also produce a slime layer that is resist to phagocytosis. *Pseudomonas* is often encountered in hospital and clinical work because it is a major cause of hospital acquired (nosocomal) infections. Its main targets are immunocompromised individuals, burn victims, and individuals on respirators or with indwelling catheters. Additionally, these pathogens colonize the lungs of cystic fibrosis patients, increasing the mortality rate of individuals with the disease. Infection can occur at many sites and can lead to urinary tract infections, sepsis, pneumonia, pharyngitis, and a lot of other problems (Stolp and GudKari, 1984).

The genus *pseudomonas* included two pigmentation groups as genetic characters, which are so-called fluorescent and non-fluorescent subgroups. Some species of *Pseudomonas* are plant pathogens like *P.putida*, others are opportunistic pathogenic for human and animals like *P.aeruginosa* , they are also play an important role in spoilage of food, dairy product, meat, poultry and eggs .If spoilage develops after storage under refrigerated condition, *Pseudomonas* usually prevails(Brooks, *et al.*,1995).

1.1.2 *Pseudomonas putida*

Pseudomonas putida is a gram negative bacteria, rod-shape with multitrichous flageli of fluorescents group of the family *Pseudomonodaceae* .Itś growth occur from 4°C or lower to 40°C, chemo-organotrophic , an ubiquitous saprophytic endowed with a remarkable adaptability to diverse environments.Itś mostly putrefaction, soil microorganism has been studied extensively as an experimented model for the biodegradation of aromatic compound (Hill and Robinson, 1975).

It was found to be the most efficient species of the genus *Pseudomonas* have the ability to degraded hydrocarbon compounds this characteristic came from Itś ability in adaptation to environmental change may be facilitated by transfer and sorting of genetic material between and within naturally occurring populations(Finette and Gibson, 1988).

Horizontal gene transfer is major mechanisms for microorganisms to acquire a new metabolic trait in a new combination as such horizontal gene transfer has important implication in the spread of antibiotic resistance and tolerate relatively high level of heavy metals (McHumgh, *et al*, 1975).

1.2 Microbial biodegradation of hydrocarbon

Microbial biodegradation of petroleum products and natural wastes is the modification or decomposition of the product by microbes to produce ultimately energy, CO_2 and water (Dragon, 1988b).

The first study revealed that the biodegradation of hydrocarbons as a sole source of energy and carbon when it was noted that the fungus *Botcrytis cinera* has the ability to utilize paraffin wax as a source of energy (AL-Hydari, 1989) .

Later it was found that other microbial genera have the ability for hydrocarbons biodegradation such as *Arthrobacter* , *Achromobacter* , *Micrococcus* , *corynebacterium* , *Acinetobacter* , *Nocardia* , *Alcaligenes* ,

streptomyces , vibrio and *Flavobacterium,* the number of microbial community that degrades hydrocarbons is proportionally increased, when the degree of pollution increases. The percentage of hydrocarbon degrading bacteria isolated from unpolluted area is 0.01%, while the percentage reaches 60-80 % in petroleum-contaminated areas (Azoulay, *et al*. 1983; Nasir, *et al*., 2002).

Pseudomonas spp. possess different enzymatic systems like monoterminal oxidation systems for aliphatic hydrocarbons, besides the catabolic pathways that cleave and oxidize aromatic hydrocarbons through ortho , para and meta pathways (AL-Hadhrami, *et al.* , 1997).

Natural wastes and hydrocarbons have degraded naturally at a slow but steady rate. However in areas contaminated with petroleum product, this natural process is not very fast enough to ensure removal of pollutants.It was recognized that, the microbial biodegradation of hydrocarbons was highly dependent on the chemical nature of the compounds within the petroleum mixture and on environmental condition (Van Hamme, *et al.*, 2003).

1.2.1 Types of biodegradable hydrocarbons

Alkanes and aromatic compounds are the predominant chemicals comprising most petroleum products, n-alkanes and aromatic compounds ranged between C10-C22 are the least toxic and most readily biodegradable compounds, while the n-alkane and aromatic hydrocarbons ranged between C5-C6 are biodegradable at low concentration by some micro-organisms because of their toxicity, but in most environments they are removed by volatilization rather than by biodegradation. Gaseous n-alkanes (C1-C4) are biodegradable but are used only by a narrow range of specialized hydrocarbon degraders. n-Alkanes and aromatic compounds above C22 have extremely low

water solubility and absorption on soil particles, their solid state makes microbial degradation of these chemicals slow (Dragon, 1988a).

The Polyaromatic hydrocarbons represent a major problem in bioremediation of polluted sites because of their low bioavailability and hence, biodegradability. Recently studies showed that their bioavailability have been increased by the use of activated carbon as supporting material and the partial oxidation of these compounds , therefore , their biodegradability will increase (Abu-Salah, *et al.* , 1996) .

Branched alkanes and cycloalkanes ranged between C10-C22 are less biodegradable than their n-alkane and an aromatic analog, branching creates tertiary and quaternary carbon atoms that constitute a hindrance to β-oxidation. The biodegradation of cyclo-alkanes synergistic cooperation of two or more microbial species and also, cyclo-alkanes of C10 and below has high toxicity (Meulenberg, *et al*., 1997).

1.2.2 Environmental condition for hydrocarbon biodegradation

A number of environmental factors affecting the biodegradation process can be summarized as follows:

- Source of energy, which is the primary factor limiting biodegradation, is the scarcity or absence of a suitable and available source of energy. Whenever available energy material is an abundant, microbes capable of using that material are usually abundant (Clark, *et al*., 1979).
- pH, in general the optimum pH for microbial biodegradation is slightly above 7 (Clark, *et al*., 1979) .

- Temperature, that has a marked effect on the rate of hydrocarbon degradation. Hydrocarbon degradation was faster at 25°C than 5°C (Atlas, 1981).
- Nutrients, that where at least 11 essential macronutrient elements must be present in proper amounts, forms and ratios to sustain the growth of microorganisms .These include nitrogen, phosphorus, sodium, sulfur and iron. therefore the availability of these elements should be optimized to achieve maximum degradation rates (Ribbons and Eaton 1982).

In addition to above factors, there are also some factors that may affect the biodegradation rate includes the moisture rate in contaminated soil, the exhaustion of dissolved oxygen, salinity and pressure (Atlas and Bartha , 1998b).

1.3 Microbial biodegradation of hydrocarbons

Several uptake mechanisms have been proposed for the biodegradation of hydrocarbons as a sole source of carbon and energy by microbial cells. These mechanisms can be classified according to the physical state of a hydrocarbon and the type of hydrocarbon degradative organism to:

- Uptake Dissolved Hydrocarbon Mechanism.
- Direct Contact Mechanism.
- Emulsification Mechanism.

The pattern of growth on liquid hydrocarbons are substantially different from those on hydrophilic substrates and are a reflection of the microbial process of uptake and transport of hydrocarbons to intercellular sites for degradation (Britton , 1995) .

Other factor affecting microbial biodegradation of hydrocarbons is biosurfactant, which it was a specialized class of compounds that are produced

by bacteria, yeast, and fungi as extra – cellular or membrane – associated surface-active compounds (Desai and Banat, 1997).

The term biosurfactant refers to any type of compounds produced by microorganisms with surface active or emulsifying properties (Fiechter, 1992).

These can be divided into low - molecular – weight molecules that lower surface and interfacial tensions efficiently and high – molecular – weight polymers that bind tightly to surface (Rosenberg and Ron, 2001).

1.4 Mechanisms for microbial oxidation of hydrocarbons

The biodegradation of hydrocarbons as a sole source of carbon and energy is attempted by a series of catabolic reactions that are catalyzed by a group of enzymes yielding in the end of the reaction energy, CO_2 and H_2O. Hydrocarbon – degradative organisms differ in their ability to oxidize different types of hydrocarbons and as follows:

1.4.1 Oxidation of aliphatic hydrocarbons

Crude petroleum is the main source of saturated and unsaturated aliphatic hydrocarbons, which are the most rapidly degraded in both laboratory cultures and in the environment (Al-Hadrami, *et al*., 1997).

a) **Monoterminal oxidation:** The initial attack on alkanes occurs by monooxygenase enzymes. These enzymes were directed at the terminal methyl group , forming a primary alcohol that in turn , is further oxidized to an aldehydefatty acid . Once a fatty acid is formed , further catabolism occur by β-oxidation sequence yielding acetyl-CoA units which are converted to CO_2 through the tricarboxylic acid cycle , so the end products of hydrocarbon mineralization are H_2O and CO_2 as it was shown in figure (1-1).

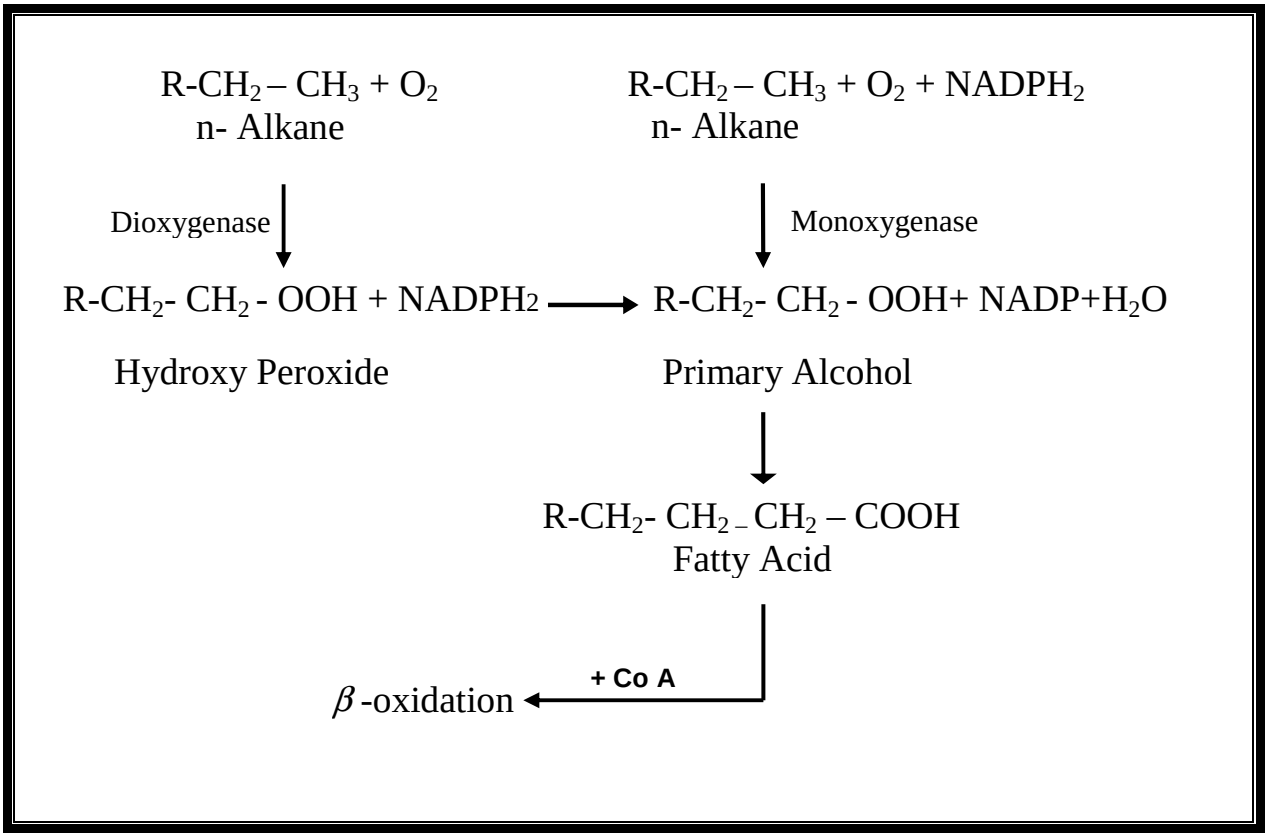

Figure (1-1): Microbial Monoterminal Oxidation of n-Alkane Compounds (Atlas and Bratha, 1998a)**.**

b) **Subterminral oxidation:** Some microorganisms attack alkanes sub terminally; that is, oxygen is inserted on a carbon atom within the chain instead of its end. In this manner, a secondary alcohol is formed first, which is then further oxidized to a ketone and finally to an ester. The ester bond is cleaved, yielding a primary alcohol and a fatty acid. The first fragment (alcohol) is oxidized through the aldehyde to a fatty acid, and with the second fragment (fatty acid), is metabolized further by β-oxidation sequence as it was shown in figure(1-2).

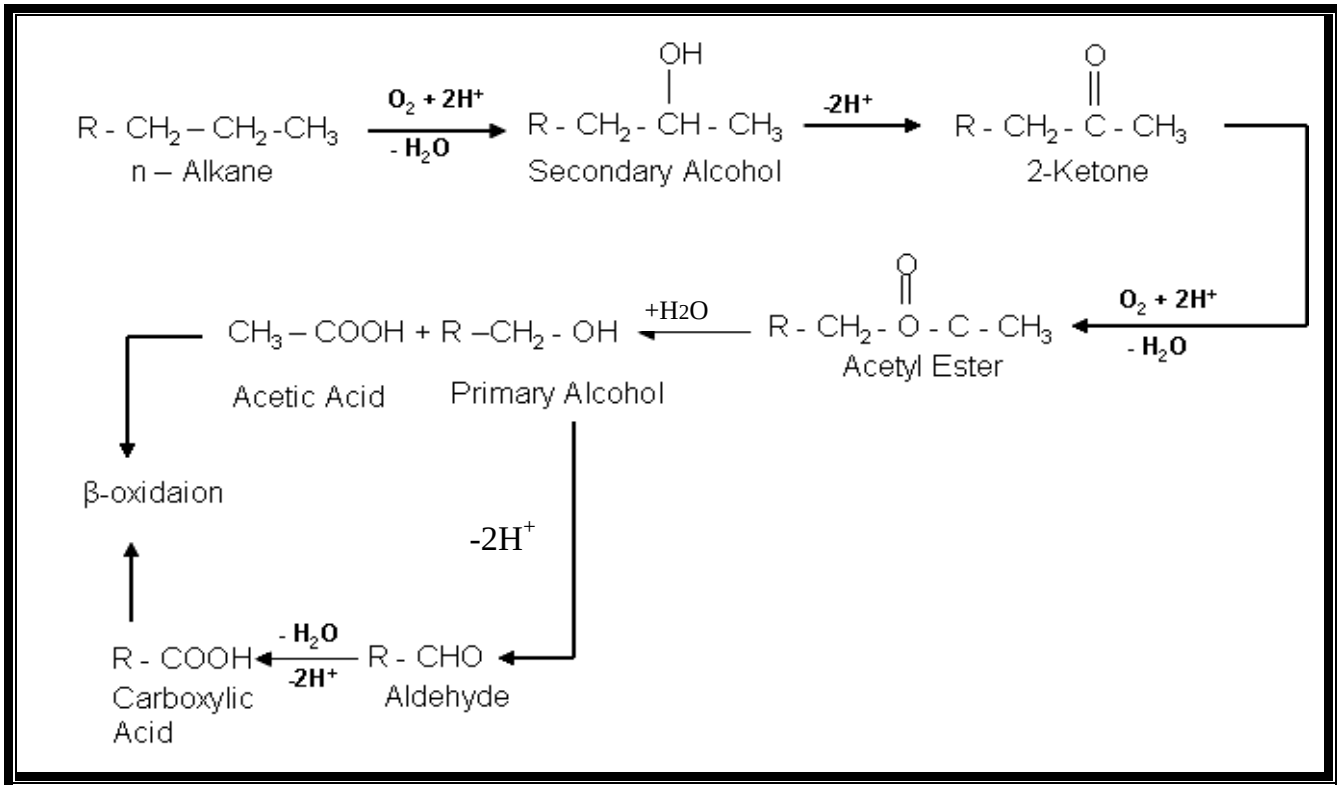

Figure (1-2): Microbial Subterminal Oxidation of n-Alkane Compounds (Atlas and Bratha, 1998a)**.**

1.4.2 Oxidation of alicyclic hydrocarbons

Alicyclic hydrocarbons are widely distributed in the biosphere, and are potential for environmental pollution. Alicyclic hydrocarbons are more resistant to biodegradation than other groups of hydrocarbons and more toxic to microbial cells, but have a high degree of volatilization that aids in their removal Alicyclic compounds having no terminal methyl groups are biodegraded by a mechanism similar to the subterminal oxidation. Hydroxylation by monoxygenase leads to an alicyclic alcohol. Dehydrogenation leads to the ketone .The hydroxyl group is oxidized in sequence, to an aldehyde and carboxyl group. The resulting dicarboxylic is further metabolized by β-oxidation as it was shown in figure (1-3).

