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Molecular Study on Thermotolerant Bacteria that Degraded Nitroaromatic Compounds

A Dissertation

Submitted to the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the degree of Doctorate of Philosophy of Science in Biotechnology

By

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بسم الله الرحمن الرحيم

فَتَبَسَّمَ ضَاحِكًا مِّن قَوْلِهَا وَقَالَ رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ وَعَلَى

وَالِدَيَّ وَأَنْ أَعْمَلَ صَالِحًا تَرْضَاهُ وَأَدْخِلْنِي بِرَحْمَتِكَ فِي عِبَادِكَ الصَّالِحِينَ

صدق الله العظيم

سورة النمل اية ١٩

بسم الله الرحمن الرحيم

رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ وَعَلَى وَالِدَيَّ وَأَنْ أَعْمَلَ صَالِحًا تَرْضَاهُ وَأَدْخِلْنِي بِرَحْمَتِكَ فِي عِبَادِكَ الصَّالِحِينَ

صدق الله العظيم

سورة النمل اية ١٩

Supervisors Certification

We, certify that this dissertation entitled "**The role of thermophilic bacteria in biodegradation of aromatic hydrocarbons**" was prepared by **"Mayada"** under our supervision at the College of Science/ Al-Nahrain University as a partial fulfillment of the degree of Doctorate of Philosophy of Science in Biotechnology.

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Dedication

To my Family

Mayada

Acknowledgement

Praise is to Allah, Lord of the whole creation. Mercy and Peace are to the Prophet Mohammad and his relatives and companions.

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Mayada

Summary

The main aim of this study was isolating and identifying thermophilic bacteria capable of utilizing aromatic hydrocarbon that form part of petroleum components. For this purpose (95) hydrocarbon-contaminated soil samples were collected from different places in Basrah and Baghdad.

Fourty eight bacterial isolates that utilize crude oil were isolated. Thirty eight mesophilic bacterial isolates showed good ability to grow on crude oil as the only carbon and energy source. These isolates were characterized as: (20) *Pseudomonas*, (3) *Staphylococcus*, (3) *Acinetobacter*, (2) *Alcaligenes*, (2) *Aeromonas*, (2) *Flavobacterium*, and (6) *Bacillus*. In addition to these genera, ten thermophilic bacterial isolates showed an excellent ability to utilize crude oil as the sole source of carbon, nitrogen and energy. Utilization of aromatic compounds (Naphthalene, Carbazole, Nitrobenzene, and ρ-Nitrophenol) of these ten isolates was studied. Results showed a diverge utilization ability.

These ten isolates cannot be distinguished by ordinary morphological, physiological and biochemical tests; therefore, they (for four selected isolates) were subjected to molecular identification through PCR amplification of 16S rDNA by using 7 primers (fd1, fd2, fd3, fd4, rd1, rp1 and rp2) which represent primers for the PCR amplification of eubacterial 16S rDNA, and followed by sequencing. The nucleotide sequence data was compared with 16S rDNA sequences of other culture on BLAST of the National Center of Biotechnology Information database (NCBI database).

According to the results of molecular identification, these isolates characterized as, 2G (Ir1) (*Geobacillus thermoleovorans*), 3A (Ir2) and

4A (Ir3) (*Anoxybacillus rupiensis*), and were deposited in the National Genebank database under the accession number, JQ912239, JQ912240, and JQ912241 respectively.

Anoxybacillus rupiensis strain Ir3 (JQ912241) was selected for its ability to utilize all the aromatic compounds efficiently and used throughout this study.

The plasmid profile of the selected isolate *A. rupiensis* strain Ir43 (JQ912241) showed that this bacterium contain large and small plasmid DNA bands.

In order to determine the role of plasmid in utilization of aromatic compounds (carbazole for example), many attempts were made to cure *A. rupiensis* strain Ir3 (JQ912241) plasmid (s) using Sodium dedocyl sulphate (SDS) and Ethidium Bromide (ET Br). Results indicated that no cured colonies (lost their ability to utilize carbazole at 70◦C) were obtained. Plasmid isolated from some of these colonies after treated with Et Br, indicated that these colonies are still harboring the large plasmid, which is difficult to cure, and the trait might be located on it.

The optimum conditions for growth of *A. rupiensis* strain Ir3 (JQ912241) were determined. The results showed that growing in LB (Luria-Broth) medium (pH 7) containing 0.5 to 1% of NaCl, and incubated with shaking (150 rpm) at 55 -65◦C for 24h. It was also found that this bacterium was able to withstand 80◦C for 90 min. The optimum conditions for growth of *A. rupiensis* strain Ir3 (JQ912241) on minimal medium (CDM) were adjusted, pH to 7 and incubated at 55-65◦C and the bacterial growth was increased with carbazole concentration increasing. This means that bacterial growth with Carbazole (CAR) as an N-source was concentration-dependent.

To confirm the ability of *A. rupiensis* strain Ir3 (JQ912241) to utilize the aromatic compounds, analytical experiments include HPLC (High performance liquid chromatography) and FTIR (Fourier transform infrared) were used. The results of quantitative analysis (HPLC) indicated that this bacterium showed as much as 99.62% consumption of carbazole, 99.4% of ρ-nitrophenole, 97.73% of nitrobenzene and 98.89% of naphthalene. Qualitative analysis of FTIR spectra showed that *A. rupiensis* strain Ir3 (JQ912241) has the ability to convert carbazole to anthranilic acid, indicating the presence of meta cleavage enzyme, this also confirmed by using 2, 3-dihydroxybiphenyl through converting the colonies color on Luria-Bertani (LB) and minimal agar plates to brown.

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List of Abbreviations

Abbreviation	Mean
BLAST	Basic local alignment search tool
CAR	Carbazole
CDM	Chemically defined medium
DMSO	Dimethyl sulfoxide
Et Br	Ethidium bromide
FTIR	Fourier Transform Infrared
HPLC	High performance liquid chromatography
LB	Luria-Broth
Naph	Naphthalene
NOD	Naphthalene dioxygenase
NCBI	National center for biotechnology information
PNP	p- nitrophenol
PAH	Polycyclic aromatic hydrocarbon
rDNA	Ribosomal DNA
SDS	Sodium dedocyl sulphate
TCA	Tricarboxylic acid cycle
TSI	Triple sugar iron agar

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Chapter One

Introduction and Literature Review

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Introduction and Literatures Review

1.1. Introduction:

Rapid industrialization and urbanization have resulted in the release of large amount of wastes into the environment causing major pollution problems. Aromatic compounds are among the most persistent pollutants in the environments. Petroleum contaminated soil and sediment commonly contain a mixture of polycyclic and heterocyclic aromatic hydrocarbons. Aromatics derived from industrial activities often have functional groups such as alkyls, halogens and nitro group (Sameera *et al*., 2011; Seo *et al*., 2009).

The elimination of a wide range of pollutants and wastes from the environment is an absolute requirement to promote a sustainable development of our society with low environmental impact. Biological processes play a major role in the removal of contaminants and they take advantages of the astonishing catabolic versatility of microorganisms to degrade or convert such compounds. New methodological breakthrough in sequencing, genomics, proteomics, bioinformatics and imaging are producing vast amounts of information. Functional genomics and meta-genomic approaches are increasing our understanding of the relative importance of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds and they were certainly accelerate the development of bioremediation technologies and biotransformation processes (Wikimedia foundation, Inc., 2012).

As a result of an advanced interest in microbial ecological research, a commonly accepted opinion that the natural habitats harboring undiscovered microorganisms (Derekova *et al*., 2008).

Hot environments are between the supporting life extreme niches that appear to have maintained some degree of pristine quality and of special biotechnological interest. These habitats have attracted broad interest because of the unique thermophilic properties of the organisms thriving in these biotopes and the description of an increasing number of new thermophilic species (Tomova *et al*., 2010; Satyanarayana *et al*., 2005).

Thermostable enzymes synthesized by thermophilic microorganisms are active at harsh conditions attending many industrial processes. Therefore isolation of novel thermophilic bacilli have received considerable attention among their scientific community in the whole world because of their biotechnological importance as they possess unique enzyme with thermal activity and stability at elevated temperature. Thermozymes from these thermophilic microorganisms, as a result of their extreme stability, have been used in a number of industrial applications such as biocatalysis, biotransformation and biodegradation (Cihan *et al*., 2011; Demirjian *et al*., 2001).

DNA-based technology provided a major stimulus for direct exploitation of thermophilic microorganism's genes by in situ detection as well as for studies of prokaryotic classification and ecology. In spectacular, it has provided a natural system for classifying the various bacterial groups, many of which are more distantly related to each other. A molecular alternative, which involves DNA extraction followed by PCR amplification and subsequent cloning of 16SrRNA genes was developed to overcome the limitation associated with cultivation approaches and has become common in ecological studies (Ferris and Ward, 1997; Hugenholtz *et al*., 1998; Ferris *et al*., 2001).

The present study was directed toward enriching indigenous bacterial isolates capable of metabolizing PAH such as naphthalene, and N-compound such as carbazole, ρ-nitrophenole and nitrobenzene from petroleum

contaminated soils in Iraq. Emphasis was given to isolate and identify thermophilic bacterial isolates with ability to attack a wide range of PAHs and N-compounds. Strains showing versatile and high degradation potential will be used for further investigations to develop a biotechnology for enhancing removal of biohazardous petrochemical pollutants.

The aims of the study are as follows:-

1. Isolation of thermophilic bacterial isolates with high capabilities for degradation of aromatic hydrocarbons.
2. Identification and characterization of bacterial isolates using traditional biochemical teste and molecular biological tools such as 16S rRNA amplification, sequencing and homology.
3. Determination of plasmid(s) profile for the efficient isolate and study the role of their plasmid(s) in biodegradation process via curing experiments.
4. Investigation of the quality and quantity changes of aromatic hydrocarbons treated with *Anoxybacillus rupeinsi*s strain Ir3 (JQ912241) using FTIR and HPLC analyses.

1.2. Literatures Review:

1.2.1. Description of *Anoxybacillus* gen. nov.

The genus *Anoxybacillus* belongs to the order bacillales under the firmicutes phulum in the domin bacteria (Chai *et al*., 2011). The genus with the type species *Anoxybacillus pushchinoensis* was separated from the genus *Bacillus* by Pikuta *et al*. (2000).

Anoxybacillus (An means without; oxy is shortened from oxygenium, oxygen; bacillus is a small rod; *Anoxybacillus* is a small rod living without oxygen). Cells are rod-shaped and straight, often ranged in pairs or chains, with

rounded ends. Gram positive, endospores are round and resistant to heating and freezing. Spores are located at the end of the cell. There is not more than one spore per cell (Pikuta *et al*., 2003). The description of this genus was corrected to be aerotolerant anaerobes or facultative anaerobes.

Euzeby (2012) mentioned that there are 16 species and 2 subspecies described in the genus *Anoxybacillus*: *A. pushchinoensis* gen. nov., sp. nov., a novel anaerobic alkaliphilic, moderately thermophilic bacterium from manure (Pikuta *et al*., 2000), *A. pushchinoensis* $K1^T$ (Pikuta *et al*., 2003), *A. amylolyticus* sp. nov., a thermophilic amylase producing bacterium isolated from mount Rittmann (Antarctica) (Poli *et al* ., 2006), *A. ayderensis* sp. nov., pertains to ayder, a hot spring in the province of Rize, Turkey (Dulger *et al*., 2004), *A. bogrovensis* sp. nov., pertains to Bogrov region, a novel thermophilic bacterium isolated from a hot spring in Dolni Brogrov, Bulgaria (Atanassova *et al*., 2008), *A. contaminans* sp. nov., polluting, spoiling, contaminating, isolated from contaminated gelatin batches (Declerck *et al*., 2004).

A. eryuanesis sp. nov., pertains to Eryuan, Yunnan province, South- West China, facultative anaerobic, alkalitolerant bacteria from hot springs (Zhang *et al*., 2011), *A. flavithermus* sp. nov., flavus, yellow; thermos, warm. Flavithermus, indicates a yellow thermophilic organism, a novel anaerobic alkaliphilic moderately thermophilic bacterium from manure (pikuta *et al.,* 2000), a new subspecies of *A. flavithermus* spp. *Yunnanensis* spp. nov., with very high ethanol tolerance. *A. gonesis* sp. nov., pertains to Gonen, a hot spring in the province of Balikesir, Turkey, a moderately thermophilic, xylose-utilizing, endospore-forming bacterium (Belduz *et al*., 2003), *A. kamchatkensis* sp. nov., pertains to Kamchatka peninsula, Far East Russia, a novel thermophilic faculitative an aerobic bacterium with a broad pH optimum (Kevbrin *et al*., 2006).

A. kestanbolensis sp. nov., pertains to Kestanbol, Turkey (Dulger *et al*., 2004). *A. mongoliensis* sp. nov., pertains to Mongolia, the country of isolation, a novel thermophilic proteinase production bacterium isolated from alkaline hot spring, central Mongolia, (Namsaraev *et al*., 2010), *A. rupiensis* sp. nov., pertains to Rupi Bassin, reffering to the place of isolation, a novel thermophilic bacterium isolated from Rupi Basin (Bulgaria) (Derekova *et al*., 2007).

A. salavtliensis sp. nov., an α-glucosidase producing, thermophilic bacterium isolated from Salavatli, Turkey (Cihan *et al.,* 2011). *A. tengchongensis* sp. nov., pertains to Tengchong, Yunnan province, South West China, faculatively an aerobic, alkalitolerant bacteria from hot springs (Zhang *et al*., 2011), *A. thermarum* sp. nov., a novel thermophilic bacterium is isolated from thermal mud in Euganean hot springs, Abano terme, Italy (Poli *et al*., 2011).

A. voinovskiensis sp. nov., refers to the Voinovskie hot springs, the place of isolation, a moderately thermophilic bacterium from a hot springs in Kamchata (Yumoto *et al*., 2004). Three species of the genus referred to as extremophile, which are *A. rupiensis* (Derekova *et al*., 2008), *A. thermarum* (Poli *et al*., 2011) and *A. kamchatkensis* (Kevbrin *et al*., 2006). Extremophiles possess application possibilities in several industrial fields, including agricultural, chemical, laundry, pharmaceutical, food, petroleum and bioremediation (Martinez *et al*., 2010).

Among the 16 species, it has been proven that five of them, such as *A. kamchatkensis* (Kevbrin *et al*., 2006), *A. flavithermus* sp. (Pikuta *et al*., 2000), *A. contaminans* (Declerck *et al*., 2004), *A. amylolyticus* (Poli *et al*., 2006) and *A. rupiensis* (Derekova *et al*., 2007), were aerobes. It is appeared that all the members of this genus show thermophilic features. The species that belong to the genus *Anoxybacillus* show 97% or more 16S rRNA sequence similarity,

while they have proven to be different species by DNA-DNA hybridization. Hence, the 16S rRNA sequences are applicable tools for classifying the genus *Anoxybacillus* in genus level, and inappropriate for species level (Colak *et al*., 2010). In the study of Inan *et al*. (2011) in which they were conducted to investigate the applicability of *rpoB* gene, which encodes the β subunit of RNA polymerase to be used as an alternative to 16S rRNA gene sequence similarity analysis in the thermophilic genus *Anoxybacillus*. The *rpoB* gene was found to provide a better resolution for *Anoxybacillus* species, with lower interspecies sequence similarities. The *rpoB* sequence similarity analysis permitted a more accurate discrimination of the species within the *Anoxybacillus* genus than the more commonly used 16S rRNA gene.

Inan *et al*. (2011) reported that *Anoxybacillus* species are widely distributed and readily isolated from geothermaly heated environments, with a continually increasing industrial interest for their thermostable gene product. Therefore isolating the new strains of this novel bacterial genus is not a taxonomical concern, but also a necessity in order to exploit its biotechnological potential completely.

1.2.2. *Anoxybacillus rupiensis*:

A novel thermophilic, strictly aerobic, Gram positive, spore forming hemo-organotrophic bacterium was isolated from hot springs in the region of Rupi basin, Bulgaria as producers of amylolytic enzymes. It was able to ferment a wide spectrum of carbohedrates such as sugars, polyols, and polysaccharides like xylan, glycogen and starch. Optimal growth was observed at 55-58 ◦C, pH at 6.0-6.5. phylogenetic analysis of the whole 16S rRNA gene sequence clustered the bacterium with the representatives of the genus *Anoxybacillus* and with *Geobacillus tepidamans*. The G+C content of the

genomic DNA was 41.7%. DNA-DNA hybridization analysis revealed low homology with the closest relatives (32.0 mol% homology to *G. tepidamans*). Fatty acid profile confirmed the affiliation of the strain to the genus *Anoxybacillus* (Derekova *et al*., 2007). It was reported that, it has the ability to produce phytase. Phytases (myo-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) catalase the hydrolysis of phytic acid (myoinositol hexakisphosphate) to the mono-, di-, tri-, tetra-, and pentaphosphates of myo-inositol and inorganic phosphate (Mohamad *et al*., 2012). Husein *et al*. (2009) mentioned that a broad range of microorganisms, including bacteria, yeast, fungi, produce phytases. Phytic acid is the primary storage form of phosphate in cereal grains, legumes, and oilseeds, such as soy, which are the principal components of animal feeds. However, monogastric animals are unable to metabolize phytic acid and largely excrete it in their manure. Therefore, the presence of phytic acid in animal feeds for chickens and pigs is undesirable, because the phosphate moieties of phytic acid chelate essential minerals and possibly proteins, rendering the nutrients unavailable. Since phosphorus is an essential element for the growth of all organisms, livestock feed must be supplemented with inorganic phosphate. Phytases are of great interest for biotechnological applications, in particular for the reduction of phytate content in feed and food. Pandey (2001) reported that enzymes used as feed additives should be effective in releasing phytate phosphate in the digestive tract, stable to resist inactivation by heat from feed processing and storage and also cheap to produce. Ibrahim and EI-diwany (2007) indicted that thermophilic bacteria are defined as bacteria capable of living at high temperature. Naturally-occurring phytases having the required level of thermo stability for application in animal feeding have not been found in nature. Therefore, it comes as no surprise that isolation and characterization and engineering of thermostable enzymes from thermophilic bacteria, as well as the search for the determinants of thermostability are hot spots of current research nowadays. Gursahani and

Gupta (2011) elucidated that the release of colored textile effluents is undesirable in the aquatic environment as they reduce light penetration, thereby affecting aquatic life and limits utilization of the water media. Microbial remediation is an alternative treatment option available other than the commonly employed physic-chemical methods to treat these toxic effluents. They investigated the potential of *Anoxybacillus rupiensis*, a thermophilic bacteria isolated from hot water springs of India to decolourize local textile effluent .

1.2.3. Extremophilic bacteria:

Extremophiles are group of microorganisms that thrives in environments previously thought to be hostile to life (Herbert, 1992).

Gomes and Steiner (2004); Mesbah and Wiegel (2008); Podar and Reysenbach (2006) elucidated that discovery and research on extremophiles and their enzymes have provided invaluable data and application possibilities in molecular and evolutionary biology, and occupy an important place in the environmental biotechnology industry. Applications span agricultural, biomedical and industrial sectors such as food laundry, pharmaceutical petroleum and bioremediation.

Thermophiles, microorganisms that grow at temperatures greater than 45 ◦C, are among the best studied extremophiles. The enzymes produced by these microorganisms are extremely thermostable and usually resistant to chemical denaturants such as detergent, chaotropic agents, organic solvent and extreme of pH (Niehous *et al*., 1999; Marchant *et al*., 2002).

Thermophiles are further subcategorized on the bases of their temperature tolerance: for instance, facultative thermophiles, can grow at temperatures between 50-65 ◦C, but also grow at 37 ◦C, obligate thermophiles have

maximum growth temperatures of 65-70 ◦C and will not grow under 40 ◦C, extremely thermophiles can grow between 40-70 ◦C with an optimal temperature of about 65 ◦C and hyperthermophiles mainly comprising of archae, can grow over 90 ◦C with a range of optimal temperature between 80-115 ◦C. (Singh, 2006).

Kikani *et al*. (2010) reported that thermophilic organisms have been less explored due to the difficulties in isolation and maintenance of pure culture. Therefore, it remains to explore their diversity and biotechnological potential from majority of the thermal habitats. As a consequence to growth at high temperature and unique macromolecular properties, thermophiles can possess high metabolic rates, physically and chemically stable enzymes and lower growth but higher end product yields than similar mesophilic species. In addition, cultivation of thermophilic at high temperature is technically and economically beneficial as it reduces risk of contamination and viscosity which leads to high degree of substrate solubility.

1.2.4. Biodegredation:

Biodegradation is a viable bioremediation technology which has become one of the most rapidly developing fields of environmental restoration, utilizing microorganisms to reduce the concentration and toxicity of various chemical pollutants, such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalate esters, nitroaromatic compounds, industrial solvents, pesticides and metals. A number of bioremediation strategies have been developed to treat contaminated wastes and sites. Selecting the most appropriate strategy to treat a specific site can be guided by considering three basic principles: the amenability of the pollutant to biological transformation to less toxic products (biochemistry), the accessibility of the

contaminant to microorganisms (bioavailability) and opportunity for optimizing of biological activity (bioactivity) (Dua *et al*., 2002).

Das and Preethy (2011) reported that currently accepted disposal methods of incineration or burial insecure landfills can become prohibitively expensive when amounts of contaminates are large. Mechanical and chemical methods that generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive. Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization of organic material into carbon dioxide, water and inorganic compounds.

Biodegradation of crude oil by microbial appears to be the natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of petroleum components to lower molecular compounds or transformed into the other organic compounds such as biosurfactants. In another words, biodegradation of crude oil contaminants can be described as the conversion of chemical compounds by microorganisms into energy, cell mass and biological products (Zhang *et al*., 2005), but conditions for down-hole applications require the use of thermopiles, resistant to organic solvent, with heat stable enzymes and reduced oxygen requirements (Van Hamme *et al*., 2003).

1.2.5. Factors influencing petroleum hydrocarbons degradation:

A number of limiting factors have been recognized to affect the bioremediation. The composition and inherent biodegradability of petroleum hydrocarbon pollutant is the first and foremost important consideration when the suitability of a remediation approach is to be assessed (Das and Preethy, 2011). Among physical factors, temperature plays an important role in biodegradation by directly affecting the chemistry of the pollutants as well as

affecting the physiology and diversity of the microbial flora (Atlas, 1975), nutrients are very important ingredients for succeful biodegradation of hydrocarbons pollutants especially nitrogen, phosphorus, and in some cases iron.

1.2.6. Bacteria capable of degrading aromatic compounds aerobically:

A feasible remedial technology requires microorganisms being capable of quick adaption to and efficient uses of pollutants of interest in a particular case in a reasonable period of time.

Sites contaminated by xenobiotics need urgent remedial solutions, the search for which has revealed a diverse range of bacteria that can utilize these xenobiotics as substrates, often mineralizing them or converting them into harmless products, and in the process helping to clean up the environment (Jain *et al*., 2005). The predominant bacteria of polluted soils belong to a spectrum of genera and species are listed in Table (1-1).

Table (1-1) Predominant bacteria in soil samples polluted with aliphatic, aromatic, polycyclic aromatic, and chlorinated hydrocarbons (Wolfgang and Martin, 2008).

Gram negative bacteria	Gram positive bacteria
Pseudomonas spp.	*Nocardia* spp.
Acinetobacter spp.	*Mycobacterium* spp.
Alcaligenes sp.	*Corybacterium* spp.
Flavobacterium /Cytophage group	*Arthobacter* spp.
Xanthomonas spp.	*Bacillus* spp.

1.2.7. Aromatic compounds in the environment:

Aromatic compounds can be defined as organic molecules that are among the most prevalent and persistant environmental pollutants, containing one or more aromatic rings, specifically benzene rings, for example. Different aromatic compounds co-exist as complex mixtures in petroleum refining and distillation sites (Cerniglia, 1992; Cheung *et al*., 2001). Aromatic hydrocarbons enter the global environment through human activities such as crude oil spillage, fossil fuel combustion and gasoline leakage as well as natural inputs like forest fire smoke and natural petroleum seepage. These hydrocarbons comprise simple aromatics like benzene and toluene as well as polycyclic aromatic hydrocarbons (PAHs) from naphthalene to pyrenes, as well as myriad alkyl-substituted isomers. Annually, large inputs of such compounds impact both aerobic and anaerobic environments such as aquifers, surface fresh water bodies, soils, and terrestrial and marine sediments (Foght, 2008).

There are three major categories: polycyclic aromatic hydrocarbons, heterocyclic, and substituted aromatics. PAHs are a group of chemicals that contain two or more fused aromatic rings in linear, angular, or cluster arrangement. Physical and chemical properties of PAHs vary with the number of rings and hence their molecular weight. Chemical reactivity, aqueous solubility and volatility PAHs decrease with increasing molecular weight (Cerniglia, 1992; Cheung *et al*., 2001). These group of chemicals are priority pollutants due to their lipophilic nature, they have the potential to bind to particulates in soil and sediments rendering them less available for microbial uptake and their degrade in soils has become a major concern to environmental researchers (Owabor and Ogunbor, 2006).

Naphthalene has often been used as a model compound to investigate the ability of bacteria to degrade PAHs as shown in Figure (1-1) because it is the

simplest and the most soluble PAH (Goyal and Zylstra, 1997). Therefore, information of bacterial degradation of naphthalene has been used to understand and predict pathway in the degradation of three- or more ring PAHs. Many bacteria that have been isolated and utilized naphthalene as a sole source of carbon and energy belong to the genera *Alcaligenes, Burkholderia, Mycobacterium, Polaromonas, Pseudomonas, Ralstonia, Rhodococcus, Sphingomonas,* and *streptomyces* (Jouanneau *et al*., 2007; Pumphrey *et al*., 2007).

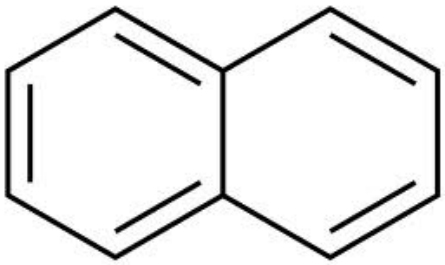

Figure (1-1): Chemical structure of naphthalene

Heterocyclic aromatic compounds including dibenzothiophene, dibenzofuran and carbazole are components of creosote, crude oils, and shale oils often co-exist in the environment with PAHs and other aromatic compounds (Max, 1974).

Dibenzothiophene is a sulfur heterocyclic compound and is quite persistent in the environment. Dibenzofuran and its substituted analogus are found in several woody plants as stress chemicals (Gottstein *et al*., 1992). Carbazole as shown in Figure (1-2) a nitrogen heterocycle, is carcinogenic and toxic (Tsuda *et al*., 1982), but has been used as an industrial raw material for the production of dyes, medicines and plastics (Benedik *et al*., 1998).

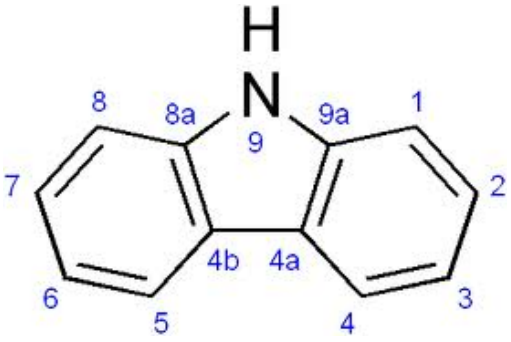

Figure (1-2): Chemical structure of carbazole

Wang *et al*. (2007) elucidated that carbazole and its derivatives are common N-compounds founds in environments contaminated by coal tar and creosote. When these N-compounds are combusted, nitrogen oxides (NOX) are released in to atmosphere, which causes acid rain and air pollution. Moreover, carbazole and its derivatives have been found to be toxic and mutagenic, and they readily undergo radical chemistry to generate the more poisonous hydroxynitrocarbazole. Soil, river sediments, and ground water polluted by carbazole have become a great threat to the environment.