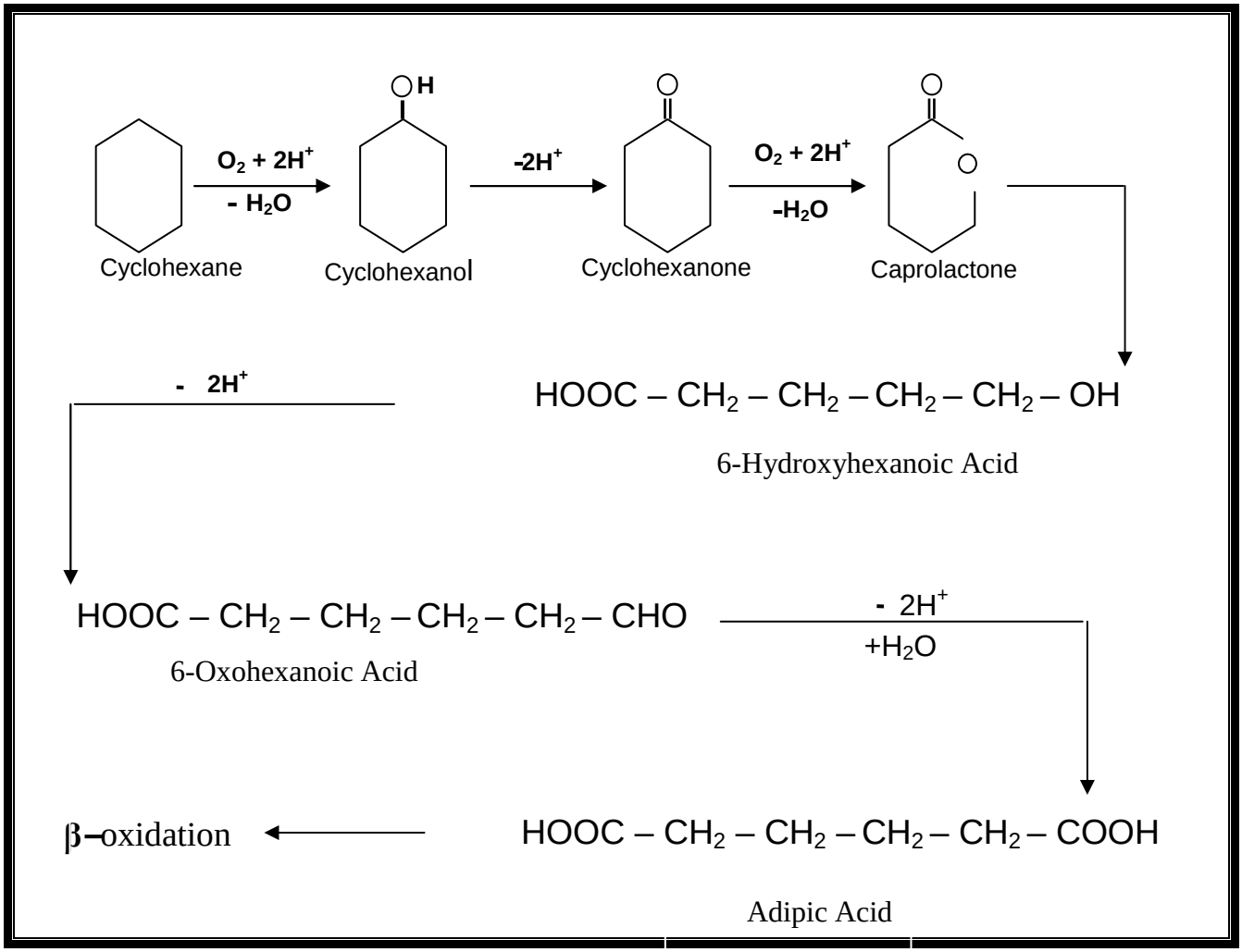

Figure (1-3): Microbial Oxidation of Alicyclic Hydrocarbons (Trudgill, 1984a)**.**

1.4.3 Oxidation of monoaromatics hydrocarbons

Monoaromatic compounds like toluene , benzene and phenol are less or never volatilization than cyclohexane these molecules are produced in huge amounts and are used in fuels , as solvents , and as starting materials for the production of plastics , synthetic fibers and pesticides (Button, 1991) .

The biodegradation mechanism of toluene by *P. putida* has been studied extensively, in this mechanism toluene is first oxidized to cis-toulene dihydrodiol through the action of toluene dioxygenase (TDO) and

dehydrogenated to form 3–methylcatechol , which is cleaved at the meta position and then converted in three steps to acetaldehyde and pyruvate. Benzene also metabolized by *P.putida* by benzene – hydroxylating enzymes(these enzymes is very similar to TDO enzyme) ,first by addition of two atoms of oxygen to form cis – benzene dihydrodiol, followed by converting dehydrodiol to catechol and further transformation to TCA cycle intermediates(Zylstra and Gibson , 1989).

Toluene dioxygenase has also been identified as the enzyme responsible for the initial step in the metabolism of phenol by *P.putida*. TDO catalyzes the monohydroxylation of phenol and benzene to catechol, as it was shown in figure (1-4).

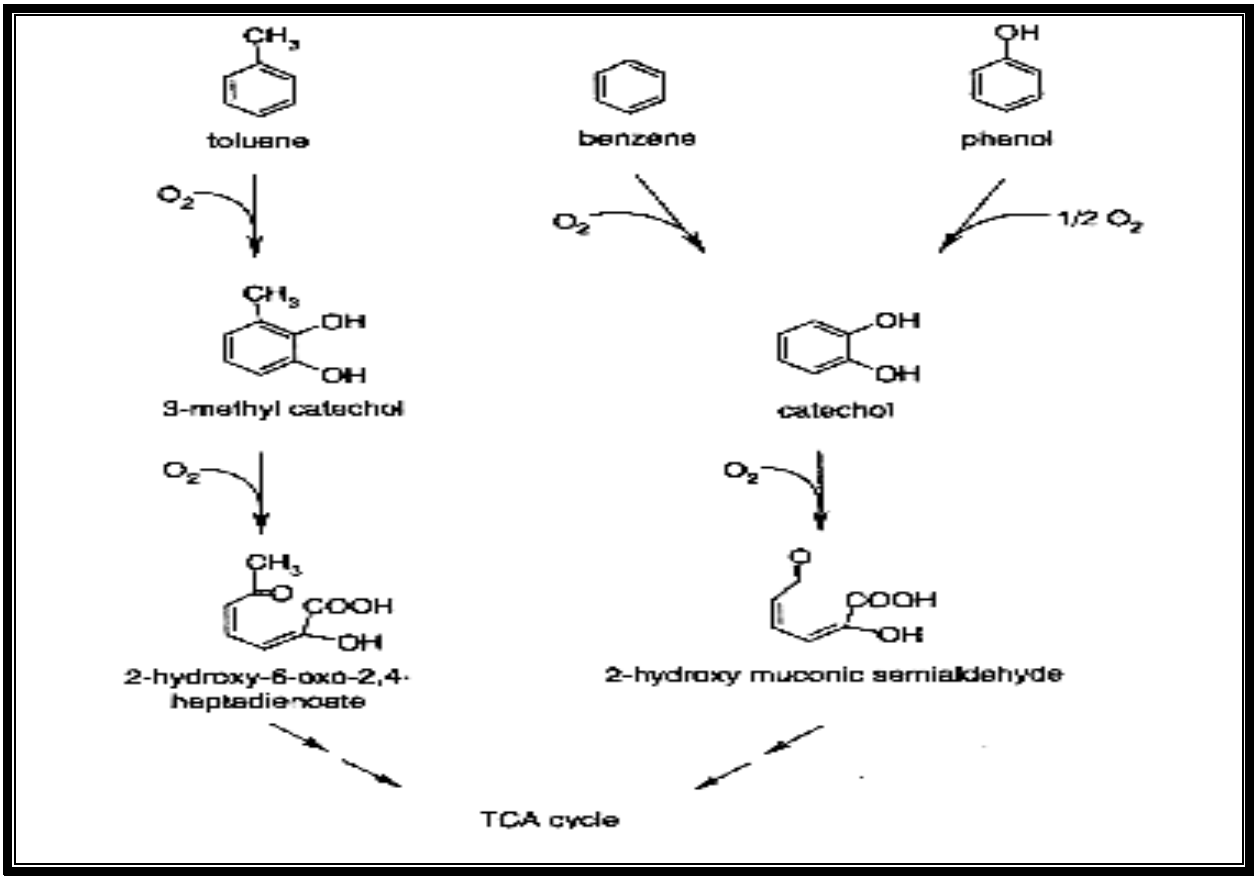

Figure (1-4): Microbial Oxidation of Monoaromatic Compounds (Lau, *et al*., 1994)**.**

1.4.4 Oxidation of furans

Furans are most frequently encountered in the plant kingdom and are used widely in industry. The microbial degradation of the furan ring received relatively little attention , and the knowledge of ring cleavage systems is confined to simple structures . Briefly , the cleavage of furan ring involves the oxidation of the ring by monoxygenase , followed by dehydrogenation to form the lactone , and cleave by an aliesterase, as it was shown in figure (1-5).

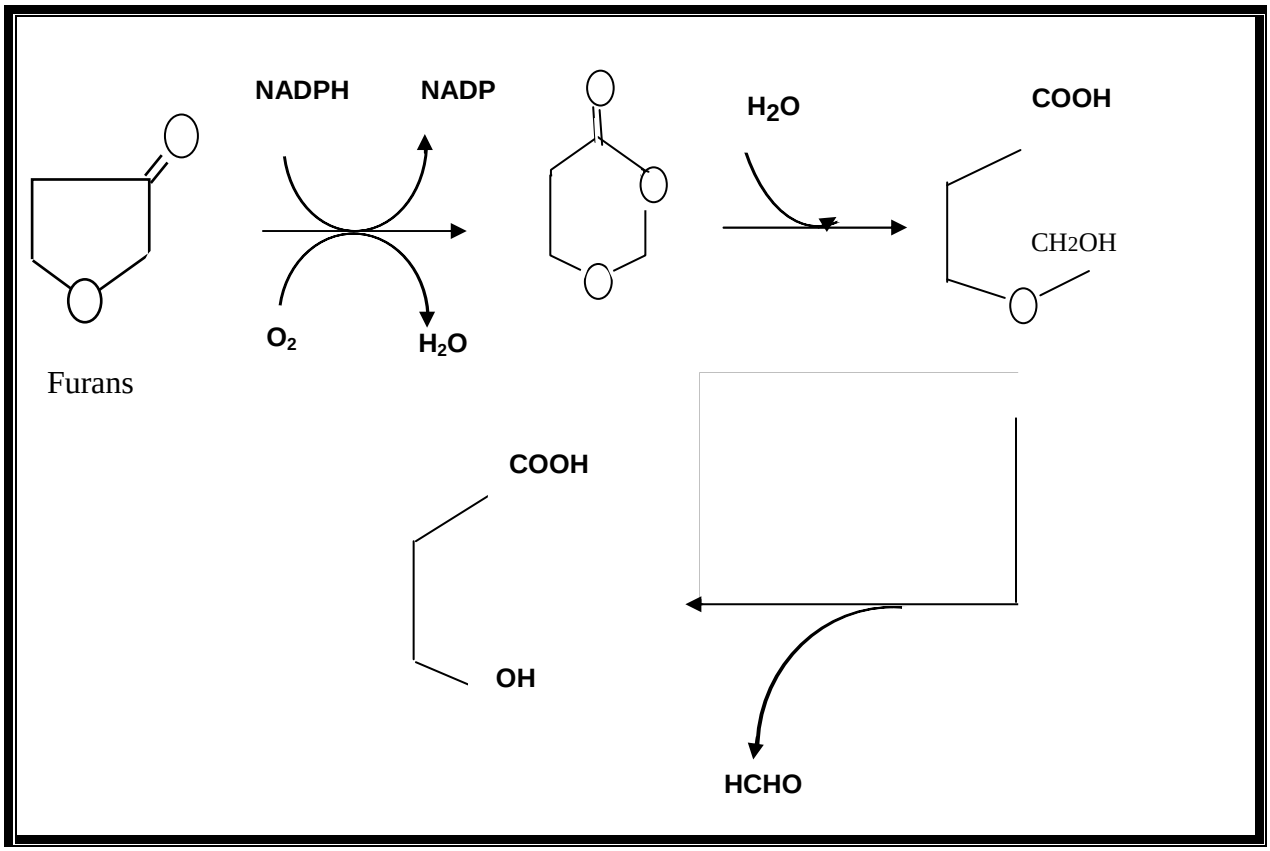

Figure (1-5): Microbial Oxidation of Furans (Trudgill, 1984b)**.**

1.4.5 Oxidation of polyaromatic hydrocarbons

The oxidation of polyaromatic hydrocarbons is more complicated than aliphatic compounds, because it requires energy to cleave the aromatic ring , as well as enzymes that participate in aromatic hydrocarbon oxidation differing from those of aliphatic hydrocarbon oxidation . The aromatic ring is opened by oxidative ortho cleavage to yield catechol and muconic acid. Muconic acid is

cleaved to yield acetyl-CoA and succinic acid. The catechol ring may be opened by meta cleavage yielding pyruvic acid and acetaldehyde as it was shown in figure (1-6).