Snyder and Saunder (1979) stated that the three major groups of nitrogen compounds in petroleum fractions boiling below 538 ◦C are pyridine derivatives (including quinolines and phenanthridines), indol derivatives (including Pyrrol, carbazole, and benzo carbazole), and aromatic amides. The presence of nitrogen compounds in oils can lead to the poisoning of catalysts used in the cracking of oil for commercial uses (Fdorak and Donald, 1984). Many researchers have used enrichment cultures to isolate prokaryotes that are capable of degrading pure nitrogen heterocycles. For examples, Watson and Cain (1975) described the metabolism of pyridine by a *Bacillus* sp. and a *Nocardia* sp., both of which use this compound as a sole carbon, nitrogen, and energy source. Finnerty *et al*. (1983) reported studies on four bacterial isolates which are able to use carbazole as their sole source of carbon, nitrogen, and energy.

Ye *et al*. (2004) illustrated that nitroaromatic compounds constitute a major class of widely distributed environmental contaminants. Compounds like nitrobenzene, nitrotoluenes, nitrophenols, nitrobenzoates and nitrate esters are of considerable industrial importance. They are frequently used as pesticides, explosives, dyes and in the manufacture of polymers and pharmaceuticals. Many nitroaromatic compounds and their conversion products have been shown to have toxic or mutagenic properties. Most of them are biodegradable in nature by various microorganisms. Nitrobenzene as shown in Figure (1-3) is produced annually in the order of 225000 metric tons and it has been estimated that as much as 2000 metric tons of nitrobenzene is discharged annually into natural waters. The presence of these aromatic xenobiotics in the environment may create serious public health and environmental problems. Some of these compounds have mutagenic or carcinogenic activity and may bio accumulated in the food chain (Donlon *et al*., 1995).

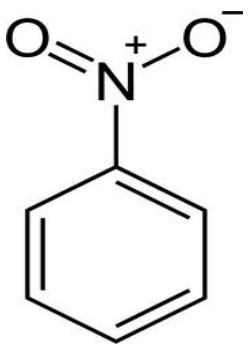

Figure (1-3): Chemical structure of nitrobenzene

1.2.8. Biodegradation of aromatic hydrocarbons:

Aromatic compounds of both natural and man-made sources abound in the environment. The degradation of such chemicals is mainly accomplished by microorganisms (Smith, 1990). Biodegradation can achieve complete and cost-effective elimination of aromatic pollutants through harnessing diverse

microbial metabolic processes. Aromatics biodegradation plays an important role in environmental cleanup and has been extensively studied since the inception of biodegradation (Cao *et al.,* 2009).

Abayomi (2006) stated that aromatic compounds carrying substituents form a special class of xenobiotics because of their recalcitrance, and they have been known to belong to the family of carcinogens and neurotoxic organic compounds.

The aromatic hydrocarbons are important components of petroleum and its refined products. Aromatic compounds are broadly defined to be benzene and other compounds that exhibit chemical behavior, benzene and substituted benzene constitute the naturally occurring aromatic hydrocarbons. Among the most important aromatic petroleum hydrocarbons are benzene, toluene, ethylbenzene, xylene and the polycyclic aromatic hydrocarbons, of which naphthalene is the simplest representative (Gibson and Subramanian, 1984).

1.2.8.1. Biodegradation of benzene:

Because all of the important aromatic hydrocarbons that occur in petroleum are derivatives of benzene, a reaction that is common to all pathways that lead to mineralization of aromatic substrates is cleavage of the benzene ring. Therefore, biodegradation pathways for aromatic hydrocarbons begin with a description of the pathways used for mineralization of benzene itself. Molecular oxygen serves a reactant in two steps in the pathways for benzene catabolism. In each of these reactions, both atoms from molecular oxygen become incorporated into the substrate. Enzymes that catalyze such reactions are called dioxygenases (Sheldon and Kochi, 1981). The stoichiometry of dioxygenase-catalyzed reactions can be written as:

S + O2 ------> SO2 (R 2-2)

Note that a hydrogen donor, such as NADH, is not always required as a co-substrate in dioxygenase-catalyzed reactions, whereas hydrogen-donating co-substrates are always required for the monooxygenase-catalyzed hydroxylation of alkanes. Ring cleavage and subsequent bacterial metabolism of benzene requires that the aromatic ring be destabilized, that is, it must be made more reactive. This is accomplished by a dioxygenase-catalyzed reaction between benzene and molecular oxygen, resulting in production of benzene dihydrodiol (i.e., *cis* -1, 2-dihydroxycyclohexa-3, 5-diene) (Ribbons and Eaton, 1982; Gottschalk, 1986). Aromaticity is restored by a dehydrogenase-catalyzed conversion of benzene dihydrodiol to catechol (i.e., 1, 2-dihydroxybenzene), which is the ring cleavage substrate. The reactions leading to catechol are shown in Figure (1- 4). The dioxygenases that catalyze hydroxylation of benzene rings require an electron-donating co-substrate in order to function. The dioxygenases that catalyze the ring cleavage reactions that are described below do not require a co-substrate. The hydroxylating dioxygenases are multicomponent systems that resemble the bacterial monooxygenases: a flavoprotein accepts electrons from NADH and passes them through a ferredoxin to the dioxygenase. The reduced dioxygenase reacts with O2 and the aromatic substrate (Gibson and Subramanian, 1984).

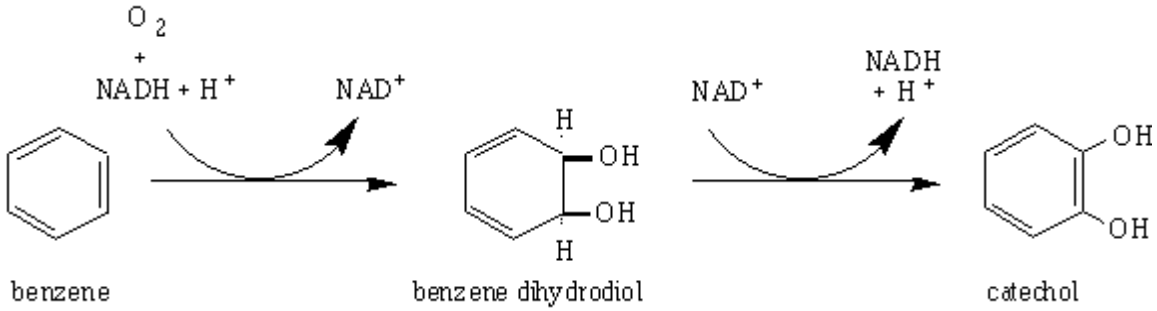

Figure (1- 4): Oxidation of benzene to catechol

(Gibson and Subramanian, 1984)

Catechol is catabolized by ring cleavage, in which the aromatic ring is broken. Ring cleavage can occur by either of two pathways: the ortho-cleavage pathway, in which the aromatic ring is split between the two carbon atoms bearing hydroxyl groups, or the meta-cleavage pathway, in which the ring is broken between a hydroxylated carbon atom and an adjacent unsubstituted carbon atom (Gottschalk, 1986). Each of these ring-cleavage reactions is catalyzed by a dioxygenase. The subsequent metabolic pathways are quite different, but they both lead to TCA cycle intermediates (acetate and succinate) or to substrates that can be easily converted to TCA cycle intermediates (pyruvate and acetaldehyde). The ortho-cleavage pathway (also called the β-ketoadipate pathway) is shown in Figure (1-5), and the meta-cleavage pathway is presented in Figure (1-6).

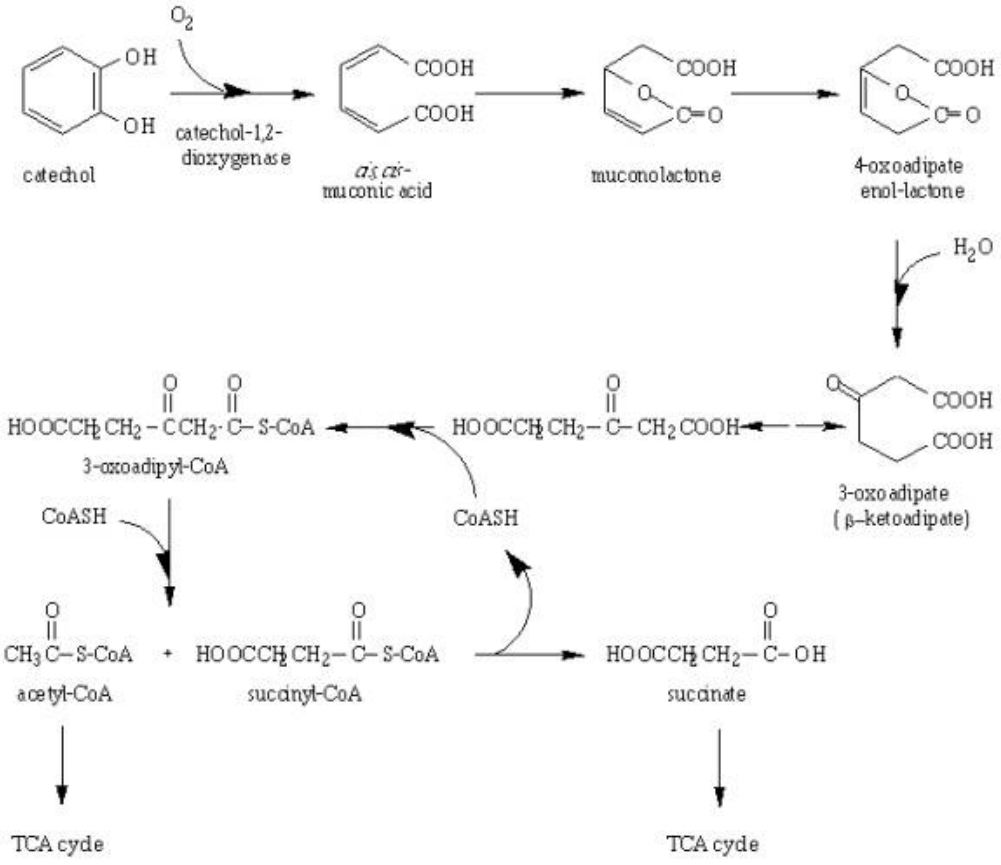

Figure (1-5): *Ortho*- cleavage pathway for catabolism of catechol (Gibson and Subramanian, 1984)

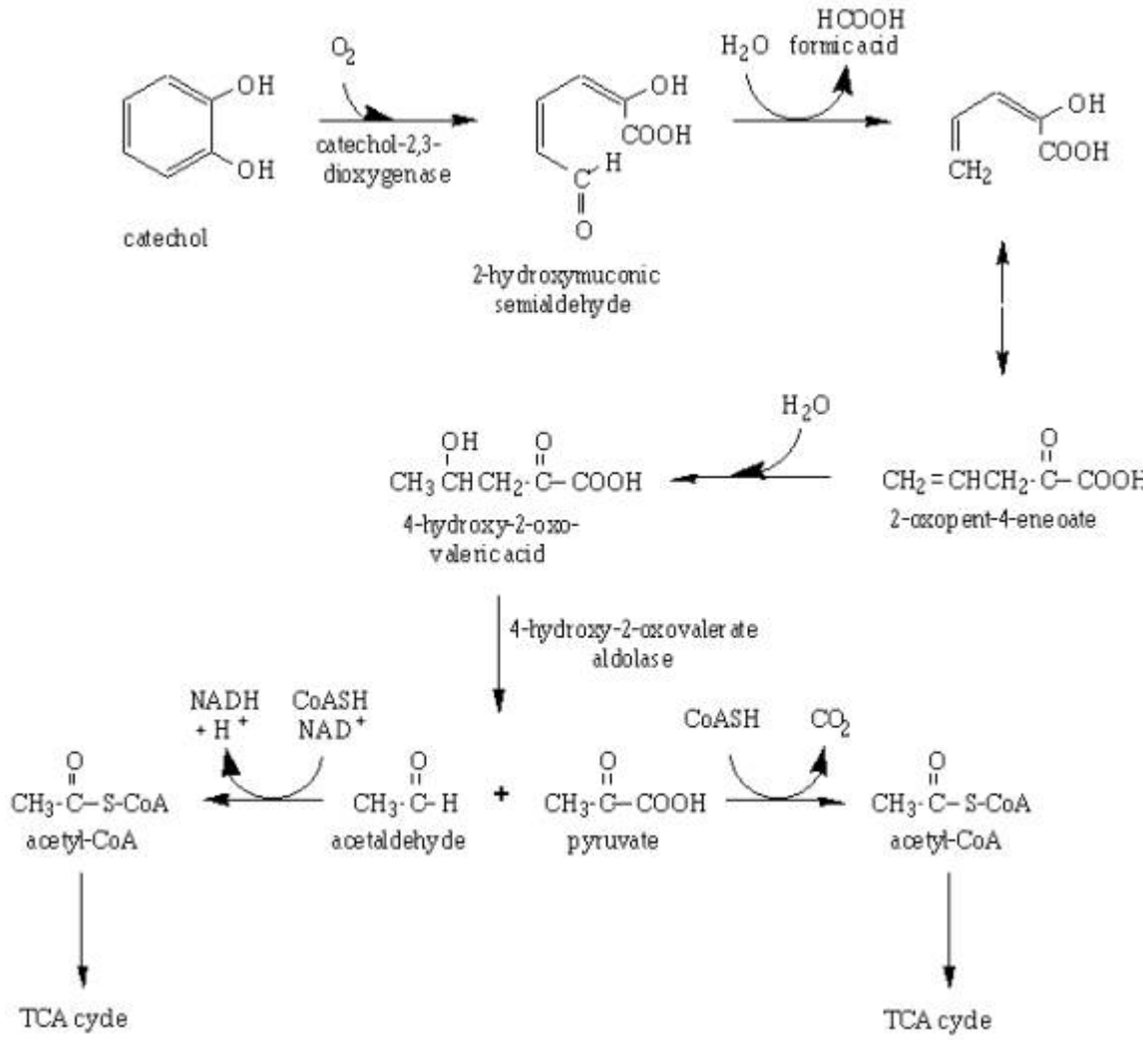

Figure (1-6): *Meta*- cleavage pathway for catechol catabolism (Gibson and Subramanian, 1984).

The aerobic degradation of aromatic compounds in general can be divided into three basic steps: (i) activation of the aromatic ring (ii) ring cleavage (iii) breakdown of the cleavage products to krebs cycle intermediates (Omokoko *et al*., 2008). This included members from the group *Pseudomonas*,

Ralstonia, Burkholderia, Sphingomonas, Flavobacterium and *Bacillus* (Stapleton *et al*., 2000).