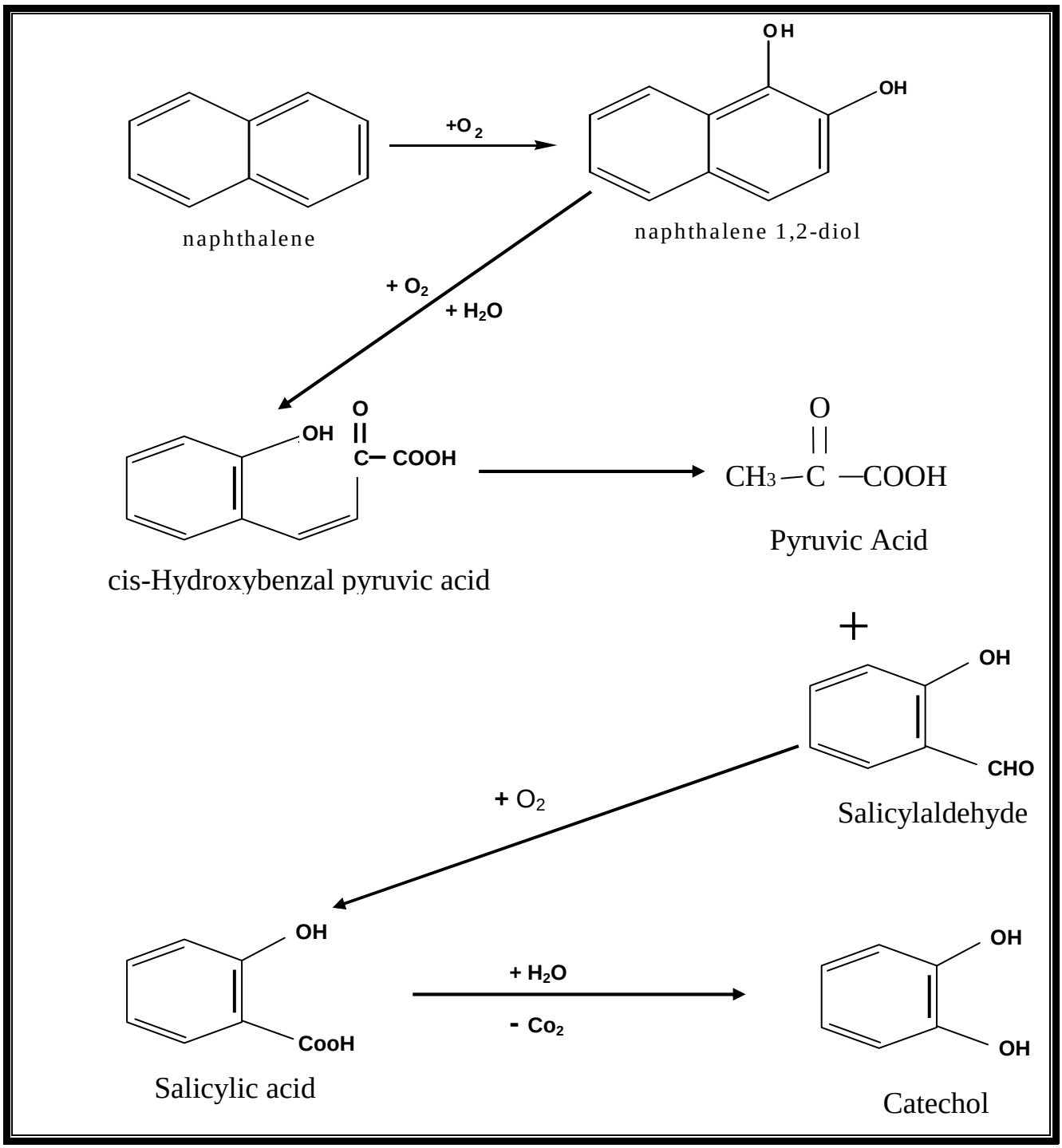

Figure (1-6): Microbial oxidation of Polyaromatic Hydrocarbons (Gibson and Subramanian, 1984)**.**

1.5 Microbial resistance for heavy metals and antibiotics

1.5.1 Microbial resistance to heavy metals

Resistance to heavy metals could be defined as the ability of certain microorganisms to grow in the presence of given heavy metal; while tolerance, is better described as survival manifested by slow growth and reduced metabolic activity (Doleman, 1986).

Heavy metal ions and metalloids are chemical compounds that can be very toxic to *P. putida* and other microbial cells in a variety of ways, e.g. through binding to essential respiratory chain proteins, oxidative damage via the production of reactive oxygen species, DNA damage, etc., it can be anticipated that soil organisms are likely to bear systems to cope with toxic metals in their environment. Although excess of metals is generally toxic, some of them are essential to life in trace amounts (cu^{2+}, Mn^{2+}, Zn^{2+}**,** and so on). Cells need to maintain certain cytoplasmic concentrations of these metals if they are to meet physiological requirements. To this end, microorganisms uses a number of mechanisms to maintain the correct equilibrium including the uptake, chelation and extrusion of metals (Rouch, *et al*., 1995).

Many systems have already been identified in bacteria that involve metallothioneins, P-type ATPases, Cation / proton antiporters and redox enzymes. Some systems, such as that encoded by the arsenic / antimony detoxification *ars*-genes, is found in *P. putida* .However; other has only been identified in certain types of bacteria (Bowen, 1979).

1.5.1.1 Mechanisms of microbial resistance to heavy metals

There are two mechanisms by which microorganism might exhibit resistance to heavy metals:

- **Non-specific resistance :**

This mechanism depends on, physiological characteristic of the microorganism, such as production of H_2S or organic acid (ex. citric acid), production of gelatinous matrix (ex. capsule). These substances reduce the effective metal concentration extracellular by either precipitation or chelation. So the resistance to the mercury in this mechanism, for many bacteria was found that microorganism produce H_2S, which inactivate and precipitate mercury as in insoluble from (HgS), or the transformation of Hg^{2+} to the volatile methyl mercury, which diffuse out the cell and growth medium as in (insoluble mercuric sulphides); ultimately reducing the bioavailability of the element and cause release of toxicity (Wood, 1984).

- **Inheritable specific type resistance :**

This mechanism controlled by determined genes carried on plasmid or transposons. This type of resistance noted in *Alcaligenes* spp., *Bacillus* spp., *Escherichia coli*, *Klebsiella* spp., *Staphylococcu* spp. and *pseudomonas* spp. In the inheritable bacterial mercury type resistance is pocess cluster of genes called *mer* operon which induced transcription and then translation to produced mercuric reductase or mercurial lyase, these enzymes can transform inorganic mercury and a few number of organomercurials (e.g. fluoresain meruic, acetate and merbromin) to Hg^0 (metallic mercury), through process called enzymetic detoxification of the metal ion to a volatile form; enzymes enter in this process called mercuric reductase (. Robinson and Tonvinen, 1984).

Microorganism shared this process can break the [Hg – C] bond in other organomercurials include (methyl mercury , phenyl mercury , p-hydroxy

mercuribenzoate and thimerosal) by the organomercurial lyase enzyme producing inorganic Hg^{2+}, which in turn reduced to Hg^{0} by mercuric reductase (Summers, 1986b) .

1.5.2 Microbial resistance to antibiotics

With increasing usage of antibiotics for treatment of diseases and in feeding and drinking water for improvement animal production, there was a respective increase in antibiotic resistant bacteria are also found , however , in environments where antibiotics are not used . Antibiotic resistance is determined by genes located on plasmid and / or chromosomes (Kelch and lee, 1978).

Chromosomal resistance could be developed as a result of spontaneous mutation in a locus that controls susceptibility to a given antibiotic. The presence of antibiotic serves as a selecting mechanism to suppress susceptible organisms and favor the growth of drug – resistant mutants. Other genetic origins are plasmids which carrying R-genes, that are conferring resistance to one or more – antimicrobial drugs and heavy metals. These genes often control the formation of enzymes capable of destroying the antimicrobial drugs. Spread of antibiotic resistance for *P.aeruginosa* through conjugation and transduction are particularly much more important than the transformation. Consequently, *Pseudomonas* spp. are resistant to a number of antibacterial agents and the mechanisms of antibiotic resistance determined by plasmids in *Pseudomonas*, generally found similar to those in enteric bacteria (Foster, 1983; Livermore, 1995).

R-factors in some cases may be non–transmissible, but they may mobilized by other plasmid which are transmissible but not carrying resistance gene (Chakrabarty, 1984).

1.5.2.1 Mechanisms of microbial resistance to antibiotics

Different mechanism by which microorganisms might exhibit resistance to antibiotics have been characterized according to their biological action:

a) Change cell membrane permeability to drug(s), like tetracycline, aminoglycoside and some chloramphenicol resistant bacteria (Huncock, 1985).

b) Producing enzymes that destroy the active drug, like *staphylococci* resistant to penicillin producing a β-lactamase wkich destroying the drug. Gram negative bacteria may be resistant to chloramphenicol acetyltransferase (Livermore, 1995).

c) Developing an altered structural target for drug like , chromosomal resistance to aminoglycoside is associated with the loss or alteration of specific protein on the 30S subunit of the bacterial ribosome that serves as binding site in susceptible organisms (Jawetz, *et al.*, 1976).

d) Developing an altered metabolic pathway that passing the reaction inhibited by the drug likes some sulfonamide – resistant bacteria which not require extracellular p-aminobenzoic acid (Brooks, *et al*., 1995).

e) Developing an altered enzyme that can still perform its metabolic function that is much less affected by drug than the enzyme in the susceptible organism (Smith and Amyes, 1984).

1.6 Genetics of *Pseudomonas putida*

1.6.1 Role of plasmids in hydrocarbon biodegradation

Plasmids are extrachromosomal elements that replicate independently and regulate their own replication. Plasmids play a significant role in bacterial adaptation and evolution; they provide gene products that can benefit the bacterium under certain conditions (Summers, 1986a).

Degradative plasmids represent a group of naturally occurring plasmids that have been distributed in a wide range of bacterial species (Farrell and Chakrabarty, 1976).

Almost all of the plasmids that have genes for hydrocarbons and xenobiotic catabolism are from gram negative bacteria, predominantly *pseudomonas* species, as described in table (1-1).

Table (1-1): Hydrocarbon degradative plasmids in different species of *Pseudomonas* (Lindow and Panopoulos,1989)

ORIGINAL HOST	PLASMID	HYDROCARBON	MOLECULAR WEIGHT(KB)
P. putida	SAL(T)	Salicylate	85
P.putida	TOL(T)	Toluene	117
P. aeruginosa	PAC25(T)	Chlorobenzotate	117
P. putida	NAH(T)	Naphthalene	83
P. putida	OCT	Alcanes	>500
P.putida	CAM(T)	Camphor	>500
Pseudomonas spp.	pQ(T)	Quinoline	320
P. aeruginosa	pQM	Methylquinoloine	225
P.aeruginosa	pKF	Chloorobiphenyls	81

T: Transmissible Plasmid.

The degradative plasmids govern the metabolism of a diverse group of aliphatic compounds (octane , decane , etc) , aromatic and polynulclear aromatic hydrocarbons (xylene , toluene , anthracene , etc) , products of their oxidative metabolism (salicylate , benzoate) , terpenes , alkaloids and chlorinated hydrocarbons . These plasmids may encode a complete degradative pathway, or partial degradative steps such as naphthalen to salicylate.The genes for the degradation of such hydrocarbons can be borne on either chromosomes or plasmids, or compound nature. For example: toluene is metabolized by some *Pseudomonas* spp. through formation of benzyl alcohol and toluic acid, such genes are known to be plasmid coded (Farrell and Charkrabrty, 1979).

Farrell and Charkrabrty (1979) have observed that TOL and NAH plasmid show an extensive homology with the antibiotic resistance plasmids R2 and PMG18 , 20% and 14% respectively .

As antibiotic resistance plasmids, some degradative plasmids like TOL-plasmid show the ability to dissociate into two plasmids , one nonconjugative (42Kb) and the other is conjugative (72Kb) called TOLΔ. This appears when TOL-plasmid was transferred by conjugation between *P.putida* and *P.aeruginosa* . TOLΔ plasmid has been hybridized with RP4 plasmid (Tetracycline resistance plasmid), and as a result the trait of tetracycline resistance has been lost, indicating that a join occur in the site of tetracycline resistance gene (Rams-G, *et al.*, 1994).

On the other hand, there are plasmids that encode a complete enzymatic pathway for hydrocarbon degradation for example: OCT-plasmid encode proteins involved in the conversion of n-alkanes to fatty acid. Some degradative plasmids like SAL , TOL and OCT are characterized by their ability to dissociate into two or more plasmids (Van Beilen, *et al.* , 1994) .

The plasmid PGE47 , enable *P.putida* to grown on alkane , when this plasmid was transferred to *E.coli* , the *alk* genes were expressed and regulated as in the original host , allowing the *E.coli* to grow well on octane . Other *Pseudomonas* degradative plasmid gene seemed to be expressed inefficiently in *E.coli*, because *E.coli* may not have gene required to complete the degradation pathway, which resulted in an accumulation of intermediates and because of their toxicity led to cell death (Chakrabarty, 1976).

1.6.2 Role of plasmids in heavy metal resistance

The genome of *P.putida* encodes an unexpected capacity to tolerate heavy metals and metalloids. The availability of the complete chromosomal sequence allowed categorization of 61 open reading frame (ORF) likely to involved in metal tolerance or homeostasis, plus seven more possibly involved in metal resistance mechanisms. Some systems appeared to be duplicated, these might perform redundant function or be involved in tolerance to different metals. Some of the metal related clusters located in gene islands with atypical genome signatures. The predicated capacity of *P.putida* to endure exposure to heavy metals is discussed from an evolutionary perspective (Furukawa and Tonomura 1973).

Studies on chromosome of *P.putida* have depend heavily on conjugation, which in turn depends in most instances on the chromosome donor ability, or

chromosome mobilizing activity of some plasmids. Plasmids conferring heavy metal and antibiotic resistances are shown in table (1-2).

Table (1-2): Conjugative plasmids of *Pseudomonas* spp. carrying Heavy metal and antibiotic resistance genes* (Rouch, 1995)**.**

Plasmids	Resistance	
	Antibiotics	Heavy Metals
R902	Am, Sm, Su	Hg
R26	Cb, Tc, Nm, Km, Sm, Su, Gm	Hg , Cd
R931	Sm, Tc,	Hg
R527	Cb, Tc, Nm, Km, Sm, Su, Gm	Hg
FP_2	—	Hg
MER	—	Hg
R3108	Sm, Su, Tc	Hg
PMG1	Gm, Sm, Su	Hg
PMG2	Gm, Sm, Su	Hg
PMG5	Tm, Su, Km	Hg
RP111	Cb, Sm, Tc, Gm, Su	Hg
PT12.2	Rif, Cam	Cu
PT23.3	Rif, Cam	Cu

* Cb, Carbenicillin; Tc, Tetracyline; Km, Kanamycin; Su, Sulfonamides; Tm, Tobramycin; Gm, Gentamicin; Sm, Streptomicin; Nm, Neomycin, Cam, Chloramphenicol; Am, Ampicillin; Rif, Rifampicin, Cu, Copper; Hg, mercury; Cd, Cadmium.

1.6.3 Relationship between heavy metal and antibiotic resistance

The relationship between heavy metal and antibiotic resistance plasmid in recent years, increasing attention has been directed towards linkage of antibiotic and heavy metal resistance. Environment exposed to heavy metal contamination often have, high incidence of antibiotic- resistant bacteria. Until the – 1960s little was known about the mechanisms of these resistance, and nothing was known about their genetic bases (Summers, 1986a).

Many studies referred that with the wide incidence of heavy metal contamination, a development in microbial resistance to antibiotic occurred, with the popular use of silver compounds for treatments of burns, it has lead to an increase in antibiotic and heavy metal resistant bacteria in hospital environment (McHumgh, *et al*, 1975).

P. putida isolated from infected plants and soil samples was found resistant to several antibiotic and heavy metals, which indicate the risky relationship between both resistance in metal – contaminated environment, that cause distribution for resistance factors for antibiotic and heavy metals (Nakahara , *et al*, 1977; Bhattacherjee, *et al,* 1988).

1.7 Curing Of Plasmid DNA

In nature, plasmid can be lost spontaneously from a very few bacterial cells, but the probability of this loss is extremely low, ranging from 10^{-5} to 10^{-7}. However, the majority of plasmids are extremely stable, and require the use of curing agents or other procedures that might increase the plasmid loss, and these form the basis of artificial plasmid elimination (Trevors, 1986).

Elimination of antibiotic resistance at high frequency is of interest to assert extra chromosomal location of genetic determinants, and obtaining a plasmid-cured derivative will allow a direct compression to be made between the plasmid

– containing and plasmid – cured cells. As a result of earlier studies it was already known that acridine orange, ethidium bromide and sodium dodecyl sulphate affect plasmid replication.Some physical factors also affects plasmid replication like elevated growth temperature and thymine starvation (Button, 1991).

Antibiotics like rifampicin, chloramphenicol and mitomycin C also have a moderate effect on plasmid replication. Some tricyclic compounds like bromethiazine and impramine were shown to have antiplasmide activity (Scott, *et al*., 1999).

Acridine have been used extensively to eliminate various extra chromosomal genetic elements such as sex factors, and R factors from bacterial cells. The efficiency of curing generally various from less than 0.1% to more than 99% depending upon the element involved the bacterial strain, and the mode of action of the curing agent. It is generally assumed that curing activity is related to the ability of these compounds to intercalate into superspiralized DNA and to inhibit its replication. Like the acridine, ethidium bromide was intercalated between base pairs of DNA and inhibits DNA – polymerase and RNA – polymerase. At the same time, they allow the replication of bacterial chromosome and the multiplication of bacteria, without the multiplication of plasmid, which is sensitive to intercalation (Meulenberg, *et al.*, 1997).

Sodium Dodecyl Sulfate as a detergent is known to act on the bacterial membrane. Snyder and Champness (1997), postulated that SDS might gain access to the membrane via the pili, which the plasmids are attached to membrane close to the pili and may thus be damaged.

Aislabie, *et al*. (1990) found that exposure to SDS lead to selection of clones completely resistant to pilus– specific phages and they concluded that non – susceptibility to SDS is strongly correlated with failure to produce pili.

The usefulness of curing agents is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids (Trevors, 1998).

2 . MATERIALS AND METHODS

2.1 Materials

The following equipments and apparatus were used in this study:

Equipments	Company(origin)
Autoclave	Express(west-Germany)
Balance	Ohans(France)
Compound Light Microscope	Olympus(Japan)
Centrifuge	Hermal Z200A(Germany)
Disstillator	GFL(Germany)
Oven	Memmert(Germany)
Minielectrophoresis apparatus	Bio Rad(Italy)
Eppendrof Bench Centrifuge	Netherler and Hinz(GMBH) (Germany)
Hot plate with magnetic stirrer	Gallenkamp(England)
Incubator	Termaks(U.K)
Micropipette	Witey(Germany)
Millipore filter unit	Millipore corp(USA)
pH-meter	Mettler Toledo(U.K)
Cool centrifuge	Harrier(U.K)
Electronic balance	Delta Range(Switzerland)
Spectrophotometer	Aurora instruments Ltd. (England)
Shaker incubator	GFL(Germany)
Tensiometer	Karl kolb (Germany)
UV-transilluminator	Vilber lourmat(France)
Vortex	Buchi(Swissrain)
Water bath	GFL(Germany)

2.2: Chemicals

The following chemicals were used in this study:

Material	Company (origin)
Benzoate, Decan, glycin, naphtholbenzene, octane, rhamnose, sodiumtoluate, tetrahydrofurane and Xylene.	Analar – England
Anthracen, calcium nitrate $Ca(NO_3)_2$, chloroform, cyclohexane ,fructose,glucose, glycerol, iodine, hydrochloric acid (HCl), magnessium chloride ($MgCl_2$), magnessium sulphate ($MgSO_4$), naphthalene, peptone, potassium dihydrogen phosohate(KH_2PO_4), potassium hydroxide(KOH), potassium iodide (KI), salicylic acid, sodium chloride(NaCl), sodium benzoate, sodium hydroxide (NaOH), Starch, trans-1-2-diphenyl ethylene, tryptose and urea.	BDH – England
Agar, borate (H_3BO_3), gelatin, maltose and trehalose.	Biolife- Italy
Tetra methyl–p–phenyl–diamine dihydro- chloride.	Biomeriux – France
amine sulphate (NH_3SO_4),cadmium sulphate ($CdSO_4.2H_2O$),crystal violet, isopropanol, safranin, hydrogen peroxide, phenol naphthol, SDS,toludine blue, toluene and zinc sulphate ($ZnSO_4.7H_2O$).	Difco -USA
Agarose,ammonium chloride (NH_4Cl) copper sulphate hydride ($CuSO_4.7H_2O$), EDTA, ethidium bromide, iron sulphate hydride ($FeSO_4.7H_2O$), magnessium sulphate hydride ($MnSO_4.7H_2O$), mercury nitrate hydride [$Hg(NO_3).2H_2O$)] and tris (hydroxyl methyl) amino methane base (Tris –base).	Fluka – Switzerland
Boric acid, bromo phenol blue and bromo thymol blue.	Riedel- Dehaeny-Germany
Cetrimide, cobalt dinitrate hydride [$CO(NO_3)_2.6H_2O$)], disodium hydrogen phosphate (Na_2HPO_4) and Ethanol.	Merck- Germany
Calcium chloride hydride ($CaCl_2.2H_2O$) and Inositol.	Oxoid – England
DNse agar, magnessium sulphate hydride ($MgSO_4.7H_2O$), maltose, potassium sulphate(K_2SO_4) and tryptone.	Sigma –USA

2.3 Antibiotic

The following antibiotic discs were used in this study:

Antibiotic	Symbol	Conc.µg/ml	Company(origin)
Ampicillin	AMP	30 u	Oxoid
Carbencillin	by	25	AL – Razzi *
cefotaxime	CTX	15	Oxoid
Chloramphenicol	C	30	AL – Razzi
Ciprofloxacin	CF	5	AL – Razzi
Cloxacillin	CLX	٣٠	AL – Razzi
Erythromycin	E	20	AL – Razzi
Gentamicin	GM	١٠	AL – Razzi
Naldixic acid	NA	٣٠	AL – Razzi
Penicillin	P	١٠	AL – Razzi
Rifampicine	Rif	٥	Oxoid
Streptomycin	S	10	Oxoid
Tetracycline	TE	٣٠	Oxoid
Ticarcillin	TC	30	AL – Razzi
Vancomycin	VA	15	Oxoid

* AL- Razzi center for diagnostic kits production/Ministry of Industry (Iraq).

2.4 Enzymes

The following enzymes were used in this study:

Enzymes	Company(origin)
Lysozyme	BDH – England
Pronase	Difco -USA

2.5 Culture Media

The following media were used during this study:

- **Mineral Salt Medium I** (Zylstra and Gibson,1989):

This medium consists of:

Constituent	Concentration (g/100 ml)
K_2HPO_4	0.02
KH_2PO_4	0.05
$MgSO_4.\ 7H_2O$	0.02
NH_4Cl	0.2
$CaNO_3.4H_2O$	0.05
$FeSO_4.7H_2O$	0.03
$MnSO_4.4H_2O$	0.006
H_3BO_3	0.005
$ZnSO_4.7H_2O$	0.01
$CuSO_4.5H_2O$	0.006
$CO(NO_3)_2.6H_2O$	0.006

The ingredients were added to 800 ml distilled water, potential hydrogen ion was adjusted to pH 7, then volume was completed to 1000 ml and sterilized by autoclaving, then 1% (V/V) of liquid hydrocarbon sterilized by filtration was added. The solid hydrocarbons sterilized by autoclaving for 5min, after that were added at a concentration of 1 %(W/V).

- **Mineral Salt Medium II** (Higham, *et al*., 1985)**:**

This medium consists of:

Constituent	Concentration (g/100 ml)
Glucose	0.01
NH_4Cl	0.03
Na_2PO_4	0.06
Mineral salt solution	0.6 ml

All ingredients were dissolved in 800 ml distilled water, potential hydrogen was adjusted to pH 7 , then volume was completed to 1000 ml and sterilized by autoclaving, after cooling 10 % of glucose sterilized by filtration was added.

- **Mineral salt solution:**

This solution prepared to be consists of:

Constituent	Concentration (g/100 ml)
$CaCl_2.2H_2O$	0.01
$Mg\ SO_4.7H_2O$	0.01
$MnSO_4.7H_2O$	0.007
$FeSO_4.7H_2O$	0.004

All ingredients were dissolved in 800 ml distilled water, potential hydrogen was adjusted to pH 7, and then volume was completed to 1000 ml and sterilized by autoclaving.

- **Carbohydrates Fermentation Medium** (Colline and Lyne, 1987) **:**

This medium consists of:

Constituent	Concentration(g/100 ml)
Peptone	0.02
NaCl	0.015
K_2HPO_4	0.03
Agar	0.03
Bromthymol blue	0.003
Carbon source	0.01

All ingredients were dissolved in 800 ml distilled water, potential hydrogen was adjusted to pH 7.1 , then volume was completed to 1000 ml and sterilized by autoclaving, after cooling .1% of carbon source sterilized by filtration was added.

- **Luria – Bertani broth** (Maniatis, *et al.*, 1982)**:**

This medium consists of:

Constituent	Concentration (g/100 ml)
Tryptone	0.01
Yeast extract	0.05
NaCl	0.05
Glucose	0.01

All ingredients were dissolved in 800 ml distilled water, potential hydrogen was adjusted to pH 7, and then volume was completed to 1000 ml and sterilized by autoclaving.

- **Cetrimide agar medium** (Stolp and Gudkari, 1984)**:**

This medium consists of:

Constituent	Concentration (g/100 ml)
Peptone	0.020
$MgCl_2$	0.045
K_2SO_4	0.01
Cetrimide	0.03
Agar	0.15

All ingredients were dissolved in 800 distilled water, potential hydrogen was adjusted to pH 7, and then volume was completed to 1000 ml and sterilized by autoclaving.

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

This medium consists of:

Constituent	Concentration (g/100 ml)
Peptone	0.02
K_2SO_4	0.01
$MgCl_2$	0.014
Glycerol	0.01
Agar	0.15

All ingredients were dissolved in 800 distilled water, potential hydrogen was adjusted to pH 7, and then volume was completed to 1000 ml and sterilized by autoclaving.

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

This medium consists of:

Constituent	Concentration (g/100 ml)
Peptone	0.02
$MgSO_4.7H_2O$	0.35
K_2SO_4	0.15
Glycerol	0.01
Agar	0.15

All ingredients were dissolved in 800 ml distilled water, potential hydrogen was adjusted to pH 7, and then volume was completed to 1000 ml and sterilized by autoclaving.

- **Urea agar medium** (Christensens media) (Colline and Lyne, 1987)**:**

This medium was prepared by dissolving 24g of urea agar base in 950 ml distilled water, potential hydrogen was adjusted to pH 7, sterilized by autoclaving, then 50 ml of 40% (w/v) filter sterilized urea was added, and mixed gently.

- **Gelatin medium** (Stolp and GudKari, 1984)**:**

Gelatin 12% (W/V) was added to trypticase Soya broth and sterilized by autoclaving.

- **DNase Medium** (Collee, *et al.*,1996) **:**

This medium was prepared by dissolving 4.2g DNase of DNase medium and in addition to 0.01g of toludine blue in 100 ml of distilled water, potential hydrogen was adjusted to pH 7.2, then it was sterilized by autoclaving.

- **Other ready made media:**
  - Simmon citrate agar (Biomeriux – France).
  - Nutrient Broth and nutrient agar (Biolife- Italy**)**.
  - Muller – Hinton agar (Sigma –USA).
  - Tryptic Soy broth (BDH – England**)**.
  - Brain heart infusion agar medium (BDH – England**)**.

These media were prepared according to the instructions of manufacturer company.

2.6 Dyes

- **Gram's stain:** It was prepared according to the procedure described by Simbert and Krieg (1981).
- **Ethidium bromide:** Stock solution (10 mg/ml) of ethidium bromide was prepared by dissolving 100mg of ethidium bromide in 10ml D.W. and sterilized by filtration throughout millipore filter (0.45µm), then it was kept in a dark place (Maniatis, *et al*., 1982).

2.7 Solutions

2.7.1 Normal saline solution

It is prepared by dissolving 8.5g of sodium chloride in 950 of D.W., then volume was completed to 1000 ml in a volumetric flask.

2.7.2 Antibiotic solutions (Atlas, *et al*., 1995)

- **Carbencillin:**

Stock solution of carbencillin (25 mg/ml) was prepared in D.W., sterilized by filtration and stored at -20˚C.

- **Penicillin:**

Stock solution of penicillin (25 mg/ml) was prepared in water, sterilized by filtration and stored at -20˚C.

- **Erythromycin:**

Stock solution of erythromycin (10 mg/ml) was prepared in solution of alcohol/water (20% V/V), sterilized by filtration and stored at -20˚C.

- **Tetracycline:**

Stock solution of tetracycline (12.5 mg/ml) was prepared in solution of ethanol/water (50% V/V), sterilized by filtration and stored at -20˚C.

2.7.3 Heavy metal solutions (Higham, *et al*., 1985)

Stock solution of each of heavy metal {cadmium ($CdSO_4.2H_2O$), copper ($CuSO_4.7H_2O$), iron ($FeSO_4.7H_2O$), mercury [Hg (NO_3).$2H_2O$)] and zinc ($ZnSO_4.7H_2O$)} was prepared in a concentration of 5 mMolar, sterilized by filtration and kept at 4°C in dark place according to Higham, *et al*., (1985) .

2.8 Reagents

1) Catalase reagent (Atlas, *et al*., 1995):

This reagent was consist of 3% hydrogen peroxide solution.

2) Oxidase reagent (1%) (Koneman, *et al*., 1992):

This reagent was prepared by dissolving 1g of tetra methyl–p–phenyl–diaminedihydro- chloride in 100ml distled water in a volumetric flask.

2.9 Enzyme

1) Lysozyme:

Enzyme solution was prepared by dissolving 50mg Lysozyme in 1ml SET buffer and kept at 4°C till it was used.

2) Pronase:

This solution was prepared by dissolving 20mg of pronase in 1ml SET buffer and kept at 4°C till it was used.

2.10 Buffers

1) SET buffer:

This solution was prepared to be consist of 75mM NaCl and 25mM EDTA, pH was adjusted to 8.0, then mixed with 20mM Tris- HCl (pH 7.5) and sterilized by filtration.

2) Sodium Dodecyl Sulphate (SDS) solution:

This solution was prepared by dissolving 1g SDS in 10ml D.W., and kept at 55 °C till it was used.

3) Tris-EDTA (TE) buffer:

This solution was prepared to be consist of 1mM EDTA and 10mM Tris-HCl, potential hydrogen was adjusted to pH 8.0, sterilized by autoclaving and kept at 4°C till it was used.

4) Phosphate buffer:

This solution was prepared by mixing 196 ml of solution A, and 804 of solution B. Both solutions A and B were prepared as the following:

- Solution A, it was prepared by dissolving 9.0 g of potassium dihydrogen phosphate in 950 ml of distilled water, then volume was completed to1000ml in a volumetric flask.

- Solution B, it was prepared by dissolving 23.8g of disodium hydrogen phosphate in 950 ml of distilled water, then volume was completed to1000ml in a volumetric flask.

5) Tris- borate EDTA (TBE) buffer 5X:

This solution was prepared by dissolving 54g Tris- base, 27.5g boric acid, and dissolved in 900ml of D.W, then 20ml of 0.5M EDTA (pH=8) was added, and the volume was completed to1000ml in a volumetric flask.