Chakrabarty (1985) found that aerobic bacteria are an excellent choice for application in oil recovery. They are known to degrade hydrocarbons and oil efficiently. Many of them produce potent surface. Active component can greatly reduce the interfacial tension between the oil-water interfaces, there by facilitating the migration of oil into flood water.

1.2.9. Mechanism of aerobic catabolism:

The aerobic catabolic includes pathways involving oxygenation reactions (enzymatic key reaction) carried out by monooxygenase or hydroxylating dioxygenases and generates dihydroxy aromatic compounds. These intermediate compound are processed through either ortho or meta cleavage leading to central intermediates such as protocatechuates, catechols, gentisates, homoprotocatechuates, homogentisates, hydroquinones and hydroxyquinols, which are further transformed to tricarboxylic acid cycle intermediate and finally channeled into the intermediates of Krebs cycle (Sinha *et al*., 2009). Oxygenases belong to the oxidoreductase group of enzymes. They participate in oxidation of reduced substrates by transferring oxygen from molecular oxygen (O_2) utilizing FAD/NADH/NADPH as a co substrate (Chandrakant *et al*., 2011).

Microorganisms are known to degrade both aliphatic and aromatic hydrocarbons as mentioned above: clusters of genes coding for the catabolism of aromatic compounds are usually found in mobile genetic elements, such as transposons and plasmid, which facilitate their horizontal transfer and therefore, microorganisms are rapidly adapted to new pollutants (Diaz, 2004).

Van Hamme *et al*. (2003) indicated that the majority of information on the genetics of PAH degradation has come from the studying of naphthalene catabolic plasmids such as NAH7 from *Pseudomonas putida* strain G7. In this well characterized system, the first operon (nahAaAbAcAdBfCED) encodes the pathway for naphthalene conversion to salicylate, and the second (nahGTH1NLoMky) codes for the conversion of salicylate via catechol meta-cleavage to acetaldehyde and pyruvate. The regulator for both operons is encoded by a third operon containing nah R, which is induced by a salicylate. Here, molecular oxygen is introduced into the aromatic nucleus via naphthalene dihydrodiol, which is subsequently converted to salicylate and then to tricarboxylic acid intermediates as shown in Figure (1-7). Naphthalene dioxygenase is now known to be a verstail enzyme, able to catalase a wide variety of reactions. Molecular and biochemical evidences show that the naphthalene plasmid degradative enzyme system could mineralize other PAHs, such as phenanthrene and anthracence.

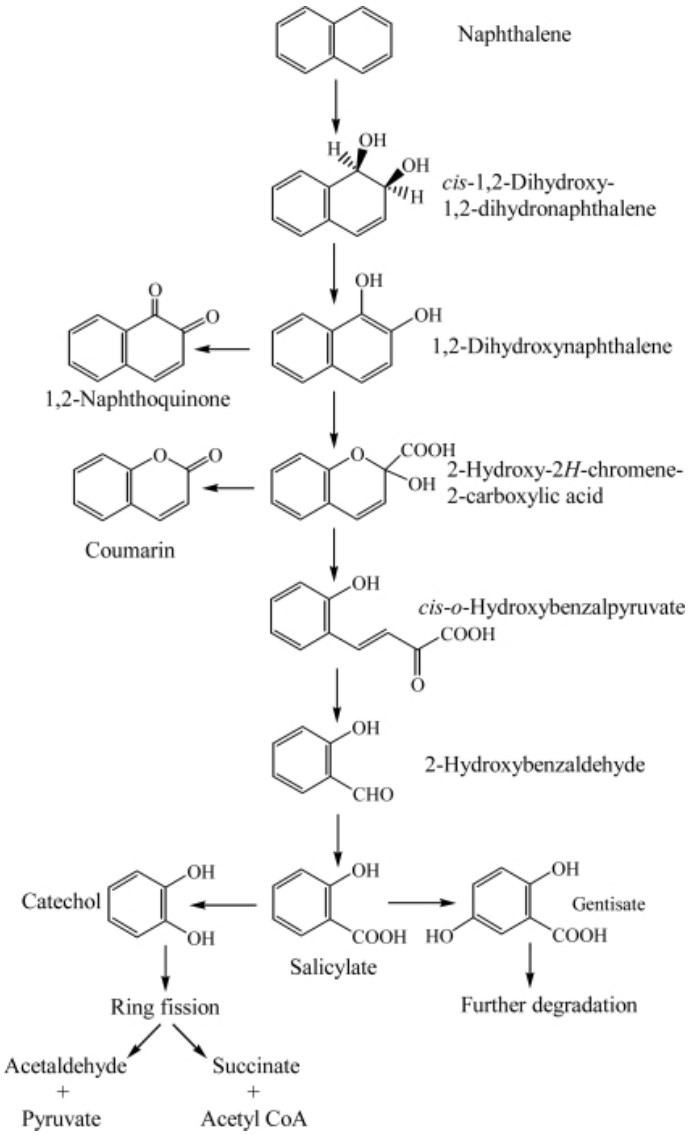

Figure (1-7): Proposed catabolic pathways of naphthalene by bacteria (Baboshin *et al*., 2008).

Carbazole (CAR) is a heterocyclic aromatic compound containing a dibenzopyrrol system as it appears in Figure (1-2), being derived from coal tar and shale oil and serious problems arise if it is released into the environment. The initial step of degradation of CAR is considered to be dioxygenation at the angular position adjacent to the nitrogen atom. The car and ant operons originally isolated from *Pseudomonas resinovorans* strain CA10 contain the genes encoding the carbazole/ dioxin- degrading enzymes and anthranilate 1, 2-dioxygenase, respectively, and are located on the plasmid pCAR1. The entire

nucleotide sequence of pCAR1 was determined to elucidate the mechanism by which the car operon may have been assembled and distributed in nature. pCAR1 is a 199,035 bp circular plasmid, and carries 190 open reading frames (Maeda *et al*., 2003).

Inoue *et al*. (2006) mentioned that the most important enzymes for CAR breakdown is carbazole 1, 9 a dioxygenase (CARDO; encoded by carAa, carAC, and carAd), the meta- cleavage enzyme (encoded by carBa and car Bb), and the meta- cleavage compound hydrolase (encoded by car C) together are involved in the conversion of carbazole to anthranilate and 2-hydroxypenta-2,4-dienoate (Figure 1-8). 2- hydroxypenta-2,4-dienoate is converted to pyruvic acid and acetyl coenzyme A by 2-hydroxypenta-2,4-dienoate hydratase (encoded by carD), 4-hydroxy-2-oxovaleate aldolase (encoded by carE), and acetaldehyde dehydrogenase (encoded by carF)

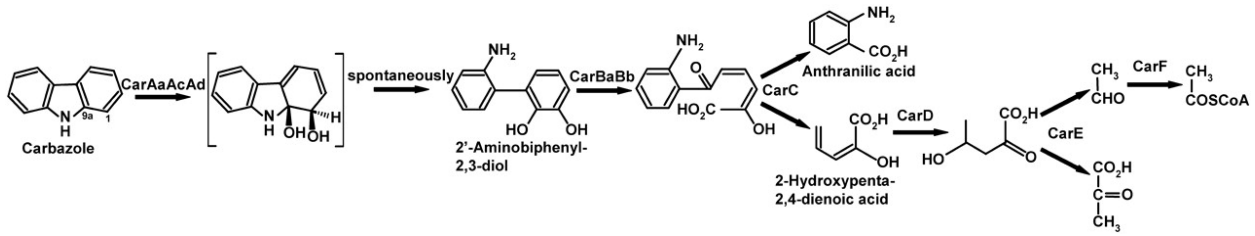

Figure (1-8) pathway for conversion of carbazole to anthranilate and 2-hydroxypenta-2, 4-dienoate (Inoue *et al*., 2006).

Microorganisms may simply transform nitroaromatic compounds to dead end product, or they may actually utilize the nitroaromatic compound as a carbon and /or nitrogen source (Yabannavar and Gerben, 1995). Several catabolic pathways are now known for this microbially mediated degraditive process, starting from a variety of nitroaromatic substrates. In general, these processes can be grouped by whether the nitro group is removed as nitrite from the parent compound or is eliminated in the form of ammonia. For example, monooxygenase have been implicated in the direct removal of the nitro group

from the aromatic ring for ρ-nitrophenol and o-nitrophenol degradation (Raymond and Alexander, 1971). ρ- nitrophenol as shown in Figure (1-9) is among the nitroaromatic compound that founds in many different environments, used on a large scale in the synthesis of the aspirin substitute acetaminophen and in the manufacture of pesticides such as parathion and methylparathion (Kitagawa *et al*., 2004).

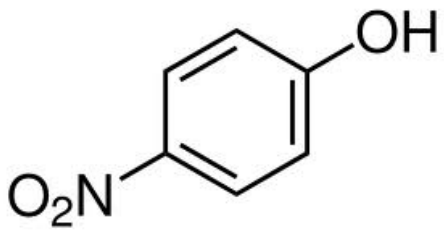

Figure (1-9) Chemical structure of ρ-nitrophenol

Dioxygenases have implicated in the direct removal of the nitro group from the aromatic ring during the degradation of 2,4-dinitrotoluene, 2,6-dinitrophenol, 2-nitrotoluene, nitrobenzene and m-nitrobenzoate (Yabannavar and Gerben, 1995).

1.2.10. Crude oil constituents:

Crude oil is a complex mixture of hydrocarbons, basically composed of aliphatic, aromatic and asphaltene fractions a long with nitrogen, sulfur and oxygen containing compound (Speight, 1999). These compounds are carcinogenic, mutagenic and have immunomodulatory effects on humans, animals and plant life (Miller and Miller, 1981; Van Gestel *et al*., 2001). The sites contaminated with hydrocarbons are ecologically important locations as one may encounter microbial flora of diverse nature, which may be potential candidates for important industrial processes. There is a plethora of cultivable microbes with the ability to utilize hydrocarbons as sole source of carbon or to

transform them to a less toxic form (Leahy and Colwell, 1990; Cerniglia, 1993; Kanaly and Harayama, 2000).

Earlier reports based on cultivable bacteria suggested that hydrocarbon-contaminated soil is predominated by Gram-negative bacteria (Macnaughton *et al*., 1999). The culture independent studies from contaminated soil samples have revealed existence of new bacterial lineages (Bakonyi *et al*., 2003). Studies with molecular tools indicate that specific groups of bacteria commonly occur in oil- contaminated environment (Watanabe, 2001).

Kilbane and Borgne (2004) mentioned that the quality of petroleum is progressively deteriorating as the highest quality petroleum deposits are preferentially produced. Consequently, the concern about the concentration of compounds/ contaminants such as sulfur, nitrogen and metals in petroleum will intensify. These contaminants not only contribute to environmental pollution resulting from the combustion of petroleum but also interfere with the processing of petroleum by poisoning catalysts and contributing to corrosion. The selective removal of contaminants from petroleum while retaining the fuel energetic value is a difficult technical challenge. New processes are needed and bioprocesses are the option. The sulfur content of crude oil can vary from 0.03 to 7.89%, sulfur present in crude oil almost exclusively as organic sulfur. The sulfur, nitrogen and metal content is preferentially associated with the higher molecular weight component of crude oils and consequently, heavy crude oils typically have higher sulfur, nitrogen, and metals content than light crude oils. Similarly, when crude oil is refined, the sulfur, nitrogen, and metals concentrate into the higher molecular weight fractions.

Kotlar *et al*. (2004) reported that nitrogen compounds are typically found in crude oil which consists of heterocycles such as quinoline and carbazole, which are examples of basic and non-basic organo-nitrogen compound respectively.

The total nitrogen content of crude oil is typically about 0.3%, but it can be as high as 5%. Basic organonitrogen compounds in petroleum usually comprise 25 to 30% of the total nitrogen and include compounds such as quinoline and pyridine. Non basic organonitrogen compounds typically comprise 70- 75%of the total nitrogen in crude oils and alkylated derivatives of carbazole are the most typically found non-basic organonitrogen compounds. The heavy metals found in greatest abundance in crude oil are nickel and vanadium. Both of them are potent inhibitors of refining catalysts. These heavy metals are typically associated with nitrogen compound.

1.2.11. Biorefining:

Petroleum refining is traditionally based on the use of physicochemical processes such as distillation and chemical catalysis that operate under high temperatures and pressures conditions, which are energy intensive and costly. Biotechnology has become an important tool for providing new approaches in petroleum industry during oil production, refining and processing (Singh *et al*., 2012).

Le Borgne and Quintero (2003) illustrated that biorefining is the use of living organism or part of them like enzymes in order to upgrade petroleum, that is, the application of bioprocesses to the fractionation and enhancing of petroleum, which might contribute to mitigate the associated pollution and up-grading of heavy crude . The microbial and enzymatic catalysis can be manipulated and used for more specific applications where the chemical processing requires several steps. It is proposed that heavy crude oil and some fractions like gas-oil, gasoline and diesel may be subjected to biorefining to get off most of the sulfur, nitrogen, toxic metals, asphaltenes which are mainly associated with this heaviest fraction in petroleum.

1.2.12. Microbial denitrogenation (Biodenitrogenation):

Nitrogen is typically found in petroleum as non-basic and basic related compounds, which contribute to acid and atmospheric contamination and also interfers with the refining processes, leading to equipment corrosion and catalyst poisoning (Montiel *et al*., 2009).

Van Hamme *et al*. (2003) found that crude oil contains about 0.5 to 2.1% nitrogen, with 70 to 75% consisting of pyrrols, indols and carbazole non basic compounds. Carbazole is a potent inhibitor of hydrodesulfurization poisons cracking catalysts, and it is toxic, mutagenic, and contributes to the formation of undesirable air-polluting nitric oxides

Nitrogenous compounds are generally eliminated from petroleum by expensive hydrotreatment under high temperature and pressures. Quinoline and carbazole are the most widely studied as regard biodegradation. Several species of bacteria that can utilize indole, pyridine, quinoline, carbazole and its alkyl derivatives have been isolated and characterized including *Alcaligenes, Bacillus, Beijerinckia, Burkholderia, Comamonas, Mycobacterium, Pseudomonas, Serratia,* and *Xanthomonas* (Bouchez *et al*., 1995; Kaiser *et al*., 1996).

The major barrier to using a microbial process to remove nitrogen from crude oil is the same as that for desulfurization, namely, the need to create an oil- water two phase system. Removal of nitrogen and sulfur requires specific attack of the C-N and C-S bonds, respectively, but not C-C bond attack, thus preserving the fuel value of the residual products. To make economic sense, denitrogenation processes need to be integrated with a crude oil desulfurization (Benedik *et al*., 1998). In other words, removal of these organonitrogen compounds will not only significantly improve the efficiency of the catalytic cracking process and result in cost saving for the refinery but also decreasing atmospheric pollutions (Kilbane, 2002).

Chapter Two

Materials and Methods

Chapter Two

Materials and Methods

2.1. Materials:

2.1.1. Equipments and Apparatus:-

The following equipments and apparatus were used during this study:

Equipment	Company (Origin)
Autoclave	Express (Germany)
Balance	Ohans (France)
Block heater	Techne (UK)
Centrifuge	Hermle Z_{200} A (Germany)
Compound Light Microscope	Olympus (Japan)
Cooling centrifuge	Harrier (U.K)
Eppendorf bench centrifuge	Netherler and Hinz (Germany)
HPLC , FTIR	Shimadzu (Japan)
Incubator	Termaks (U.K)
Laminar air flow hood	Heraeus (Germany)
Micropipette	Witey (Germany)
Minimal electrophoresis apparatus	Bio Rad (Italy)
Oven	Gallen Kamp Sayo(U.K)
pH-meter	Metler Toledo (U.K)
Sensitive balance	Metler AE $_{260}$ (Switzerland)
Shaker incubator, water bath, distillator	GFL (Germany)
Spectrophotometer	Aurora Instrument Ltd (England)
T-5000 thermo cycle PCR machine	Biometra (UK)
UV-transilluminator	Cross-linker (France)
Vortex	Giffin(England)

2.1.2. Chemicals:

All chemicals and biochemicals were analytical grade of polycyclic aromatic hydrocarbon (naphthalene), heterocyclic aromatic hydrocarbon (carbazole) and nitroaromatic compounds (nitrophenol and nitrobenzene) were purchased from BDH- England and Merk- Germany.

Other chemicals, biochemicals and cultural media were purchased from Difico (France), Fluka (Switzer-land) and Sigma (USA). Molecular biology reagents and enzymes were purchased from Promega (USA) and Qiagen (Germany).

2.1.3. Antibiotics

The following antibiotics discs were used during this study:

Antibiotics	Abbreviation	Concentration(µg/disc)
Ceftazidine	CAZ	30
Clarithromycin	CLR	15
Ciproflaxacin	Cip	5
Bacitracin	BA	10
Cephalexin	CL	30
Gentamycin	CN	10
Oxacillin	OX	1
Nitrofurontoin	F	300
Pipracillin	PRL	100
Nalidixic acid	NA	30
Cefotaxime	CTX	30
Amikacin	AK	30
Aztreonam	ATM	30
Penicillin	P	10

Antibiotics	Abbreviation	Concentration(µg/disc)
Vancomycin	VA	30
Tobramycin	TOB	10
Chloramphenicol	C	30
Tetracycline	TE	30
Erythromycin	E	15
Carbenicillin	CAR	100

2.1.4. Media:

2.1.4.1. Ready to use media:

These media were prepared according to the manufacturing companies and sterilized by autoclaving at 121°C for 15 min:

Medium	Company (Origin)
Brain heart infusion agar	Difco (U.S.A)
Simmon citrate media	
Triple sugar iron (TSI) agar	
Urea agar base	Biolife (Italy)
Nutrient broth	Oxiod (England)
Nutrient agar	

2.1.4.2. Laboratory prepared media

- **Skim milk agar medium (Sneath *et al*., 1986)**

  This medium was prepared by dissolving 5g of skim milk in 50mL of distilled water (D.W.) and sterilized by autoclaving, and then 2g of agar were dissolved in 50ml D.W. and sterilized by autoclaving, cooled into 45C◦, mixed together then distributed into sterilized plates.

- **Peptone Water Medium (Atlas *et al*., 1995)**

  This medium was prepared by dissolving thoroughly 20g peptone and 5g of NaCl in a quantity of D.W., the volume was completed to 1L and pH was adjusted to 7 before distributing into test tube (10ml each) and autoclaving.

- **Methyl Red Voges-Proskauer (MR-VP) Medium (Atlas *et al*., 1995)**

  This medium was prepared by dissolving peptone (5g) and K_2HPO4 (5g) in 900 ml of D.W.; pH was adjusted to 7.6, the volume was completed to 950 ml with D.W. and sterilized by autoclaving, then 50 ml of 10% glucose solution(sterilized by filtration) was added.

- **Urea Agar (Collee *et al*., 1996)**

  It was prepared by adjusting pH of the urea agar base to 7.0 and autoclaved, after cooling to 50˚C, 50 ml of 40% urea (previously sterilized by filtration) was mixed with it and distributed into sterilized test tubes (20ml each), then kept slant.

- **Nitrate medium (Atlas *et al*., 1995)**

  This medium was composed of 5g peptone supplemented with 0.2g KNO_3 in 1L of D.W., pH was adjusted to 7, and distributed into tubes, then sterilized by autoclaving.

- **Gelatin medium (Cruickshank *et al*., 1975)**

  This medium was prepared by dissolving 12g of gelatin and completed to 100ml of nutrient broth medium, then distributed in tubes and sterilized by autoclaving.

- **Cetrimid agar (Stolp and Gadkari, 1984)**

This medium is composed of the following components (g/L):-

Peptone	20
$MgCl_2$	4.5
K_2So_4	10
Cetrimide	0.3
Agar	15

The components were dissolved into part of the D.W., then the pH was adjusted to 7.2 and the volume completed with D.W. to 1L before sterilized by autoclaving at 121˚C for 15 min.

- **Semi solid agar (Collee *et al*., 1996)**

  It was prepared by dissolving 0.4% of agar in nutrient broth or peptone water, in which the final medium should be quite clear and transparent. Then 10ml was dispensed in test tubes, sterilized by autoclaving and left to stand in a vertical position.

- **Luria-Bertani (LB) medium (Nazina *et al*., 2001).**

  This medium was prepared by dissolving tryptone (10g), yeast extract (5g) and NaCl (5g) in 950ml D.W., pH was adjusted to 7, then the volume was completed to 1L, sterilized by autoclaving, cooled to 45◦C then 1 ml of 0.1M $MnCl.H_2O$(autoclaved separately) was added.

- **Minimal medium is a chemically defined medium (CDM) (AL-Dousary, 2004).**

  Chemically defined media were prepared from the following stock solutions as given in Table (2-1):

Table (2-1): Minimal medium is a chemically defined medium (CDM):

Stock solutions	Volume added (ml) per 1L medium					
	Control medium	Carbazole medium	ρ-Nitrophenol medium	Nitrobenzenemedium	Naphthalenemedium	Oil medium
Phosphate buffer (1M)	50	50	50	50	50	50
NH_4Cl (1M)	5	-	-	-	5	-
Carbazole(100mM)	-	10	-	-	-	-
ρ-Nitrophenol (100mM)	-	-	10	-	-	-
Nitrobenzene(100mM)	-	-	-	10	-	-
Naphthalene(100 m M)	-	-	-	-	10	-
Crude oil	-	-	-	-	-	10
$MgSO_4.7H_2O$(1 M)	1	1	1	1	1	1
$CaCl_2.2H_2O$(0.3 M)	1	1	1	1	1	1
Trace element	1	1	1	1	1	1
Vitamine solutions (VL7)	1	1	1	1	1	1
Glucose (1M)	10				-	-
Distilled H_2O	931	936	936	936	931	936
Final volume	1L	1L	1L	1L	1L	1L

2.1.5. Preparation of stock solutions

2.1.5.1. K-phosphate buffer (1M, pH 7.2)

K_2HPO_4	140 g/L
KH_2PO_4	27 g/L

2.1.5.2. NH_4Cl (1M) 53.48 g/L

2.1.5.3. Mg SO_4. $7H_2O$ (1M) 248 g/L

2.1.5.4. $CaCl_2.2H_2O$ (0.3M) 44 g/L

2.1.5.5. Trace elements (Van Hamm *et al*., 2000)

$FeSO_4.7H_2O$	2.1g/L
$ZnSO_4$. $7H_2O$	150mg/L
$MnSo_4.H_2O$	85mg/L
$CuSo_4.5H_2O$	37 mg/L
$CoCl_2.6H_2O$	200mg/L
$Na_2MoO_4.2H_2O$	40mg/L
$NiCl_2.6H_2O$	20mg/L
H_3BO_3	20mg/L

2.1.5.6. Vitamins (Pfennig, 1978):

Cyanocobalamin (B_{12})	25mg/250ml
Thiamin-HCl	75mg/250ml
Nicotinic acid	50mg/250ml
Biotin	40mg/ 250ml

2.1.5.7. Glucose solution (1M) 180 g/L

- **2.1.5.8. PAH and N-compounds (AL-Dousary, 2004).**

Naphthalene (Naph)	(100mM in DMSO)	0.640 g/50ml
Carbazole (CAR)	(100mM in DMSO)	0.835 g/50ml
Nitrobenzene	(100mM in DMSO)	0.765 g/50ml
ρ-Nitrophenol	(100mM in DMSO)	0.965 g/50ml

The basal medium, which consisted of phosphate buffer (pH 7.2), was autoclaved and the other components of the media were added from sterile stock solutions as indicated in Table (2-1). Aromatic compounds solutions, vitamins solutions, trace elements solution and glucose solutions were sterilized by filtration, crude oil was sterilized by tyndallization whereas all other stock solution were sterilized separately by autoclaving.

2.1.6. Reagents solutions, Buffers and dyes

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

This solution consists of 3% hydrogen peroxide.

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

This solution is freshly prepared and consists of 1% tetramethyl-*p*-phenylenediaminedihydrochloride in D.W.

2.1.6.3. Methyl red indicator (Collee *et al*., 1996)

This indicator was prepared by mixing the following components

Methyl red	0.05g
Ethanol 95%	150ml
D.W.	100ml

2.1.6.4. Barritt's reagent (Collee *et al*., 1996)

It consists of two solutions:

Solution A: Potassium hydroxide (40%)

Solution B: It was prepared by dissolving 5 g of α-naphthol into 100 ml of absolute ethanol.

Equal volumes of solution A and solution B were immediately mixed before use.

2.1.6.5. Nitrate test reagent (Atlas *et al*., 1995).

This reagent consists of two solutions:

Solution A: it was prepared by adding 0.8g of sulfanilic acid to 5N acetic acid up to 100ml.

Solution B: it was prepared by adding 0.5g of dimethyle- α-naphthylamine to 5N acetic acid up to 100ml.

Equal volumes of solution A and solution B were immediately mixed before use.

2.1.6.6. Kovac's reagent (Atlas *et al*., 1995)

Isoamyl alcohol	150ml
ρ-Dimethyl-aminobenzaldehyde	10g
Concentrated HCl	50ml

ρ-Dimethyl-aminobenzaldehyde was dissolved in isoamyl alcohol with heating in a water bath at 50^{o}C, and acid was slowly added. The reagent was prepared in small quantities and stored in the refrigerator.

2.1.6.7. Gram stain (Atlas *et al.*, 1995)

It was composéd of 4 reagents:

1-A primary stain- crystal violet.

2-Mordent- iodine solution.

3-A decolorizing agent-an organic solvent(alcohol).

4-A secondary stain- safranin.

2.1.6.8. Ethidium bromide solution (10 mg / ml) (Sambrouk and Russell, 2001)

It was prepared by dissolving 0.2g of ethidium bromide in 20ml distilled water and stirred on magnetic stirrer for four hours to ensure that the ethidium bromide was dissolved, then it was filtrated, and stored in a dark bottle at 4ºC, until used.

2.1.6.9. Potassium phosphate buffer (pH=7.0) (Atlas *et al*., 1995).

KH_2PO_4 50mM

K_2HPO_4 50mM

2.1.7. Kits

2.1.7.1. Genomic DNA isolation by Wizard genomic DNA purification kits solutions (www.promega.com), USA.

2.1.7.2. Plasmid isolation by gene JET™ plasmid miniprep kit solutions (www.fermentas.com).

2.1.7.3. Plasmid isolation by Pure Yield™ plasmid miniprep kit solutions (www.promega.com),USA.

2.1.7.4. Plasmid and Genomic extraction buffers and solutions.

2.1.7.4.1. TE buffer (Sambrouk and Russell, 2001)

EDTA	1mM
Tris-OH	10mM

PH was adjusted to 8.0 and sterilized by autoclaving.

2.1.7.4.2. SET buffer (Sambrouk and Russell, 2001)

EDTA	25mM
Tris-HCl	20mM
NaCl	75mM

PH was adjusted to 8.0 and sterilized by autoclaving.

2.1.7.4.3. NaCl solution (5M) in distilled water (Kieser, 1995).

2.1.7.4.4. SDS solution

It was freshly prepared by dissolving 10g in 100ml of sterile distilled water.

2.1.7.4.5. Lysozyme solution (Kieser, 1995).

This solution was freshly prepared by dissolving 50mg lysozyme enzyme in 1ml of sterile distilled water.

2.1.8. Curing experiments:

2.1.8.1. Curing solutions:

It was prepared as stock solutions of SDS (10% w/v) and Ethidium bromide (5000µg/ml) in distilled water.

2.1.9. Electrophoresis buffer

-Tris-Borate-EDTA (5X) (TBE), pH 8.0 (Sambrouk and Russell, 2001)

Tris base (2M)	54 g
Boric acid	27.5 g
EDTA (0.5 M)	20 ml
D. W. to volume	1000 ml

- Ethidium bromide (Sambrouk and Russell, 2001).

It was prepared in concentration 10µg/ml in distilled water and store at 4 ◦C.

- Gel loading buffer (6X) (Sambrouk and Russell, 2001).