6) Loading buffer:

This buffer solution was prepared to be consist of bromophenol blue (0.25%), and sucrose (40%) in D.W.

2.11 Methods

2.11.1 Sterilization methods (Colline and Lyne, 1987):

- **Autoclaving:** Normal saline solution and culture media were sterilized by autoclaving at 121 °C for 15 min. and 15 Ib/In, while solid hydrocarbons were sterilized by autoclaving for 5 min.
- **Filter sterilization:** Antibiotic solutions, heavy metal solutions, liquid hydrocarbon, carbon source, urea, ethidium bromide were sterilized throughout Millipore filter (0.45µm).
- **Oven sterilization:** Plates, tubes and pipettes were sterilized at 160°C for 1.5 hours.

2.11.2 Samples collection

Samples of oil-contaminated soils were collected from different locations in Baghdad governorate during the period of 1/9/2004 to 15/11/2004, in these locations the most contaminants were hydrocarbon compounds like crud oil, kerosene, engine oil, benzene and gasoline. Contaminated soil samples were puts in labeled nylon bags, and transferred to the laboratory to isolate *Pseudomonas putida*.

2.11.3 Soil sample preparations

Ten gram of each sample to 90ml D.W. in conical flasks, mixed vigorously, and let to stand for a few minutes. Serial dilutions were made (from 10^{-1} to 10^{-5}).

2.11.4 Isolation of *Pseudomonas* spp.

One hundred microliter of suitable dilution (2.9.3) was spreaded on nutrient agar and then transferred to cetramide agar plates, and incubated at 30°C for 48hrs, then different single colonies were transferred separately on King A and King B plates, to accurate differentiatiation of *pseudomonas* spp (Holt, *et al*., 1994a).

2.11.5 Identification of local isolates

Each of the local isolates grown on cetrimide, King A and King B plates, fluorescent under UV-transilluminator and non pigmented were subjected to the following tests:

A. Morphological, Cultural and Physiological Characteristics:-

- Morphological characteristic of colonies (Collee, *et al*., 1996).

Shape, color, viscous growth, size and edge of the colonies were studied on plates of Brain Heart infusion agar after incubation at 30°C for 48hr.

- Growth on cetrimid agar.

This test was achieved according to Green wood, *et al.*, (1997). Cetrimid agar was used as a selective medium for isolation of *pseudomonas* spp., 100 µl of fresh cultures of each isolate was spreaded on cetrimid agar plates and incubated at 30°C for 48hrs.

- Growth on King A.

This test was achieved according to Cruick Shank, *et al.*, (1975). 100 µl of fresh cultures of each isolate was streaked on King A medium and incubated at 30°C for 48hrs.to examine the isolates ability in pyocyanin pigment production.

- Growth on King B

This test was achieved according to Cruick Shank *et al.*, (1975). 100 µl of fresh cultures of each isolate was streaked on King B medium and incubated at 30°C for 48hrs. Then the plates were exposed to U.V.- light to examine the isolates ability in fluorescent pigment production.

- Gram's stain

This test was achieved according to Harely and Prescott (1996). Single colony of each isolate was transferred by a loop to a clean slide. Smeard and stained with crystal violet,then it was treated with iodine, decolorized with ethanol (95%), and counter stained with safranin, then they were examined under a light microscope for cell form, clustering and staining ability.

- Motility test.

Motility test was achieved according to hanging-drop procedure (Collee, *et al*., 1996), in which the motility of the test organism, their shape, approximate size, and general structure can be observed.

- Growth temperature.

  To examine the range of temperature for growth, Bacterial isolates were grown on brain heart infusion agar plates and incubated at 4°C, 25°C,37°C,40°C and 42°C for 24 hrs.

B. Biochemical Tests:

- Catalse Test (Atlas *et al*., 1995)

This test was achieved according to Maza, *et al.*, (1997).A single colony was placed onto a clean glass microscope slide with a sterile toothpick, then a drop of hydrogen peroxide (3%) was placed onto the colony. The production of gaseous bubbles indicates the presence of catalase.

- Oxidase test**.**

This test was achieved according to Maza, *et al.*, (1997). Filter paper was saturated with oxidase reagent, then touch of colony of each isolate to be tested was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates a positive result.

- Citrate utilization test**.**

This test was achieved according to Atlas *et al*., (1995). simmon citrate slants were inoculated with a single colony of each isolate separately, and incubated at 30°C for 18 hours. Appearances of blue color indicate a positive result.

- Gelatin hydrolysis test**.**

This test was achieved according to Harely and Prescott, (1996). Gelatin tubes were stabbed, with different local isolates spreadly and were incubated at 30°C for five days. Liquefaction of gelatin indicates a positive result.

- Urease test**.**

This test was achieved according to Atlas, *et al*., (1995). Christensen urea agar slants were inoculated with each local isolate spreadly, incubated at 30°C for 24- 48hrs.Appearances of pink color indicate a positive result.

- DNase test**.**

This test was achieved according to Collee, *et al.*, (1996). DNAse agar plates prepared as in (2.5) were inoculated with each isolate spreadly, and incubated at 30°C for 24-48 hrs. Developing of a rose pink zone around the area of the growth indicate a positive result.

- Carbohydrates Fermentation test.

This test was achieved according to Colline and Lyne, (1987). Tubes of Carbohydrates Fermentation medium prepared as in (2.5) were stapped with single colony of each isolate spreadly, incubated at 30°C for 72 hrs. Presence of yellow color indicates a positive result.

2.12 Maintenance of Bacterial isolates

Maintenance of bacterial strains were performed according to Maniatis, *et al.*, (1982) and Sambrook, *et al.*, (1989) as the following:

2- Short –term storage.

Bacterial isolates were maintained for few weeks on Luria – Bertani agar plates. The plates were tightly warped in Para-film and stored at 4°C.

3- Medium term storage.

Bacterial isolates were maintained for few months by stabbing nutrient agar in screw-capped tubes containing 5-8ml of nutrient agar medium and stored at 4°C.

4- Long –term storage.

Bacterial isolates can be stored for many years in medium containing 15% glycerol at low temperature without signification loss of viability. This was done by adding 0.15ml of sterilized glycerol to 0.85 ml of an exponential phase of each isolate in small screw-capped tubes and stored at -20°C.

2.13 Growth curve of *P.putida* H6 isolate

The selected *P.putida* H6 isolate was grown in Luria – Bertani broth at 30^{o}C in a shaker incubator (180 rpm) for 18 hrs, and then 500ml Erlenmyr flasks containing 100 ml of nutrient broth were inoculated with 1 ml (1%) of the above culture. Flasks were incubated in a shaker incubator (180 rpm) at 30^{o}C for 24 hrs. During this period, samples (2ml) were taken every two hours to measure the O.D. using spectrophotometer at wave length 600 nm (Rodrigues and Tait, 1983).

2.14 Inoculum's preparation

Fresh cultures of local isolates were grown in nutrient broth until mid exponential phase (O.D. =0.67), then 0.5 ml aliquots of culture media were taken to inoculate 50 ml fresh medium containing flasks.

2.15 The ability of locally isolated *Pseudomonas putida* in biosurfactant production

To examine the ability of local isolates of *P. putida* in biosurfactant production , 0.5 ml of different hydrocarbon sources (crud oil, Engine oil ,kerosene, Soya bean oil and sun flower oil) where added separately to conical flasks containing 49 ml of mineral salt medium-I, each flask was inoculated with 500 µl aliquots of the freshly prepared inoculum of each isolate of *P. putida* separately . then they were incubated in shaker incubater (180 rpm) at 30°C for 48 hours.

After incubation, supernatant was collected by centrifugation (4000rpm for 10 hrs) for surface tension measurement using tensiometer (DuNoüy). Before the measurement, tensiometer were calibrated with distilled water (72 mN m^{-1}), chloroform (27.1 mN m^{-1}), and glycerol (64 mN m^{-1}). Then the obtained reading of each sample was recorded at the moment in which surface – immersed ring

breaks away from the liquid surface. Surface tension readings were then recorded (Vater, *et al*., 2002**)**.

2.16 The ability of local isolates of *Pseudomonas putida* in hydrocarbon biodegradation

To examine the ability of local isolates of *Pseudomonas putida* in hydrocarbon biodegradation, 0.5 ml of suitable liquid hydrocarbon (cyclohexane, decane, naphthol, octan, phenol, tetrahydrofuran, toluene and xylene) or 0.5 gm of solid hydrocarbon (anthracene, naphthalene, naphthol benzene, sodium benzoate, sodium toluate, salicylic acid and trans-1,2-diphenylethylene) were added to 49 ml of mineral salt medium-I prepared as in point (2.5) in conical flask, then they were inoculated with 500 µl aliquots of the freshly prepared inoculum of each isolate separately [prepared as in point (2.12)].Then growth was observed after incubation at 30° C for 48 hours. (Zylstra and Gibson, 1989).

2.17 Heavy Metals Resistance

Different heavy metals (cadmium sulfate, copper sulfate, ferrous sulfate, mercuric nitrate and zinc sulfate) were used in a concentration of 5 mM. Then the minimum inhibitor concentration was determined for each heavy metal by inoculating 10 ml of Luria broth containing serial concentration of each heavy metal (5mM, 10mM, 15mM, 2omM and 25mM respectively) with 100 µl aliquots of the freshly prepared inoculum of each isolate of the separately (control positive was medium with inoculum, while control negative was medium with heavy metals). Then they were incubated in shaker incubater (180 rpm) at 30°C for 48hrs (Higham, *et al*., 1985). The minimum concentration of each heavy metal that inhibits growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

2.18 Antibiotic sensitivity

This test was done by spreading 0.1ml of mid exponential phase of *Pseudomonas putida* H6 on Muller-Hinton agar plates, then disks of different antibiotics were fixed on the plate surface, and they were incubated at room temperature for 1hour in order to let the medium to absorb the antibiotic. Plates were then incubated at 30°C for 18 hours. After that, inhibition zones appeared around each of the antibiotic disk was measured in millimeter (Atlas, *et al*., 1995).

2.19 Plasmid profile of *P.putida* H6

2.19.1 Extraction of plasmid DNA

DNA Extraction was done according to salting out procedure which described by Pospiech and Neuman (1995).

2.19.2 Bacterial culture preparation

Suspension of 0.1 ml cell was inoculated into 30ml LB-broth medium, and incubated in shaker incubater (180rpm) at 30°C for 24hrs.Then they were centrifuged for 10 minutes at 4000 rpm, washed twice and resuspended in 5ml SET buffer.

2.20.3 Procedure

1- One hundred microliter of freshly prepared Lysozyme solution was added to 5ml of cells suspended in SET buffer and incubated for 45 minutes at 37°C.

2- One hundred and forty microliter pronase solution was added and mixed by inversion then 600µl of 10% SDS was added, mixed thoroughly and incubated for 2hrs at 37°C with occasional inversion.

3- Two ml of 5M NaCl was added, mixed by inversion to precipitated chromosomal DNA.

4- Five ml of chloroform was added and mixed by inversion for 30 minutes at 20°C.

5- Tubes were then centrifuged for 15 minutes at 20°C (6000rpm).

6- Supernatant was transferred to fresh tube, then 0.6 volume of isopropanol was added and mixed by inversion, after 30 minutes DNA was spooled by sealed pasture pipette. The spooled DNA was rinsed by 5ml of 70% ethanol; air dried, and dissolves in 5 ml of TE buffer.

2.20.4 Agarose gel preparation

Agarose gel was prepared by dissolving 0.7 gm of agarose in 100 ml of TBE buffer 1x, and heated on hot plate till all agarose crystals were dissolved. After cooling to 50°C it was poured gently in the apparatus tray and cooled to 25°C. Then the tray containing agarose gel was transferred and immersed in apparatus tank containing TBE buffer solution.

2.20.5 Electrophoresis of extracted DNA samples

Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells in the gel. Generally, gels were run for 2-3 hrs at 5 volt/cm and the gel buffer added up to the level of horizontal gel surface.

Agarose gel was then stained by immersing in 0.5 µg/ml ethidium bromide for 30-45 minutes.

2.20.6 Identification of DNA bands

DNA bands were visualized by UV-illumination at 302 nm on an UV-transilluminator. Gels were destained in distilled water for 30-60 minutes to gel ride of background before photographs were taken.

2.21 Curing of plasmid DNA

Curing of plasmid DNA was achieved according to Trevors, (1986) and as follows:

1) Gradual concentrations of ethidium bromide (400, 500, 600, 700, 800, 900 and 1000) µg/ml were added separately to sterilized test tubes containing 5ml of Luria – Bertani broth separately.

2) Test tubes were then inoculated with 100µl of freshly prepared culture of *P.putida* H6 in a mid log phase, then tubes were incubated at 30°C for 24 hours. Growth density of different tubes were observed visually and compared with the control to determine the effect of curing agent on bacterial growth. The sub-lethal concentration for ethidium bromide was determined.

3) Serial dilution of growth culture containing ethidium bromide for sub-lethal concentration was taken and spreaded on nutrient agar plates, incubated overnight at 30°C to allow the growth of bacterial colonies. After incubation the resulted colonies were replica plated (using toothpick) on BHI agar plate (master plate) and BHI agar plates containing antibiotic to which the original isolate is resistance.

4) Plates were incubated at 30°C for 24hr. After that, colonies changed in its antibiotic sensitivity was detected and selected to examine plasmid curing by extraction of plasmid DNA according to procedure described in (2.17.3).

3. Results and Discussions

3.1 Isolation of *Pseudomonas putida*

Results in table (3-1) showed that there were seventy isolates were obtained from the total soil samples after spreading on nutrient agar plates. Among the total isolate (70 isolates), only ten isolates were able to grow on citramide agar plates, which gives an indicator about the presence of isolates belong to *Pseudomonas* spp. (Holt, *et al,* 1994b).

Table (3-1): Local isolates from different hydrocarbon contaminated soil samples in Baghdad governorate.

Source of sample	No. of samples	No. of isolates	Growth on Citramide agar
Kerosene Fuel Stations	2	7	1
Al-Duarah Oil Refinery	3	20	2
Fuel Stations	4	18	2
Industrial Area-1	3	13	2
AL-Jadiria (Al-Nahrain University Fields)	1	4	1
Oil Fuel Stations	1	3	1
Industrial Area-2	2	5	1
Total	16	70	10

In addition to ,60 isolates belong to other genera such as *Arthrobacte, Achromobacter, Bacillus, Micrococcus, corynebacterium, Acinetobacter, Nocardia, Alcaligenes, streptomyces, vibrio* and *Flavobacterium* were also obtained from contaminated soil samples and this results agreed with Nasir (2002) and Tuleva (2005).

3. 2 Identification

Local isolates that were able to grow on cirtrimde agar plates, which may be suspected to be belonged to *Pseudomonas* spp. were further identified according to morphological characteristics and biochemical tests. For the former, colonies of each isolate that were plated on BHI agar show different morphological characteristic of *Pseudomonas* spp. such as mucoidal growth, smooth with flat edges and elevated center, whitish or creamy in color.

Microscopical examination of each isolate showed they were all having single cell or in chains non-spore forming, gram negative and rode shape. Motility test of those local isolates using hanging drop method showed that they were all motile except two isolates (H4, H8) as it was shown in table (3-2).

Table (3-2):Morphological characteristics of local isolates of *Pseudomonas* spp grown on citramide agar at 30°C for 48hr.

Characteristic	Isolates									
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Colony color	creamy	white	white	creamy	white	white	white	creamy	white	creamy
Spore forming	-	-	-	-	-	-	-	-	-	-
Gram stain	-	-	-	-	-	-	-	-	-	-
Cell shape	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Motility	+	+	+	-	+	+	+	-	+	+

All these results are in accordance with that mentioned by Clause and Berkeley (1986).

3. 3 Growth on differential media

In order to identify *Pseudomonas putida*, isolates that have ability to grow on citramide were replated on differential media for *P.putida*, which they were king A and king B. king A medium give an indicator about the ability of the isolates to produce of water-soluble pyocianin pigment, while king B medium can identify the isolates that able to produce yellow/green fluorescent pigment after subjection to UV-light. Results in table (3-3) showed that only five isolates (H_2, H_3, H_5, H_6 and H_9) were pyocyanin-negative and fluorescent pigment-positive. From these results it can be concluded that those isolates may be related to *P. putida* as it was mentioned by Stanier, *et.al*. (1966), Champion, *et.al*.(1980) and Pallerony, (1984).

Table (3-3): Growth ability of locally isolated *Pseudomonas* spp. on differential medium at 30°C for 18 hrs.

Isolates	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
King A	-	-	-	+	-	-	-	+	-	+
King B	-	+	+	+	+	+	-	+	+	-

3.4 Biochemical Tests

Biochemical tests were done for the five isolates (H_2, H_3, H_5, H_6 and H_9) that have the features of *P.putida*. Table (3-4) showed that these isolates were given a positive result for catalase production, oxidase production, citrate hydrolysis, urease hydrolysis and DNAse production. In addition, these isolates had no ability to produce gelatinase production. Growth temperature ranged between 4°C to 40°C. these isolates were also able to ferment Benzoate, fructose, glucose, glycin and maltose as a carbon source. while they were unable to utilize inositol, rhamnose, starch and trehalose as a carbon source. In fact, these results were agreed with that recorded by Palleroni and Doudoroff (1972) and Holt (1994a).

3.5 Growth Curve

Growth characteristics of selected isolate *Pseudomonas putida* H6 was grown in Luria – Bertani broth at 30°C in shaker incubator. Results mentioned in figure (3-2) showed that lag phase take about 4 hours followed by

logarithmic phase (Log) which takes about 10 hours, in this phase optical density (cell number) increase in constant rate (cells number reaches approximately 12×10^5 cell/ml in the mid log phase). after about sixteen hours cells division slowed down and then they were entered stationary phase(cells number reach approximately 22×10^8 cell/ml) which characterized as a constant number of cells and low growth. This experiment was done to determine the extent of logarithmic (log) phase in order to be used as a basic information required in designing physiological and genetic experiments as well as for working with homogenous culture.

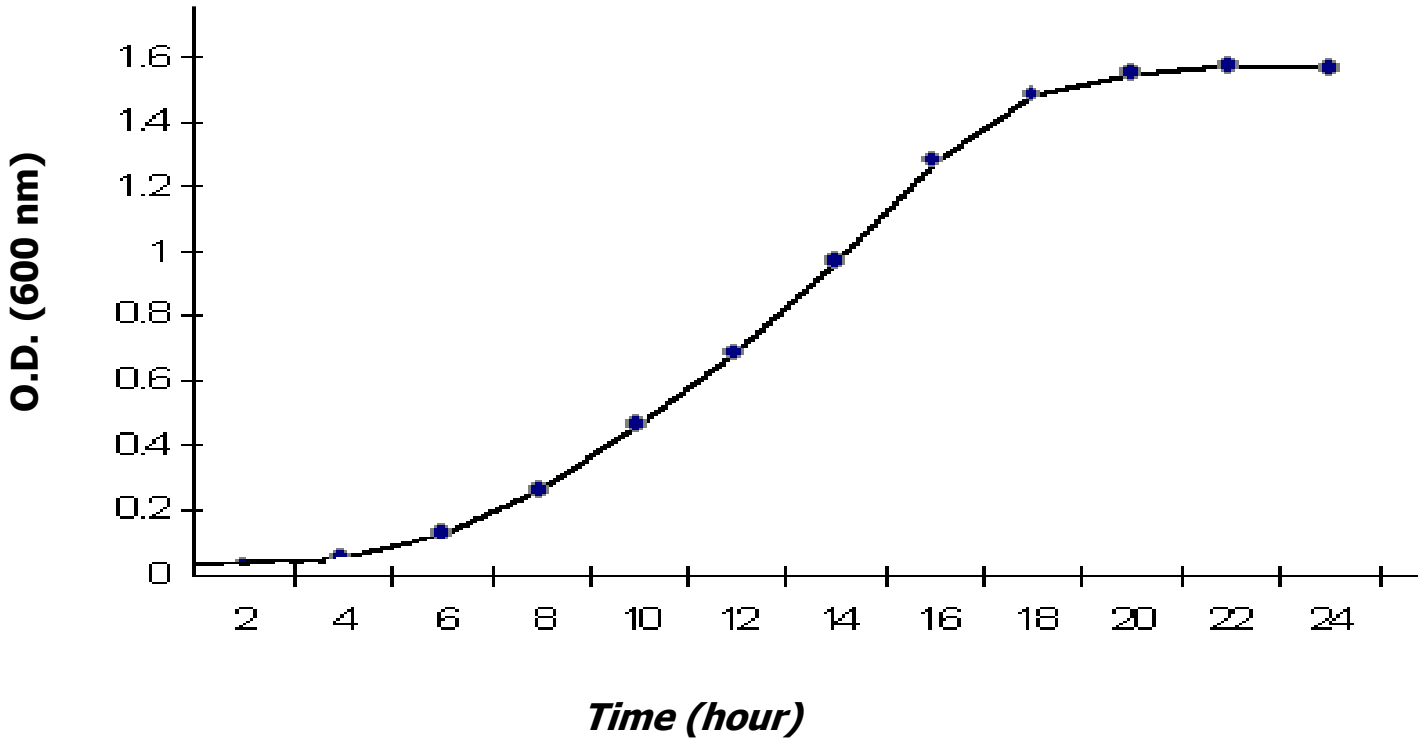

Figure (3-2): Growth Curve for *Pseudomonas putida* H6 grown in Luria–Bertani broth with shaking (180rpm) at 30˚C.

Table (3-4): Biochemical and physiological characteristic of *Pseudomonas putida* isolates.

Test	Isolates				
	H2	H3	H5	H6	H9
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Citrate hydrolysis	+	+	+	+	+
urease	+	+	+	+	+
DNase	+	+	+	+	+
Gelatinase	-	-	-	-	-
Growth in 4°C	+	+	+	+	+
Growth in 25°C	+	+	+	+	+
Growth in 37°C	+	+	+	+	+
Growth in 40°C	+	+	+	+	+
Growth in 45°C	-	-	-	-	-
Carbohydrate fermentation from:					
Benzoate	+	+	+	+	+
fructose	+	+	+	+	+
glucose	+	+	+	+	+
glycin	+	+	+	+	+
Inositol	-	-	-	-	-
maltose	+	+	+	+	+
rhamnose	-	-	-	-	-
starch	-	-	-	-	-
trehalose	-	-	-	-	-

3.6 Detection the ability of *P.putida* for biosurfactant production

Different sources of edible and nonedible oils were used to examine the ability of the local isolates(H_2, H_3, H_5, H_6 and H_9) in biosurfactant production as a first step of bacterial biodegradation of these compounds. For this purpose crude oil, engine oil, kerosene, soybean oil and sunflower oil were used by culturing each isolate of *P. putida* in mineral salt medium containing 1% of each of these oils separately as a source of carbon and energy, while the blank was mineral salt medium containing 1% of each of these oils with out growth.

Biosurfactant production was detected by lowering the surface tension of cell-free supernatant of each isolate culture after incubation with shaking (180 rpm) at 30°C for 48 hours. Table (3-5) showed that all of these isolates can lowering surface tension through its ability in biosurfactant production but in different degrees.

Table (3-5) Surface tension (mN/m) of cell free supernatant of local isolates of *P.putida* grown in different oil sources*.

Compound	Blank	Isolates				
		H2	H3	H5	H6	H9
Crude oil	42	36	38	36	35	36.5
Engine oil	34	32.5	32	33	28	31
Kerosene	45	41	40	40.5	35	38
Soya bean oil	46	36.5	35	33	29	36
Sun flower oil	47	34	34	35	27	30

*Cultures were incubated with shaking (180 rpm) at 30°C for 48 hrs.

Among the other results we can see that *P. putida* H6 was seemed to be the efficient one in biosurfactant production and then lowering surface tension of cell free supernatants of different oil compounds.

Due to the efficiency of surface-active compounds produced by the *Pseudomonas* spp., a number of studies were conducted towards this field (Jain, *et al*., 1991; Zhang and Miller, 1994). It was shown that reduction of surface tension is considered as a selection criterion for biosurfactant-producing capacity of microorganisms in a liquid medium (Cooper, *et al*., 1989; Carrillo, *et al*., 1996; Randhir, 1997).

Gurjar, *et al*., (1995) patently showed that maximum emulsification activity was found to be culture density-associated in crude oil-containing

medium. Desai and Banat (1997) demonstrated that, the production of biosurfactant by *P. putida* during growth on gasoline.

From these results it can be concluded that *P. putida* H6 was also the efficient in lowering of surface tension throughout production of biosurfactant to promote hydrocarbon biodegradation. For these reason *P. putida* H6 was selected among the other local isolates to study some of its other specific biocharacteristics dealed with hydrocarbon biodegradation (Banat, 1995; Lin, 1996).

3.7 The Ability of local isolates of *Pseudomonas putida* for hydrocarbon biodegradation

Results in table (3-6) showed that all these isolates were able to degrade octane, decane (small chain n-alkane), and benzoate (single ring aromatic compound). Their ability to degrade benzoate and small chain n-alkane was very great in comparison with their ability to degrade other hydrocarbons. In addition, all these isolates showed a good ability to degrade aliphatic hydrocarbons such as octane and decane because of their simple compositions, less toxicity and easy to degrade (Britton, 1995; Al-Hadhrami, *et al*., 1997).

Table (3-6) Growth of locally isolates of *Pseudomonas putida* in mineral salt medium containing different hydrocarbons*.

Hydrocarbon	Isolates				
	H2	H3	H5	H6	H9
Anthracene	-	-	-	++	-
Cyclohexane	-	+	-	++	-
Decane	+	++	+	+++	++
Naphthalene	+	+	-	++	+
Naphthol	+	+	-	++	+
Naphthol benzene	+	+	-	++	+
Octane	++	+++	++	+++	++
Phenol	-	-	-	+	-
Salicylic acid	+	++	++	++	+
Sodium benzoate	++	+++	++	+++	++
Sodium toluate	-	++	++	-	+
Tetrahydrofuran	-	-	-	-	-
Toluene	-	++	++	-	+
Trans-1-2-diphenyl-ethylene	-	-	-	+	-
Xylene	-	+	+	+	+

***** Cultures were incubated with shaking (180 rpm) at 30°C for 48 hrs.(O.D.=600 nm)
(-) No growth, OD less than 0.05; **(+)** Low growth, OD= 0.05 -0.15.
(++) Moderate growth, OD= 0.16-0.25; **(+++)** High growth, OD= 0.26-0.35.

Furthermore, all of these isolates showed a high ability to degrade benzoate, since benzoate is the easiest aromatic hydrocarbon could be degraded by bacteria and utilized by different metabolic pathways (Gibson and Subramanian, 1984; Seeger, *et al*., 1999).

Among these isolates *P. putida* H6 was the best in the ability of utilizing all hydrocarbon compounds except toluene and xylene in comparison with the other isolates (H2, H3, H5 and H9) that show different abilities for utilizing various hydrocarbon compounds as a sole source of carbon and energy. In addition, these have no isolates able to degrade phenol and anthracene. Phenol has a high bactericidal activity that make it′s biodegradation by microbial cells limited (Abu-Salah, *et al*., 1996), or oxidation of phenol, as a first step in the biodegradation, lead to products even more toxic than phenol (Ramos and Timmis, 1987).

It was observed from the results that the biodegradation rate of aromatic hydrocarbons ceased whenever the chemical structure of these aromatic hydrocarbons became more complicated. It was also noticed that all the isolates were able to degrade Salicylic acid (intermediate compounds), and only the isolate H6 was the only isolate that has the ability to degrade polynuclear aromatic hydrocarbon (anthracene) and trans-1, 2-diphenylethelene. These results were agreed with Kanaly and Harayama, (2000) who refers that the ability of microorganism to degrade hydrocarbon compounds is decreased with the increase of complexity of these hydrocarbons.

On the other hand environmental persistence of polynuclear aromatic hydrocarbons is made longer with the increasing numbers of rings of these compounds, for example the biodegradation half-life in soil and sediment of the three ring anthracene molecule ranged from 16 to 126 days while for the

compound that have five ring like benzo [α] pyrene may the half-life range from 229 to 1400 days (Banerjee, *et al.,* 1995).

This may explained the ability of *Pseudomonas putida* H6 to degrade anthracene, naphthalene and salicylic acid either by the same metabolic pathway, or by utilizing anthracites and naphthalene by one metabolic pathway and salicylic acid by another one.

From the other results obtained in this study, only two isolates (H3, H6) were able to degrade cyclohexane (alicyclic compound). Alicyclic compounds are characterized by their high toxicity toward microbial cells and their complex structure, therefore the biodegradation of alicyclic compounds as a sole source of carbon and energy synergistic support of two or more microbial species. *Pseudomonas* spp. are the most common bacteria that show a good ability to degrade such compounds in environments (Dragon, 1988b).

None of the five isolated of *Pseudomonas putida* show a considerable ability to degrade tetrahydrofuran. The failure of these isolates to degrade tetrahydrofuran, as a sole source of carbon and energy may be because of the antimicrobial activity of this compound. However, Trudgill (1984a) was referred to the biodegradation of tetrahydrofuran by *Pseudomonas* spp.

There is no doubt that the chemical structures of various hydrocarbon compounds not only have an extensive effect on their biodegradation by different bacterial isolates, but also the genetic diversity of these isolates affect the biodegradability of these hydrocarbons, as well as some of bacterial species may contain different genetic elements like plasmids or transposons carrying genes for biodegradative proteins that might participate in diverging the utilization of hydrocarbons (Ramos and Timmis, 1987; Chakrabarty, 1996; Nasir, *et al.*, 2003).

3.8 Heavy metal resistance

Because of the nature of the contaminated environments of selected as a regions isolation for *P. putida*. Ability of *P. putida* H6 in heavy metal resistance was examined against several types of heavy metal such as cadmium (Cd^{2+}), copper (Cu^{2+}), iron (Fe^{2+}), mercury (Hg^{2+}) and zinc (Zn^{2+}) while control positive was the mineral salt medium and inoculum and control negative was the mineral salt medium with heavy metals. Results in table (3-7) showed that *P. putida* H6 was resist to all of these heavy metals with slight difference (resistance to $Zn^{2+} > Fe^{2+} > Cu^{2+} > Cd^{2+}$ and Hg^{2+}) after incubation with shaking (180 rpm) at 30°C for 48 hours. This result indicated that most of *P. putida* which utilizing hydrocarbons can resist heavy metals. Higham, *et al.*, (1985) who mentioned that most *P. putida* strains were hydrocarbon utilizing and resist to heavy metal.