Bromophenol blue	0.25 % (w/v)
Sucrose in D.W.	40% (w/v)

2.1.10. Gel extraction by QIAquick gel extraction kit solutions (www. QIAGEN. Com), Germany.

2.1.11. Polymerase chain reaction (PCR) solutions:

2.1.11.1. PCR master mix (2X) (Intron Biotechnology, Inc.South Korea)

DNA polymerase (5U/µl)	2.5U
dNTPs	2.5mM each
PCR reaction buffer	1X
Gel loading buffer	1X

2.1.11.2. Primers (Integrated DNA Technologies, http//www:idtdna.com):

The following primers were used in this study to identify the aromatic degrading bacteria.

Primer name	Primer sequence 5'----3'	Uses	References
fD1	CCGAATTCGTCGACAACAGAG TTTGATCCTGGCTCAG	Most eubacteria	Weisburg *et al*., 1991
fD2	CCGAATTCGTCGACAACAGAG TTTGATCATGGCTCAG	Enteric and relatives	
fD3	CCGAATTCGTCGACAACAGAG TTTGATCCTGGCTTAG	Borrelia spirochetes	
fD4	CCGAATTCGTCGACAACAGAA TTTGATCTTGGTTCAG	Chlamydiae	
rD1	CCCGGGATCCAAGCTTAAGGA GGTGATCCAGCC	Many eubacteria	
rP1	CCCGGGATCCAAGCTTACGGTT ACCTTGTTACGACTT	Enteric(and most eubacteria)	
rP2	CCCGGGATCCAAGCTTACGGCT ACCTTGTTACGACTT	Most eubacteria	

2.2. Methods

2.2.1. Sterilization methods

Three methods of sterilization were used**:**

(A) Moist heat sterilization (autoclaving).

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

(B) Dry heat sterilization

Electric oven was used to sterilize Glasswares and other at 160◦C for 3 hours and 180◦C 2 hours.

(C) Membrane sterilization (filtration)

Millipore filtering was used to sterilize heat sensitive solutions by using Millipore filter paper (0.22 μm) in diameter.

2.2.2. Hydrocarbon contaminated soil samples:

Samples of soils contaminated with hydrocarbon compounds were collected from fifty different sites in Basrah and Baghdad. The soils samples (200g) were collected randomly from the top soil layer (15cm in depth). Soils samples were transferred to the laboratory using sterile plastic bags to isolate thermophilic aromatic hydrocarbons degradable bacteria.

2.2.3. Bacterial isolation:

The microbial selection procedures were performed in minimal medium (CDM) (2.1.4.2), constituted of minerals and the crude oil as sole source of carbon, nitrogen and energy. This experiment was designed to enrich indigenous bacterial communities growing at different temperatures (37 ◦C, 60 ◦C, and 75 ◦C). This was followed by isolation and purification of pure cultures capable of utilizing individual aromatic compounds, naphthalene (polycyclic aromatic hydrocarbon), carbazole (N-heterocyclic aromatic compound) and ρ-nitrophenol, nitrobenzene (nitroaromatic compounds).

One hundred milliter of chemical defined medium (CDM) were dispensed in the(250 ml)Erlenmeyer flasks, supplemented with one milliter of crude oil as sole source of carbon, nitrogen, and energy. Then one percent (w/v) of soil samples were added to the flasks and incubated at each temperature (37 ◦C, 60 ◦C and 75 ◦C) on a rotary shaker (150 rpm). After 3 days of incubation,

each flask was supplemented again with crude oil. After 7 days incubation, samples 0.1ml of appropriate dilution were spread on plate of LB agar plates, incubated at each temperature for 24hrs. A single colony was picked with a sterile loop to prepare a pure subculture in a fresh Lb agar plates by streaking. The purity of the selected colonies was checked by microscopic examination.

2.2.4. Maintenance of bacterial isolates:

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

1. Short term storage:

Bacterial isolates were maintained for few weeks on LB agar slants, they were tightly wrapped with parafilm, and then stored at 4◦C.

2. medium- term storage:

Bacterial isolates were maintained as stab cultures for months. Such cultures were prepared in small screw- capped bottles containing 2-3ml of LB agar medium and stored at 4◦C.

3. Long term storage:

Single colony was cultured in LB broth and incubated for 24hrs. And 8.5 ml of bacterial culture was then mixed with 1.5ml of glycerol. This technique was used for long term storage.

2.2.5. Identification of isolates

In order to identify the selected isolates, the following characteristics were studied:

2.2.5.1. Microscopic and morphological characteristics (Atlas *et al.,* 1995).

The morphology, size, shape and margin of the bacterial isolates were studied. On the other hand, a loop full of bacterial suspension was fixed on a

slide, and stained by gram stain to examine gram reaction, shape and spore forming of the isolated bacteria.

2.2.5.2. Biochemical Tests:

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

A single colony of different isolate was placed onto a clean glass microscope slide with a sterile toothpick, and then a drop of hydrogen peroxide (3%) was placed onto the colony. Production of gaseous bubbles indicated a positive result.

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

This test was done by using moistened paper with few drops of a freshly prepared solution of tetramethyl-ρ-phenylenediaminedihydro-chloride. Aseptically, a clump of cells was picked up from the slant growth with a sterile wooden stick and smeared on the moisten paper. Developments of a violet or purple color within 10 seconds indicate a positive result.

- **Nitrate reduction test (Atlas *et al*., 1995)**

A single colony of each bacterial isolate was used to inoculate 5ml of nitrate media, and then test tubes were incubated at 37◦C 55◦C for 24hrs. After incubation, 0.1 ml of the test reagent (2.1.6.5) was added to each tube. The immediate formation of red color indicates a positive result.

- **Gelatin liquefaction test (Cruickshank *et al*., 1975)**

Tubes of gelatin media were inoculated with a single colony of bacterial isolates and incubated overnight at 37◦C or 55◦C, then transferred to refrigerator for an hour. Liquefaction of tubes indicated positive result.

- **Motility test (Collee *et al*., 1996).**

Semisolid agar medium was inoculated with each bacterial isolates using a straight wire to make a single stab down the center of the tube to about half

the depth of the medium. Motile bacteria typically give diffuse, hazy growth that spreads throughout the medium rending it slightly opaque.

- **Indole Test (Collee *et al.*, 1996)**

Peptone water was inoculated and incubated at 37◦C or 55◦Cfor 48 hours. Fifty microlitter of Kovac's reagent (2.1.6.6) was added and mixed gently. A positive result was recorded by the appearance of a red ring on the surface of liquid media.

- **Methyl Red Test (Collee *et al*., 1996)**

MR-VP broth was inoculated and incubated at 37◦C or 55◦C for 48 hours. Five drops of methyl red reagent was added and mixed. Positive results are bright red and negative ones are yellow.

- **Voges-Proskauer Test (Collee *et al*., 1996)**

MR-VP broth was inoculated and incubated at 37◦C or 55◦C for 24 hour. Then 1 ml of Barrett's reagent a (2.1.6.4) and 3 ml of Barritt's reagent B (2.1.6.4) were added to 5 ml of cultured broth and shacked for 30 seconds. The formation of pink to red color represents a positive test for acetone.

- **Citrate Utilization Test (Atlas *et al*., 1995)**

Simmon's citrate agar slants were inoculated with bacteria, and incubated at 37◦Cor 55◦C for 24 hr. The changing of color to navy blue indicates a positive result, whereas the green color means a negative result.

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

Urease activity was detected by inoculating the surface of Christensen urea agar slants with the bacterial growth and incubated at 37◦C or 55◦C for 24 hours. The appearance of a red-violet color indicates a positive result while a yellow-orange color indicates a negative result.

- **Triple Sugar Iron (TSI) Test (Atlas *et al*., 1995)**

Isolates were cultured on TSI agar slants by stabbing into the bottom and streaking on surface, and then incubated for (24-48) hours at 37◦C or 55◦C. The results could be summarized as follows:

Slant / Butt	Color	Utilization
Alkaline / acid	Red / Yellow	Glucose fermented; peptones assimilate
Acid / acid	Yellow / Yellow	Glucose, lactose and sucrose fermented
Alkaline / alkaline	Red / Red	glucose, lactose and sucrose not fermented/ peptones assimilated

The formation of black precipitate is an indication of H_2S production, whereas pushing the agar to the top indicates CO_2 formation.

2.2.6. Utilization of pure aromatic compounds.

Thermophilic bacterial isolates were grown on the respective compounds as the sole source of carbon and energy for naphthalene or as carbon, nitrogen, and energy source for carbazole, ρ-nitrophenol and nitrobenzene in order to study their ability to degrade them. 50ml of chemically defined medium (2.1.4.2) distributed in 100ml Erlenmeyer flasks. The flasks were sterilized by autoclaving at 121◦C for 15 min, 1mM of aromatic compounds (sterilized by filtration) were added.

All the flasks were inoculated with 1% of fresh culture (18hrs), and incubated under shaking (150rpm) at 55◦C for 2 days. Cell density was determined by measuring the optical density at 600nm.

2.2.7. Molecular identification:

Molecular identification of bacterial isolates was achieved according to the analysis of DNA sequences and degree of similarity of 16S rRNA. Bacterial isolates that show thermophilic ability were selected and propagated individually in LB broth at 55◦C to obtain fresh cultures for extraction of genomic DNA.

2.2.7.1. Extraction of genomic DNA with wizard genomic DNA purification kit:

1. One ml of overnight culture was transferred to 1.5 ml centrifuge tube. Centrifugation at 13000rpm for 2min to pellet the cell.
2. Six hundred microliter of nuclei lysis solution was added. Gently pipette until the cells is resuspended.
3. The tube was incubated at 80◦Cfor 5min to lyse the cells, and then cool to room temperature.
4. RNase solution (3µl) was added to cell lysate, for mixing the tube was inverted 2-5 times, incubated at 37◦C for 15-60min, cool the sample to room temperature.
5. Two hundred microliter of protein precipitation solution was added to the RNase –treated cell lysate, vortex vigorously
6. The sample incubated on ice for 5min. centrifugation at 13000-16000rpm for 3min.
7. The supernatant containing the DNA was transferred to clean 1.5ml microcentrifuge tube containing 600µl of isopropanol. Gentle mix by inversion until the thread like strands of DNA form a visible mass. Centrifuge at 13000-16000rpm for 2min.
8. The supernatant was pour off and the tube was drained on a clean absorbent paper, 600µl of 70% ethanol was added, the tube was gently inverted several times to wash the DNA pellet.

9. The tube was centrifuged at 13000-16000rpm for 2min, carefully aspirate the ethanol.
10. One hundred microliter of DNA rehydration solution to the tube and rehydrate the DNA by incubating at 65◦C for 1hrs.

2.2.7.2. Quantization of DNA concentration (Sambrouk and Russell, 2001):

Purity and concentration of DNA solution was measured by using Biophotometer device, by adding 8µl of DNA solution to 72µl of D.W. in a quartz cuvete and the absorbency at 260 and 280nm was measured after calibration with D.W. at 260nm and 280nm respectively. Pure DNA has an A_{260}/A_{280} ration of 1.7-1.9. The concentration of double strand DNA (µg/ml) was measured directly by the device.

2.2.7.3. Agarose gel electrophoresis (Sambrouk and Russell, 2001):

Agarose gels (0.7%, 1%, and 1.5%) were run horizontally in Tris-borate-EDTA (TBE 1X). Sample of DNA was mixed with1/10 volume of the loading buffer and added to the wells of the gel. Generally, gel was run for (50min., 1hrs. 1.5hrs. and 2 hrs.) at 5V/cm and the gel buffer added up to the level of horizontal gel surface. Agarose gel was stained with ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 min. DNA bands were visualized by UV illumination at 302 nm on a UV transilluminator. Gels were distained in distilled water for 30-60min. to get rid of background before photographs were taken.

2.2.7.4. PCR for amplification of 16S rDNA of thermophilic bacterial isolates:

The 16S rDNA gene of the purified thermophilic bacterial colonies was amplified by using primers specific for Eubacteria (2.1.8.2).

The PCR reaction was performed by adding the following:

PCR reaction	50µl Rxn
2x PCR master mix solution	25µl
Template DNA(genomic DNA)	1-2µl
Primer (F:10 pmol/µl)	1µl
Primer (R: 10 pmol/µl)	1 µl
Distilled water	21-22µl
Total reaction volume	50µl

The following PCR thermal cycling program was used:

	Temp	Time	Cycle No.
Initial denaturation	94◦C	30min	1
Denaturation	94◦C	30sec	35
Annealing	60◦C	30sec	
Extension	72◦C	1min	
Final extension	72◦C	10min	1

The PCR assays were examined by Agarose gel electrophoresis.

2.2.7.5. Purification of DNA fragment with QlAquick gel extraction kit (Ql Alquick spin Handbook/ Germany).

1. The DNA fragment was excised from the agarose gel with a clean, sharp scalpel.
2. The gel slice was weigh in a colorless tube and 3 volumes of buffer QG was added to 1 volume of gel (100mg- 100µl)
3. The tube was incubated at 50◦C for 10min or until the gel slice was completely dissolved.
4. One volume of isopropanol was added to the sample and mix.

5. A QIAquick spin column was placed in a 2ml collection tube, the sample was applied and centrifuged for 1min.
6. Of the buffer QG 0.5ml to the column and centrifuge for 1min.
7. From the washing buffer (PE) 0.75ml was added to the column and centrifuged for 1min, discard the flow-through and place the column back to the same tube.
8. Centrifuge the column in a 2ml collection tube for 13000rpm.
9. The spin column into a clean 1.5ml micro centrifuge tube.
10. For elusion 50µl of buffer EB buffer was added to the center of the column, centrifuged for 1min.

2.2.7.6. 16S rDNA gene sequencing:

The purified PCR fragment (16S rDNA) was sequenced by Macrogene Inc., (Seoul, Korea) using ABI, 3310 automated sequencing system. The obtained sequences were analyzed using the genebank[66] National center for Biotechnology Information (NCBI).

2.2.8. Antibiotic sensitivity test of the bacterial isolate 4A (Atlas *et al.*, 1995):

The disc diffusion method was used to test the antibiotic sensitivity of the selected isolate.

A sterile cotton swab was dipped into the inoculums (freshly culture, 18hrs) and the entire surface of the LB agar plates were swabbed three times by rotating the plate approximately 60C◦ between streaking to ensure even distribution then the disc of antibiotic were applied and incubated at 55◦C. the zone of the inhibition was observed after incubation for 24hrs.