On the other hand results in table (3-7) showed that the minimal inhibitory concentration for both Cadmium and Mercury was 5 mM, while it was 10 mM for Copper, Iron and Zinc respectively. These MICs were greater than those for other strains of *Pseudomonas* spp. and other hydrocarbon biodegrading strains resulted in different studies (Nelson, *et .al*., 2002). Several principal sites of heavy metal complex formation in biological systems have been proposed, including accumulation in the cell wall, carbohydrate or protein polyphosphate–heavy metal complexation, complexing with the carboxyl group of the peptidoglycan in the cell wall or entering into cells via energy – dependent mechanism (NaKahara, *et.al*., 1977).

Table (3-7): Heavy metal resistance of *P. putida* H6 termed by growth density and minimal inhibitory concentration (mM) *.

Heave Metal	O.D.	MIC (mM)
Cadmium	0.12	5
Copper	0.17	10
Iron	0.22	10
Mercury	0.1	5
Zinc	0.3	10

*****Cultures were incubated with shaking (180 rpm) at 30°C for 48 hrs.
Low growth (O.D. =0.1 - 0.2). Moderate growth (O.D. = 0.21 - 0.3).
High growth (O.D. = 0.31 - 0.4).(O.D.=580 nm)

3.9 Antibiotic sensitivity test

Heavy metal resistance of *P.putida* that mainly associated with plasmid(s) is also responsible for antibiotic resistance (Clark, *et al.*, 1979; Summers, 1985).

The standard disk diffusion method was used to determine the sensitivity of *P. putida* H6 to several antibiotics. Table (3-8) showed that the resistance of this isolate to different antibiotics varied according to the nature of antibiotic. However, this isolate was resistance to ampicillin,

carbencillin, cloxacillin and penicillin the resistance could be attributed to hydrolysate this group β-lactamase or to lack of penicillin binding protein (PBP) or the change of permeability to the drug (Avesion, *et al.*, 2000).

These results were in agreement with Hollowag, *et al.*,(1979) and Scott *et al.*, (1999), who found that most species of *Pseudomonas* were resistance to a broad spectrum of penicillin's including ampicillin, carbencillin, pipracillin and others, which was due to β-lactamase production, but these antibiotic when combined with β-lactamase inhibitors became active against different isolates in vitro. Ferer (1995) found that the major mechanism of resistance to penicillin and ampicillin in gram-negative bacteria causing clinical significant in the expression of β-lactamase, of which there are several classes including plasmid encoded and chromosomally encoded enzymes.

Resistance of *P. putida* H6 to chloramphenicol could be happen by inactivate the antibiotic throughout secretion of chloramphenicol acetyl transferase (CAT), and the resistance to erythromycin may be results from an alteration (methylation) of the rRNA receptor as it was mentioned by . Jawetze, *et al.*, (1976).

Table (3-8): Resistance of *P. putida* H6 isolate, to different antibiotics*.

Antibiotic	Symbol	Sensitivity
Ampicillin	AMP	R
Carbencillin	py	R
cefotaxime	CTX	S
Chloramphenicol	C	R
Ciprofloxacin	CF	S
Cloxacillin	CLX	R
Erythromycin	E	R
Gentamicin	GM	S
Naldixic acid	NA	S
Penicillin	P	R
Rifampicine	Rif	S
Streptomycin	S	R
Tetracycline	TE	R
Ticarcillin	TC	R
Vancomycin	VA	S

*Cultures were incubated at 30°C for 18 hrs . R: Resist. S: Sensitive.

Furthermore, table (3-8) showed that this isolate was sensitive to cefotaxime, Ciprofloxacin, Rifampicine, Vancomycin, aminoglycosid (Gentamycin and Naldixic acid).

The multidrug resistance to several antibiotics could be due to the permeability of the outer membrane, which might prevent the entry of antibiotic into the cell, or due to certain mutations that occur as a result of overuse and misuse of antibiotics. Plasmids that carrying resistance gene plays an important role in transferring the multidrug resistance among bacterial strains (Snyder and Champness, 1997; Stock and Wiedemann, 2001).

3-10 Plasmid profile of *P.putida* H6

Among different isolates, *P.putida* H6 was selected to study plasmid profile because it was the best in hydrocarbon biodegradation and lowering surface tension of cell-free supernatants, in addition to it's ability in heavy metal resistance. For this purpose, salting out procedure was used for extraction of plasmid DNA according to Pospiech and Neumann (1995),

Figure(3-3) showed that there are two plasmid DNA bands on agarose gel, these results represents that *P.putida* H6 harboring two small plasmids that may be associated with the chromosome in conferring to biosurfactant production, hydrocarbon biodegradation and the resistance to heavy metals and antibiotics. These results correspond with Nelson, (2002) who found that *P.putida* contain two plasmids responsible for antibiotic and heavy metals resistance beside the large plasmid that responsible for hydrocarbon biodegradation.

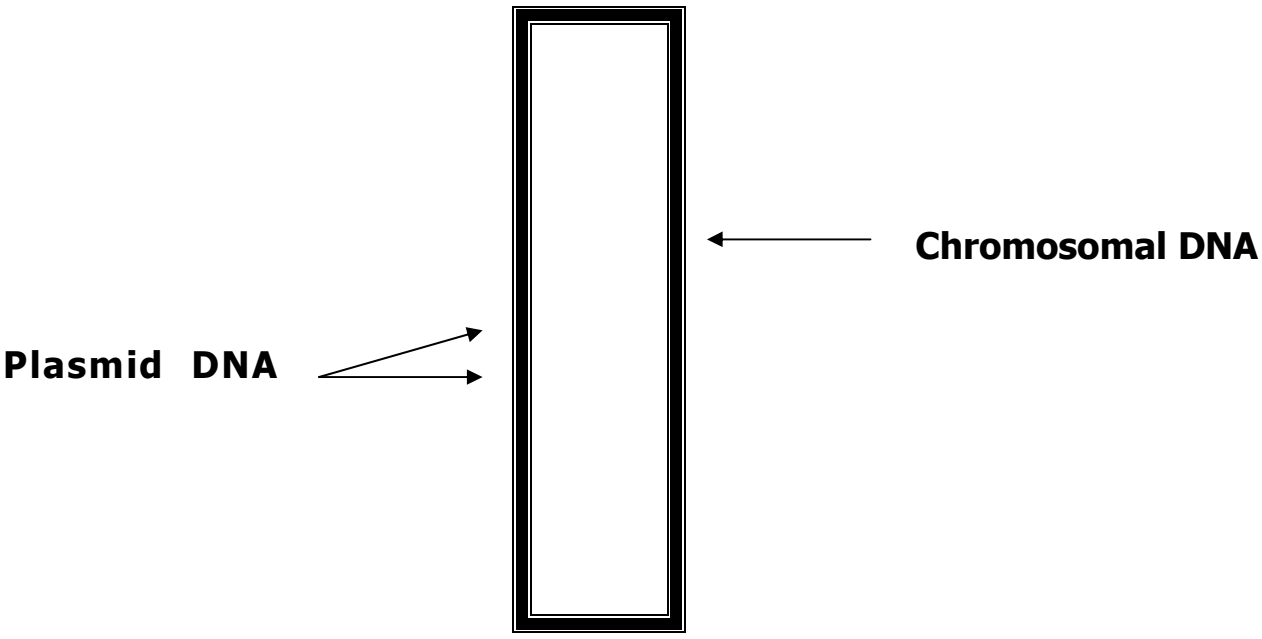

Figure (3-3) Plasmid profile of *P.putida* H6 migrated on agarose gel (0.7 %) in TBE buffer at 5 v/cm.

3.10.1 Curing of plasmid DNA

In order to know the relationship between the two plasmids of *P.putida* H6 and it is ability in biosurfactant production and hydrocarbon biodegradation and heavy metal resistance, these two plasmids were cured using ethedium bromide as an intercalating agent according to procedure described by Trevors, (1986) , the mode of action of ethedium bromide in curing plasmid DNA based on it's ability to inhibit DNA replication of plasmid DNA during cell division without any effect on the chromosomal DNA replication, which leads to the presence of plasmid-less cell in the next

generations. Furthermore ethedium bromide was a good agent in curing of plasmid DNA, if it compared with physical and other chemical agents (Hohn and Korn, 1969).

P.putida H6 was grown in Luria – Bertani broth containing gradual concentrations of ethedium bromide to determine the sub-lethal concentration. Results in table (3-9) showed that the sub-lethal concentration of ethedium bromide was 700 µg/ml. Culture bacteria containing this concentration was diluted and spread on brain heart infusion agar and incubated at 30˚C for 24 hours. Fifty of the resultant colonies were selected and replica-plated on Muller – Hinton agar plates containing carpencillin, erythromycin, penicillin and tetracycline separately. The changed from py^r, E^r, P^r and TE^r to py^s, E^s, P^s and TE^s were selected and suspected to be cured, then they were selected and examined for the ability in biosurfactant production, hydrocarbon biodegradation and heavy metals resistance.

Table (3-9): Determination sub-lethal concentration of Ethidium-bromide affects growth of *P.putida* H6*.

Ethidium-Bromide (µg/ml)	100	200	300	400	500	600	700	800	900	1000
Growth	+++	+++	+++	+++	++	++	+	-	-	-

***** Cultures were incubated with shaking (180 rpm) at 30°C for 48 hours.
(-) No growth. **(+)** slightly growth. **(++)** Moderate growth. **(+++)** Heavy growth.

Table (3-10) showed that eight colonies (16%) of fifty were lost their antibiotic resistance to one or more of tested antibiotics. Two of these (H66 and H68) were lost the resistance to one antibiotic(tetracycline), three (H61, H62) and (H67) were lost the resistance to two antibiotics (carbencillin, penicillin) and (erythromycin, tetracycline), one (H63) was lost the resistance to three antibiotics(carbencillin, , penicillin, tetracycline),. Two cured strains (H64 and H65) were lost the resistance to four antibiotics(carbencillin, erythromycin, penicillin, tetracycline).

The results indicated that Erythromycin and tetracycline resistante genes are carried on different plasmids (two plasmids), while carbencillin and penicillin resistant genes are carried on one plasmid, because no cured isolate was obtained which lost one of them only.

Table (3-10): Cured colonies of *P.putida* H6 that lost the resistance to antibiotics after incubation with ethidium-bromide, for 24 hours at 30°C.

Antibiotic	H61	H62	H63	H64	H65	H66	H67	H68
Carbencillin	S	S	S	S	S	R	R	R
Erythromycin	R	R	R	S	S	R	S	S
Penicillin	S	S	S	S	S	R	R	R
Tetracycline	R	R	S	S	S	S	S	R

R: Resist. S: Sensitive.

To determined the cured cells, *P.putida* H64 (cured colony) was selected. Figure (3-4) showed that the cured isolate H64 was lost both small plasmids that it contains after curing with ethedium bromide. On the basis of this result the cured isolate *P.putida* H64 was used to determine its ability in biosurfactant production, hydrocarbons biodegradation and heavy metal resistance.

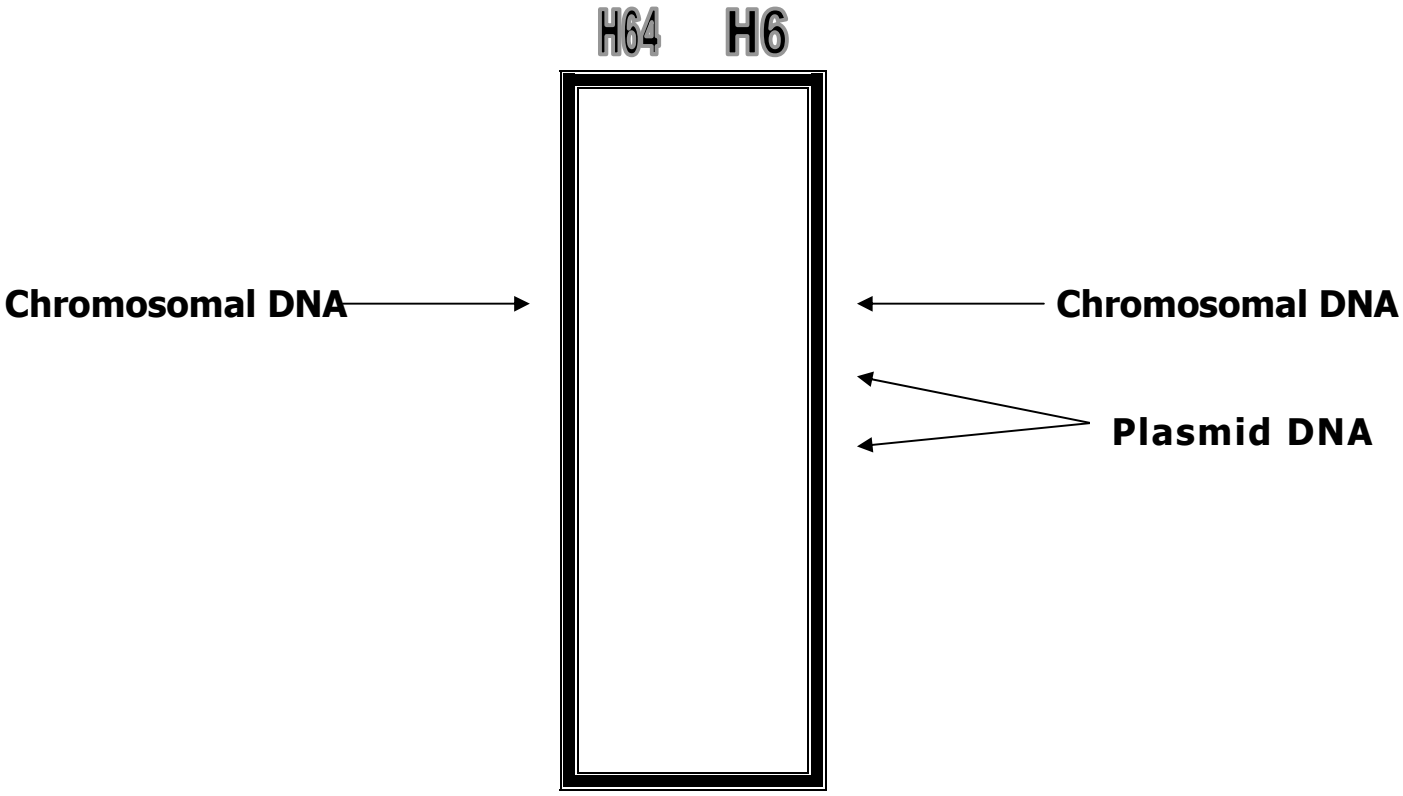

Figure (3-4) Plasmid profile for the wild type of *P. putida* H6 and the cured *P. putida* H64 on agarose gel (0.7 %) in TBE buffer at 5 v/cm.
Lane (H6): wild type
Lane (H64): cured type

3.10.2 The ability of cured cells in biosurfactant production , hydrocarbon biodegradation and heavy metal resistance.

In order to determine whether the cured isolate lost other important traits like biosurfactant production, hydrocarbon biodegradation and heavy metals resistant, *P.putida* H64 was tested for their ability in biosurfactant production, hydrocarbon biodegradation and heavy metals resistance. The results table (3-11) indicated that these two small plasmids in *P.putida* H6 are not responsible for biosurfactant production because *P.putida* H64 is still capable to lowering the surface tension of cell-free supernatant of edible and nonedible oils. This result indicated that biosurfactant production trait in *P.putida* H6 may be located on the chromosome.

Table (3-11) Surface tension (mN/m) of cell free supernatant of cured *P. putida* H64 grown in different oil sources*.

Compound	Crude oil	Engine oil	Kerosene	Soya bean oil	Sun flower oil
Blank	42	34	45	46	47
***P. putida* H6**	35	28	35	29	27
***P. putida* H64**	35	28	35	29	27

*Cultures were incubated with shaking (180 rpm) at 30°C for 48 hrs.

This results was agreed with Saifour (1998) who found that the cured isolates of *Pseudomonas spp.* did not lose their biosurfactant productivity,

but they lost some ability to degrade crude oil. Results in table (3-12) showed that *P.putida* H64 is still able to degrade different hydrocarbons except salicylic acid and xylene which may be conferred by plasmid DNA that lost in curing experiment. These results confirmed that the small plasmids of *P.putida* had no effect on biosurfactant production while it might have a regulatory role on crude oil degradation or a role in degradation of some other hydrocarbons.

Table (3-12) Growth of cured *P. putida* H64 in mineral salt Medium containing different hydrocarbons*.

Compounds	*P. putida* H6 growth	*P. putida* H64 growth
Anthracene	++	++
Cyclohexane	++	++
Decane	+++	+++
Naphthalene	++	++
Naphthol	++	++
Naphthol benzene	++	++
Octane	+++	+++
Salicylic acid	+	-
Sodium benzoate	+++	+++
Trans-1-2-diphenyl ethylene	+	+
Xylene	+	-

***** Cultures were incubated with shaking (180 rpm) at 30°C for 48 hrs.
(-) No growth, OD less than 0.05; **(+)** Low growth, OD= 0.05 -0.15.
(++) Moderate growth, OD= 0.16-0.25; **(+++)** High growth, OD= 0.26-0.35.

On the other hand, table (3-13) showed that the cured isolate *P.putida* H64 was lost resistance traits to two heavy metals (mercury and cadmium) which means that these two traits were also plasmid born, while it still resist to other heavy metals (copper, iron and zinc) which may be referred that these traits are chromosomal located.

The genome of *P.putida* encodes an unexpected capacity to resistance heavy metals and metalloids. Computer–assisted and searches of the genome sequence identified a number of putative genes involved in the regulation, uptake, extras ion and chelation of metals in *P.putida* and the location of these genes occurs in two large clusters close to the origin of replication region might result in increased resistance. (Robinson and Tonvinen, 1984; Dolman, 1986).

Table (3-13): Heavy metal resistance of cured *P. putida* H64 termed by growth density and minimal inhibitory concentration (mM)*.

Heavy metal	Cadmium	Copper	Iron	Mercury	Zinc
Growth	-	0.17	0.22	-	0.3
MIC (mM)	5	10	10	5	10

* Cultures were incubated with shaking (180 rpm) at 30°C for 48 hours.
(-) No growth. **(+)** slightly growth. **(++)** Moderate growth. **(+++)** Heavy growth.

Previously, Lee *et.al.*, (2001), confirmed that the resistance genes were carried on a plasmid.

Conclusions

1- Soil samples contaminated with oil hydrocarbons represents suitable environments for isolation of *Pseudomonas putida*.

2- Among different isolates of *Pseudomonas putida*, *P.putida* H6 was the best one in biosurfactant production, hydrocarbon biodegradation, and it was found that this isolates was resist to Cadmium, Copper, Iron, Mercury and zinc.

3- Ethedium bromide is an effective agent in curing of *P.putida* plasmid DNA.

4- Biosurfactant production trait and biodegradation of anthracene, cyclohexane, decane, naphthalene, naphthol, naphthol benzene, octane, phenol, sodium benzoate and trans-1-2-diphenyl-ethylene were chromosomally located while salicylic acid and xylene traits are located on plasmids in *P.putida* H6.

5- Resistance to copper, iron and zinc are chromosomally located while resistance to cadmium and mercury are located on plasmids.

Recommendation

1- Determination the optimum conditions for hydrocarbon degradation and biosurfactant production by *P. putida* H6.

2- Developing the ability of *P.putida* H6 biosurfactant production by mutagenesis or cloning.

3- Using *P.putida* H6 in pure cultures and /or mixed cultures in treatment of polluted sites with different oil and hydrocarbon.

4- Further study on microbial resistance to heavy metals and hydrocarbons biodegradation and the relationship between them have will be done.

5- Further studies on microbial resistance to heavy metals and antibiotic resistance.

INTRODUCTION

Pseudomonas putida has been recognized for many years as a common inhabitant of soil sand and fresh water, It was found that it's comprised an average of six percent (ranging up to14%) of all culturable isolates in roots of seven species of common weed and it's described as “ubiquitous”, the strains of this bacteria which isolated from infected plants and soil samples was found to be resistant to several antibiotic and heavy metals, this may indicated the risky relationship between both resistance to metals and contaminated environment, that cause distribution for resistance factors for antibiotic and heavy metals (Palleroni, 1984).

It must also considered a potential pathogen in human, as it has been isolated from a number of clinical samples (Stolp and Gud Kari, 1984).

It was known to microbiologists for their ability to degrade aromatic hydrocarbons such as toluene, camphor and naphthalene, through degradative pathways often associated with plasmid which have been examined extensively by biochemical and genetic means. In nature, it would be expected to play a significant role in carbon mineralization along with other *Pseudomonads*, that had a characteristic ability to degrade a wide variety of carbon sources, and it does not require special growth factors (Azoulay, *et al.*, 1983).

Pseudomonas putida was genetically developed to get a modified strain which carrying genes coding for enzymes that mediate the degradation of xylene, toluene, octane and camphor in to carbon CO_2 and H_2O. These genes are born on plasmids and were manipulated by general microbiological and genetic engineering techniques, to get a super bug strain that contains all four sets of genes (Brooks, *et al.*, 1995).

P. putida was found to be the most efficient species of the genus *Pseudomonas* have the ability to degrade hydrocarbon, it has been associated with other metal oxidation and biosurfactants production (Dragon, 1988a; Zhang, and Miller, 1994). Because of the importance of such microorganism in detoxification and relief pollution in different environments, so this study was aimed to:

- Isolation and identification of *Pseudomonas putida*.
- Screening local isolates of *P. putida* and select the efficient one based on it̓s ability in biosurfactant production and hydrocarbon biodegradation.
- Determine the genetic nature of the genes that responsible for the biosurfactant production, hydrocarbon biodegradation and the resistance to heavy metals.

List of Abbreviations

Abbreviations	Means
Cetrimide	Cetyltrimethylammonium bromide.
CAT	Chloramphenicol Acetyl Transferase .
DNA	Deoxyriboneuclic Acid.
DNase	Deoxyribonuclease.
D.W.	Distilled water.
EDTA	Ethylene Diamine Tetra Acetic Acid.
g	Gram.
MIC	Minimum Inhibitory Concentration.
ml	Milliliter.
mM	Millimolar.
mNm^{-1}	Millinuton per meter.
MT	Metallothioneins.
nm	Nanometer.
O.D.	Optical Density.
ORF	Open Reading Frame.
PBP	Pencillin Binding Protin
RNA	Riboneuclic Acid.
RNase	Ribonuclease A.
rpm	Round per minute.
SDS	Sodium Dodecyl Sulfate.
TBE	Tris -Borate Ethylene Diamine Tetra Acetic Acid.
TCA	Tri – Carboxylic Acid
TDO	Toluene Dioxygenase.
TE	Tris - Ethylene Diamine Tetra Acetic Acid.
v/v	Volume by volume
w/v	weight by volume
U.V.	Ultraviolet Light
%	Percent.
°C	Degree Celsius.
β	Beta
µl	Micro liter

List of Contents

List of Figures

List of Tables

SUMMARY

Sixteen soil samples contaminated with different hydrocarbons were collected from seven locations in Baghdad governorate. From these samples there were 70 isolates were obtained and ten of them were subjected to morphological and physiological studies and biochemical tests. Five of them (Symboled H2, H3, H5, H6 and H9) were characterized as *Pseudomonas putida*.

The ability of local isolates of *P. putida* for biosurfactant production, hydrocarbons biodegradation and heavy metal resistance were studied. Results showed that *P. putida* H6 was the best ,because of its high efficiency for biosurfactant production and lowering the surface tension of cell-free supernatants when it was grown in mineral salt medium containing crude oil , engine oil , kerosene , soyabeen oil and sunflower oil as sole source for carbon and energy. It was also shown that this isolate was the efficient in biodegradation of aliphatic hydrocarbons (octane and decane), and aromatic hydrocarbons (Anthracene, cyclohexane, naphthalene, naphthol, benzene, phenol, salicylic acid, sodium benzoate, trans-1-2-diphenyl-ethylene, xylene). Further more *P. putida* H6 was able to resist heavy metals. It was found that this isolates was resist to cadmium, copper, iron, mercury and zinc.

Plasmid profile of *P. putida* H6 was studied through the extraction of plasmid DNA by salting-out procedure. Results of agarose gel electrophoresis showed that this isolate harboring two small plasmids that may be responsible for some of the biocharacteristics of this isolates. So, curing of plasmid DNA was achieved using ethidium bromide, then cured cells that lost the antibiotic resistance to carbencillin ,erythromycin ,penicillin and tetracycline were selected and used to study its ability in biosurfactant production, hydrocarbons biodegradation and heavy metal resistance. Result showed that this cured cells (Symboled *P.putida* H64) was still the efficient in biosurfactant production, and lowering surface tension in its cell-free supernatants. This result refers to biosurfactant trait was chromosomally encoded.

It was also found that this cured cell was lost its ability in biodegradation of salicylic acid and xylene, which may be refers to plasmid origin of these traits, while it was still efficient in biodegradation of the other aliphatic and aromatic hydrocarbons.

In addition, cured cells of *P. putida* H64 were lost its ability in the resistance to cadmium and mercury, which may be also, refers that resistance traits are plasmidly encoded, while it was also still efficient to resist other heavy metals.

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Haider

الإهداء...

إ لى النبي محمد المصطفى سيد عالم الإمكان...

إلى النبي محمد باقر علوم الإنس والجـــان...

إلى النبي محمد ذي الجود والإحســـــان...

إلى النبي محمد قامع الظلم والعـــــدوان...

إلى النبي محمد صوت الحق بكـــــــوفان...

إلى أساتذتي ومن علمني من علوم القـــرآن...

اهدي جهداً مليئاً بالنقصان...

أرجو به الرضا من الرحمان...

حيدر

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Republic of Iraq
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ISOLATION AND IDENTIFICATION OF *PSEUDOMONAS PUTIDA* AND STUDY ITS ABILITY TO DEGRADE HYDROCARBONS COMPOUNDS

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
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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

عزل وتشخيص بكتيريا *Pseudomonas putida* ودراسة قابليتها على تحليل المركبات الهيدروكاربونية

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم

في التقانة الاحيائية

من قبل

«حيدر صادق عبدالحسن»

بكلوريوس تقانة احيائية / كلية العلوم

جامعة النهرين (2003)

أيلول ٢٠٠٦

شعبان ١٤٢٧

Conclusion
&
Recommendation

CHAPTER 1

Literature Review

CHAPTER 2

Materials & Methods

CHAPTER 3

Results & Discusion

References

بِسمِ اللهِ الرّحَمنِ الرّحِيمِ

﴿وَاللّهُ اَخرَجَكُم مِن بُطُونِ اُمَّهَاتِكُم لا

تَعلَمُونَ شَيئاً وَجَعَلَ لَكُمُ السَمعَ وَالاَبصَارَ

وَالاَفِئدَةَ لَعَلّكُم تَشكُرُون﴾

صَدَقَ اللهُ العلي العَظِيم

سورة النحل اية ٧٨

Introduction

الـخلاصـة

جمعت ١٦ عينة من عينات التربة الملوثة بالمركبات الهيدروكاربونية المختلفة من سبعة مواقع في محافظة بغداد. تم الحصول على ٧٠ عزلة، أخضعت جميعاً للاختبارات الكيمو حيوية والدراسات المظهرية والفسلجية وقد شخصت خمس من هذه العزلات (رمز لها H9, H6 , H5, H3, H2) على انها *P.putida* من بين المجموع الكلي للعزلات .

درست قابلية هذة العزلات من حيث قدرتها على انتاج المستحلبات الحيوية، الاختزال الحيوي للمركبات الهيدروكاربونية ومقاومتها للمعادن الثقيلة وقد اظهرت النتائج ان العزلة *P.putida* H6 كانت هي الافضل، فقد وجد ان لهذه العزلة الكفاءة العالية في انتاج المستحلبات الحيوية وخفض الشد السطحي لرائق مزارعها الخالي من الخلايا البكتيرية بعد تنميتها في وسط الاملاح المعدنية الحاوي على الزيت الخام، زيت المحركات، الكيروسين، زيت فول الصويا و زيت زهرة الشمس مصدراً وحيداً للكاربون والطاقة. كما وجد ان لهذه العزلة الكفاءة العالية ايضاً في الاختزال الحيوي للمركبات الهيدروكاربونية الاليفاتية (ديكان و الأوكتان) والاروماتية (ألانثراسين، الهكسان الحلقي، النفثالين، النفثول ، النفثول بنزين، الفينول، حامض الساليسليك، البنزين، بنزوات الصوديوم، الاثيلين ثنائي الفينول و الزايلين). ولدى دراسة قابلية العزلة *P.putida* H6 في مقاومة المعادن الثقيلة فقد وجد بانها كانت مقاومة لكل من الكادميوم ،النحاس ،الحديد ،الزئبق والخارصين.

درس النسق البلازميدي للعزلة المحلية *P.putida* H6 وذلك باستخلاص الدنا البلازميدي بطريقة الترسيب الملحي وقد اشارت نتائج الترحيل الكهربائي على هلام الاكاروز الى وجود حزمتين بلازميتين صغيرتين مما يشير الى احتواء هذه العزلة على بلازميدين صغيرين قد يكونان مسؤلين عن بعض الصفات الحيوية التي تتميز بها هذه العزلة وعلى هذا الاساس تم تحييد الدنا البلازميدي باستخدام صبغة بروميد الاثيديوم وقد تم انتفاء احدى المحيدات التي فقدت صفة المقاومة لكل من الكاربنسلين ،الارثرومايسين، البنسيلين والتتراسايكلين. ودرست قابليتها على انتاج المستحلبات الحيوية، الاختزال الحيوي للمركبات الهيدروكاربونية وقابليتها على مقاومة المعادن الثقيلة. وقد اشارت النتائج الى ان هذه المحيدة البكتيرية *P.putida* H64 لازالت تتميز بكفائتهاعلى انتاج المستحلبات الحيوية وخفض الشد السطحي لرائق مزارعها الخالي من الخلايا البكتيرية، مما يشير الى ان صفة انتاج المستحلبات الحيوية يشفر لها كروموسومياً. كما وجد ايضاً بأن هذه المحيدة البكتيرية قد فقدت صفة اختزال حامض الساليسليك والزايلين مما يشير الى ان صفة اختزال هذين المركبين هما صفتان بلازميدية في حين احتفضت بكفائتها في الاختزال الحيوي لبقية المركبات الهيدروكاربونية الاليفاتية والاورماتية . أما فيما يتعلق بقابليتها المحيدة البكتيرية H64 *P.putida*في مقاومة المعادن الثقيلة فقد وجد بأنها فقدت صفة المقاومة لكل من الكادميوم والزئبق مما يشير ايضاً الى ان هاتين الصفتين هما من الصفات التي يشفر لها بلازميدياً في حين احتفضت بكفائتها ايضاً في مقاومة المعادن الثقيلة الاخرى.

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:	**Signature:**
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I hereby certify upon the decision of the examining committee

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

Signature

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Date: 2006-09-

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

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Date: 2006-09-