2.2.9. Plasmid extraction of *A. rupiensis* strain Ir3 (JQ912241):

The plasmid DNA was isolated by using Salting out method, Gene JET ™ plasmid miniprep kit (Fermentas) and pure yield ™ plasmid miniprep kit (Promega) as follows:

2.2.9.1. Salting out method (kieser, 1995):

1. Culture of bacteria grown in 20ml of LB broth was pelleted by centrifugation at 6000 rpm for 15min.
2. The pellet washed with 3ml of SET buffer, and the cells were resuspend with 1.6ml of SET buffer, and then freshly prepared lysozyme (final concentration 1mg/ml) was added and incubated at 37◦C for 30 min.
3. One ml of 10%SDS was added and mixed by inversion, then incubated at room temperature for 30min.
4. Two ml of 5M NaCl was added and mixed by inversion at room temperature.
5. An equal volume of chloroform was added, mixed by inversion for 15min. then centrifuged (6000rpm at 4◦C) for 20min.
6. The aqueous phase was transferred to another sterile tube, and 0.6 volume of isopropanol was added and mixed by inversion. It was kept at room temperature for 5min.Then, centrifuged at 13000 rpm for 15min at 4◦C.
7. The isopropanol was discarded and the precipitated DNA dissolved in 100µl TE buffer and stored at -20◦C.

2.2.9.2. Plasmid isolation kit:

2.2.9.2.1. Gene JET™ plasmid miniprep kit (Fermentas).

1. The pelleted cells were resuspended in 250µl of resuspension solution.

2. Lysis solution (250µl) was added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.
3. Neutralization solution (350µl) was added and mixed immediately and thoroughly by inverting the tube 4-6 times.
4. Centrifugation for 5min to pellet debris and chromosomal DNA.
5. The supernatant was transferred to the Gene JET^{TM} spin column by decanting. Centrifuge for 1min and discard the the flow-through.
6. Five hundred microliter of the wash solution was added to the Gene JET^{TM} spin column centrifuge for 30-60sec and discards the flow through. This step was repeated.
7. The Gene JET^{TM} spin column was transferred into new 1.5 microcentrifuge tube.
8. Fifty microliter of the elution buffer was added to the center of the Gene JET^{TM} spin column membrane, incubate for 2min at room temperature and centrifuge for 2 min, to elute the plasmid DNA.
9. The purified plasmid was stored at -20◦C.

2.2.9.2.2. Pure Yield^{TM} plasmid miniprep (Promega) kit:

1. Six hundred microliter of bacterial culture grown in LB medium was transferred to a 1.5ml microcentrifuge tube.
2. Cell lysis buffer (100µl) was added and mixed by inverting the tube six times.
3. Of the cold (4-8◦C) neutralization solution (350µl) was added and mixed thoroughly by inverting the tube.
4. Centrifugation at maximum speed in a microcentrifuge for 3min.
5. The supernatant was transferred to a pure yield^{TM} minicolumn.
6. The minicolumn was placed into a pure yield^{TM} collection tube, and centrifuged at maximum speed for 15seconds.
7. The flowthrough was discarded, and the minicolumn was placed into the same pure yield^{TM} collection tube.

8. Two hundred microliter of endotoxin removal wash was added to the minicolumn, centrifuged at maximum speed for 15 sec.
9. Four hundred microliter of the column wash solution was added to the minicolumn, centrifuged at maximum speed for 30sec.
10. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube, and then 30µl of the elution buffer was added to the minicolumn matrix and let to stand for 1min at room temperature.
11. Centrifugation at maximum speed for 15 sec to elute the plasmid DNA.
12. The eluted plasmid DNA was stored at -20◦C.

2.2.10. Role of plasmid in degradation of aromatic hydrocarbons:

2.2.10.1. Curing of plasmid DNA:

Curing experiments were performed on bacterial isolate *A. rupiensis* strain Ir3 (JQ912241) by using two types of curing agents (SDS and Et.Br) according to Trevors, (1986).

Bacterium was grown in 5ml of LB broth to mid log phase, then 0.1 ml (300×10^6) inoculums of fresh culture (18hrs) were inoculated in a series of 5ml fresh LB broth containing various concentration of SDS (0%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%) or Et.Br (0, 70, 80, 90, 100, 125, 150, 175, 200, 225, 250) µg/ml. All tubes were incubated with shaking (150rpm) at 55◦C for 24-48hrs.

The growth density of different tubes was observed by nicked eye and compared with the control to determine the effect of SDS and Et.Br on bacterial growth. The lowest concentration of each curing agent that inhibited the growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of either SDS and Et.Br that still allowed bacterial growth, and diluted appropriately, then 0.1ml from proper dilutions were spreaded on LB agar plates and incubated overnight at 55◦C to score the survived colonies. These colonies (50 colonies) were replica plated (using sterile toothpick) on LB agar plate (master plate) and on minimal agar plate (CDM) (2.1.4.2) containing carbazole (N-heterocyclic aromatic compound)

If colonies were able to grow on the master plate but not on the selective medium containing the aromatic compound, it means that the cells of this colony are cured cells that lost the ability to grow on aromatic compound as a sole source of carbon, nitrogen and energy. The DNA has been isolated from the produced cells, and gel electrophoresis was performed in order to compare between the resulting bands with the original one.

2.2.11. Optimization of growth conditions of *A. rupiensis* strain Ir3 (JQ912241):

2.2.11.1. Determination of the optimum pH.

The Luria-Bertani (LB) medium was adjusted in different PH values (5, 6, 7, 8, and 9) to determine the optimum PH. Then cultures were incubated in a shaker incubator (150rpm) at 55◦C for 24hrs. Cell density was determined by measuring the optical density at 600nm.

2.2.11.2. Determination of the optimum temperature:

The pH of the LB broth was adjusted to get a final value of 7 before autoclaving. The optimum temperature was determined by measuring the optical density of growth at temperatures ranging from 35◦C to 70◦C. Cell density was determined by measuring the optical density at 600nm.

2.2.11.3. Growth at different sodium chloride concentrations.

The experiments were carried out in Earlnmeyer flasks (250ml) containing 100ml of LB broth with a final salt (NaCl) concentration of 0.5%, 1%, 2%, 3%, 4%, and 5.0%, flasks were inoculated using 1ml of 18hrs bacterial cultures. Flasks were then incubated at optimum temperature 55◦C in shaking incubator 150 rpm. Cell density was determined by measuring the optical density at 600nm.

2.2.11.4. Studying the death curve (Thermal death time) of *A. rupiensis* strain Ir3 (JQ912241):

Using thermal death time method, *A. rupiensis* strain Ir3 (JQ912241) grow in LB broth was subjected to water bath at 80 degree Celsius for ninety minutes. A sample of culture was taken at time 0**,** 15, 30, 45, 60, 75, and 90 minutes. The 0.1ml of bacterial culture was quadrant streaked on LB agar plates and then incubated at 55◦C for 24h.

2.2.11.5. Growth of *A. rupiensis* strain Ir3 (JQ912241) at different carbazole concentrations:

The experiments were carried out in Erlnmeyer flasks (250ml) containing 100ml of CDM with glucose (1%) and different carbazole concentrations, 0.2, 1, 5, and 10mM in addition to control flask that contain NH_4Cl as a source of nitrogen . The flasks inoculated by 1ml of 18hrs growth culture, then incubated at 55◦C in shaking incubator (150rpm). Cell density was determined by measuring the optical density at 600nm.

2.2.12. Tracing the bacterial consumption of aromatic compounds.

Chemically defined media (100ml in 250 ml flask) containing 1mM of different aromatic compounds (naphthalene, carbazole, ρ-nitrophenol and nitrobenzene) were inoculated with fresh culture of efficient bacterial isolate *A. rupiensis* strain Ir3 (JQ912241) and incubated at 55◦C for 7 days shaking at 150 rpm. After incubation, centrifugation at 13000 rpm for 10min at 4◦C. The

resulting cell-free supernatant was analyzed by HPLC for tracing the consumption of the aromatic compounds.

2.2.12.1. HPLC analysis

Cell- free culture supernatant was analyzed to trace the utilization of different aromatic compounds naphthalene and the N-containing groups namely nitroaromatics and N-heterocyclic compounds by using HPLC system. 10µl of supernatant were injected in C_{18} column (5 µM, 4.6*250mm, supelcosil Lc-2010AHT) and the following conditions were followed:

The solvent system used (60% acetonitrile in H_2O) was run at flow rate of 1ml/min. for UV detection of naphthalene, carbazole, ρ-nitrophenol and nitrobenzene, the UV detector was adjusted to 295nm. Under these conditions the observed retention time for authentic samples of naphthalene, carbazole, ρ-nitrophenol and nitrobenzene were 3.5min, 3 min, 3min, and 3min, respectively.

2.2.13. FTIR analysis.

One hundred milliliter of CDM in 250ml Erlenmeyer flasks, supplemented with crude oil (sterilized by tyndallization) 0%, 1%, 2%, 5%, 10%, 20% and 30% (v/v). All the flasks were inoculated with 1% of fresh culture of efficient bacterial isolate *A. rupiensis* strain Ir3 (JQ912241), (18hrs), and the inoculated flasks, as well as uninoculated controls were incubated in shaker incubator (150 rpm) at 55 ◦C for 30 days. Growth was monitored daily by plate count on LB agar plate medium.

2.2.13.1. Extraction procedure:

Each inoculated flasks and uninoculated controls were extracted twice by using separating funnel with 50ml of Diethyl ether. The extracts were combined, dried over (1-2) gm of Na_2So_4, filtered through (Whitman no.1) filter paper. The extracts were but in the refrigerator until the time of analysis

with Fourier Transform Infrared Spectrophotometer-prestige 21, by using polystyrene as a standard materials for calibration of the instrument (Al-Haidary, 1977).

Analysis by FTIR was used to determine the changes in the functional groups. This analysis was performed at the department of chemistry of Al-Nahrain University.

2.2.14. *Meta*- cleavage enzyme test:

The *Meta* cleavage enzyme activity was examined by monitoring the conversion of 2, 3-dihydroxybiphenyl to 2-hydroxy-6-oxo-6-phenyl-hexa-2, 4-dienoic acid. The isolate *A. rupiensis* strain Ir3 (JQ912241) was grown on LB (containing 1mM carbazole) and CDM agar plates, sprayed with 2, 3-dihydroxylphenyl-acetone solution. A change in colony color from yellow to brown indicating *meta*-cleavage activity was sought (Maeda *et al*., 2009).

Chapter Three

Results and Discussions

Chapter Three

Results and Discussion

3.1- Isolation of aromatic hydrocarbons degrading bacteria:

In order to isolate aromatic hydrocarbons degrading bacteria, a total of ninety five hydrocarbons contaminated soils samples were collected from different places in Basrah and Baghdad. Forty eight bacterial isolates were obtained. These isolates have the ability to utilize the crude oil as a sole source of carbon and energy. Thirty eight isolates were mesophilic and characterized as *Pseudomonas* (20 isolates), *Staphylococcus* (3 isolates), *Acinetobacter* (3 isolates), *Alcaligenes* (2 isolates), *Aeromonas* (2 isolates), *Bacillus* (6 isolates) and *Flavobacterium* (2 isolates), as shown in Table (3-1).

Leahy and Colwell (1990) reported biodegradation of petroleum oil by different bacterial genera: *Achromobacter, Arthrobacter, Acinetobacter, Alcaligenes, Bacillus, Flavibacterium, Nocardia, Pseudomonas* and *Rhodococcus*.

In addition to these isolates and due to industrial interest, a thermophilic aerobic spore forming, thermostable enzymes producers were favorable; therefore, it was aimed to isolate only representatives of *Bacillus* group. Ten isolates have been obtained, and these isolates showed thermophilic character in addition to their ability to utilize the crude oil as a sole source of carbon, nitrogen, and energy. These isolates assigned as 9SM, 2G, 3A, 4A, 12SM, 6A, 13SM, 14SM, 21SM and 34SM, as shown in Table (3-2).

Table (3-1) Morphological, physiological and biochemical characteristics of mesophilic bacterial isolates:

Test		Bacterial isolates						
		Pseudomonas	***Staphylococcus***	***Acinetobacter***	***Alcaligenes***	***Aeromonas***	***Bacillus***	***Flavobacterium***
Colony color		Green	Yellow	White	Non pigmented	Yellow	White	Cream to orange
Cell shape		Rod	Cocci	Coco bacilli	Rod	Rod	Rod	Rod
Gram stain		-	+	-	-	-	+	-
Catalase		+	+	+	+		+	+
Motility		+	N	N	+	+	+	+
Oxidase		+	-	-	+	+	+	+
Urease		N	-	+	N	N	N	N
Growth on cetrimide		+	N	-	N	N	N	N
Gelatinase		N	N	-	N	N	+	N
Growth at 42 C		N	N	+	N	N	+	N
Nitrate reduction		N	N	+	+	+	+	N
Citrate utilization		+	-	+	N	N	-	N
MR		+	+	-	N	N	-	N
VP		-	-	-	N	N	+	N
Indol		-	-	N	-	N	-	+
Mannitol		N	+	N	N	N	N	N
TSI	**H_2S**	-	-	-	N	N	-	N
	CO_2	-	-	-	N	N	-	N
	Acid	Alk/alk	+	Alk/alk	N	N	Alk/alk	Alk/alk

+: positive result (-) : negative result N: not tested

TSI: triple sugar iron agar MR: methyl red

VP: voguspreskauer

Table (3-2) Morphological, physiological and biochemical characteristics of thermophilic bacterial isolates:

Samples	Gram stain	Cell shape	Catalase	Oxidase	Nitrate reductase	Growth at		
						55◦C	65◦C	70◦C
9SM	+ve	Short rod	-	+	-	+++	++	-
2G	+ve	Long rod	-	+	+	+++	+++	+++
3A	+ve	Long rod	+	+	+	+++	+++	+++
4A	+ve	Long rod	+	+	+	+++	+++	+++
12 SM	+ve	Short rod	+	+	+	+++	+++	+++
6A	+ve	Long rod	+	+	+	+++	+++	+++
13SM	+ve	Short rod	-	+	-	+++	++	-
14SM	+ve	Long rod	+	+	_	+++	+	-
21SM	+ve	Short rod	-	+	_	+++	++	++
34SM	+ve	Short rod	+	+	_	+++	++	++

(+): positive result (-): negative result (+++) excellent growth

(++) good growth (+) moderate growth (-) no growth

Growth at 55◦C, 65◦C, and 70◦C

These ten isolates cannot be distinguished by ordinary morphological, physiological and biochemical tests; therefore, 16SrDNA sequence data (for four isolates) were used for detection the genotypic characterization of these thermophilic bacteria. These four isolates (2G, 3A, 4A, and 6A) have been tentatively identified as a new strains belonging to family Bacillaceae depending on partially sequencing of their 16S rDNA .

Oil contaminated soils were chosen for isolation of thermophilic aromatic degrading bacteria since petroleum oil contains aromatic compounds that are toxic for most life forms (Koukkou, 2011).

The results also revealed the presence of different thermophilic bacilli in thermal Iraq soils, some of which are phylogenetically novel ones as shown in Figures (3-1a, b), and (3-2 a, b). Investigation on their aromatic hydrocarbons and carbohydrate degrading activity demonstrated presence of biotechnologically valuable enzyme producers. Since most microbes from nature (about 99%) are difficult to cultivate (Colwell, 1997), our screening probably under estimated *Bacillus* group diversity of aromatic hydrocarbons degraders.

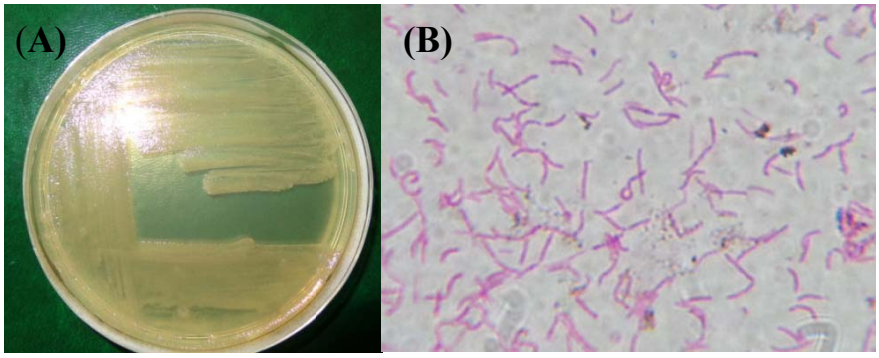

Figure (3-1): The macroscopic (A) and microscopic (B) appearance for *Anoxybacillus rupiensis* Ir3 (JQ912241).

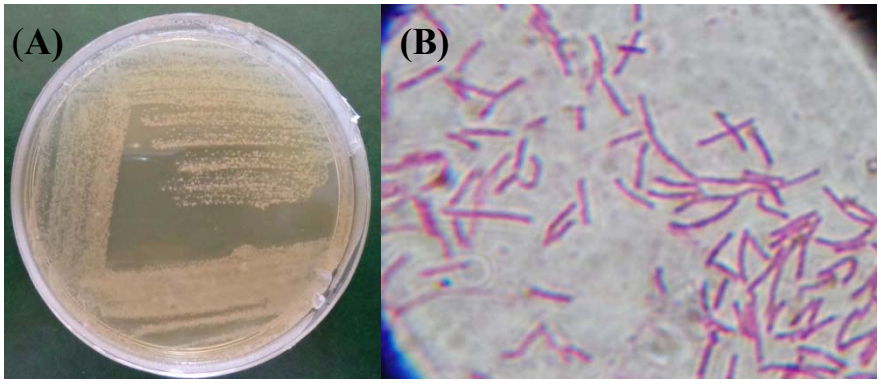

Figure (3-2): The macroscopic (A) and microscopic (B) appearance for *Geobacillus thermoleovorans* Ir1 (JQ 912239*).*

3.2-Ability of thermophilic bacterial isolates to utilize aromatic compounds:

Bacterial isolates were tested for their ability to utilize individual aromatic compounds as shown in Figure (3-3).

The results indicated that the most efficient bacterial isolate was 4A, which gave the best result in its ability to utilize all the aromatic compounds; therefore, this isolate was used for further study. Other isolates showed a

divergence in their ability to utilize aromatic compounds. There is no doubt that the chemical structures of aromatic compounds not only have an extensive effect on their utilization by different bacterial isolates, but also the genetic diversity of these isolates affect the biodegradability of aromatic compounds. As well as, some of bacteria may contain different genetic elements like plasmids or transposons, harboring biodegradative genes that might participate in diverging utilization of aromatic compounds (Chakrabarty, 1996; Nasir*et al*., 2003).

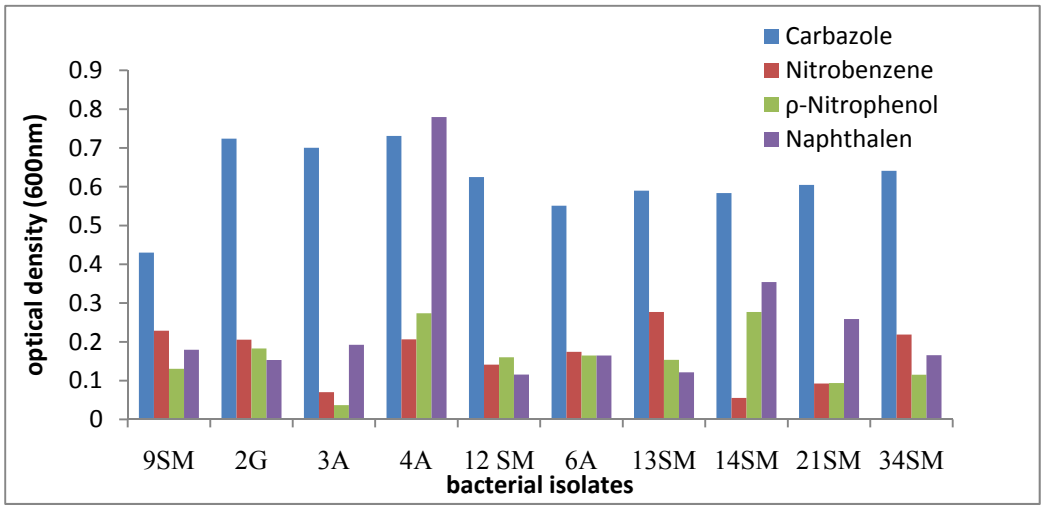

Figure (3-3) The growth density (600nm) of the thermophilic bacterial isolates on aromatic compounds after 2 days incubation at 55◦C.

3.3- Molecular identification of thermophilic isolates:

3.3.1- Isolation of genomic DNA:

In order to amplify 16S rDNA for thermophilic isolates, genomic DNA of the four isolates 2G, 3A, 4A, and 6A was extracted to provide a PCR template for the amplification.

Purity ratios of the extracted DNA samples were (1.8- 1.9) which indicate a high purity, since a pure DNA preparation has expected A_{260}/A_{280} ratio of 1.8 which are based on the extinction coefficients of nucleic acids at 260nm and 280nm (Sambrouk and Russel, 2001).

Such results were also observed when the DNA samples were analyzed by gel electrophoresis, in which sharp DNA bands were detected indicating purified DNA samples as shown in Figure (3-4).

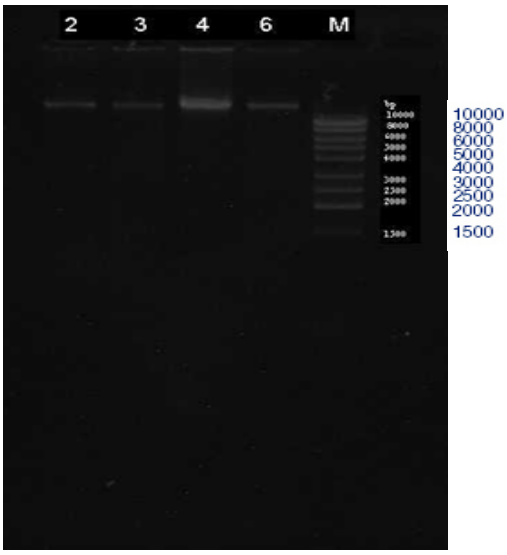

Figure (3-4): Gel electrophoresis for genomic DNA of bacterial isolates. Electrophoresis was performed on (1%) agarose gel and run with 5V/cm for 1.5 hrs.2: 2G, 3:3A, 4:4A, 6: 6A.M: molecular marker 10kb (mass ruler DNA ladder, high range).

3.3.2-Amplification, sequencing and 16S rDNA-based phylogenetic affiliation analysis:

The identity and purity of the thermophilic bacterial isolates (2G, 3A, 4A and 6A) were carried out at (Hamdi Mango Center for Scientific Reserch in Jordan) and were checked by amplification and sequencing of eubacterial 16S rDNA gene. Primers for amplification of eubacterial 16S rDNA gene were used (2.1.8.2). In presence of primers fD1, fD2, fD3, fD4, rD1, rP1 and rP2, which

are universal primers that bind at the conserved 5ó and 3ó ends of 16S rDNA of eubacteria,1500bp PCR fragments were obtained as shown in Figure (3-5) and this indicates that the four isolates (2G, 3A, 4A and 6A), were affiliated to eubacteria.

Partial sequence around the obtained PCR fragments with forward and reverse primers was performed and compared with 16S rDNA nucleotide sequences present in gene bank, using the standard basic local alignment search tool (BLAST). The results of this BLAST search showed the highest percentage of sequence similarity with both 5ó and 3ó ends of the same gene from the four bacterial isolates (Table3-3).

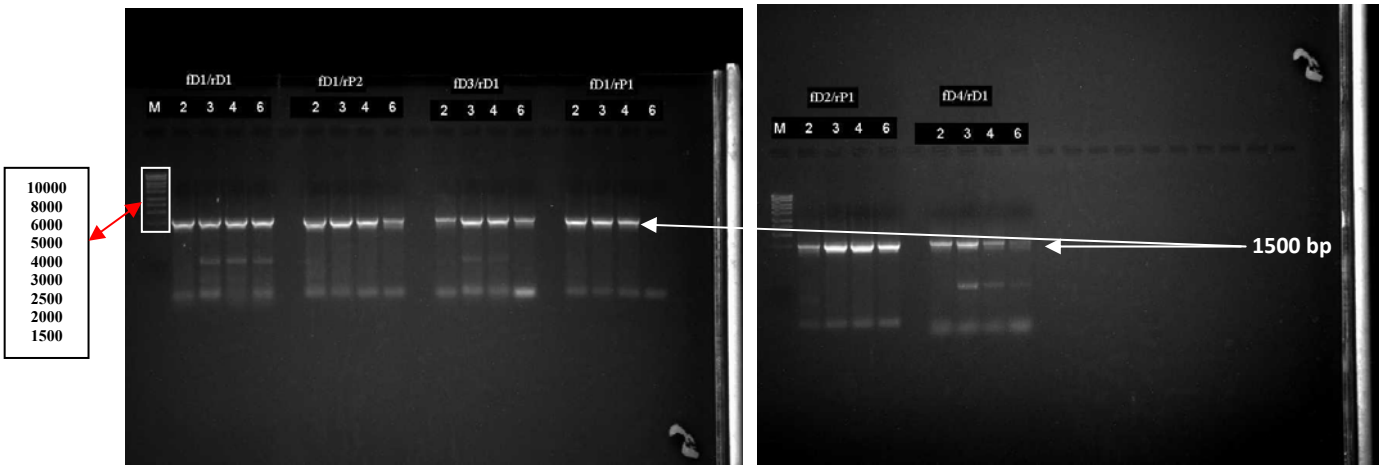

Figure (3-5): Gel electrophoresis for amplification of 16S rDNA gene using eubacterial specific primers fD1, fD2, fD3, fD4, rD1, rP1 and rP2. Electrophoresis was performed on (1.5%) agarose gel and run with 5V/cm for 1 hr.M: Mass Ruler High range. DNA ladder 2: 2G, 3: 3A, 4: 4A, 6: 6A.

Table (3-3): Database search homology of 16S rDNA genes of bacterial isolates compared with 16S rDNA sequences in gene bank.

Bacterial isolates	16S rDNA end and number of identical bases	Percentage of sequence identities with	Accession number
2G	5ó 661/664bp 3ó 397/398bp	*Geobacillus thermoleovorans*99%	Eu21465.1
3A	5ó 303/308bp 3ó 544/545bp	*Anoxybacillus* sp 99%	HQ696615.1
4A	5ó 349/359bp 3ó 621/626bp	*Anoxybacillus* sp 97%	HQ696615.1
6A	5ó 102/ 116bp 3ó 396/396bp	*Anoxybacillus* sp 94%	HQ696615.1

A comparison of generated sequences with those in Gene Bank database indicated that the majority of the identified aromatic hydrocarbons degrading isolates from oil contaminated soils in Iraq belong to bacteria domain, and the most dominant bacteria belong to the class Gram positive bacteria, *Clostridium, Bacillus* subphylum, group of *Bacillus* –like genera. The heterogeneity of the genus *Bacillus* become readily apparent after advancing of sequencing technology and Ash *et al*. (1991) was the first who initiated its reorganization. Based on the large number novel endospore forming bacteria described in the 1990s when extreme environments were explored, Zeigler (2001) introduced the term *Bacillus* sensulato for *Bacillus* like genera. Currently, thermophilic aerobic spore-forming bacteria are classified into groups, 1 (one genus) and 5 (seven genera, composing majority thermophilic bacilli, including *Geobacillus, Anoxybacillus* and *Brevibacillus*) in *Bacillus* group (Bae *et al*., 2005). The results showed that *Anoxybacillus* with its 3 isolates was the most predominant genus with aromatic hydrocarbons degradation activities in these sites, in

addition to the representatives of the genera *Geobacillus*. And since there are no previous local studies concerning these two genera, therefore the present study may be the first record for *Geobacillus* and *Anoxybacillus*.

The four selected isolates were sequenced (Appendix 1) and the sequence data was analyzed by BLAST and the nearest match from gene bank data was reported. Sequences were deposited in the gene bank. DNA sequencing and phylogenetic analysis revealed that all the isolates obtained from oil contaminated soils in Iraq showed 88% to 100% similarity with the sequence within the gene bank. The closet phylogenetic neighbors according to the 16S rRNA sequence data for the four isolates 2G, 3A, 4A, and 6A were *Geobacillus thermoleovorans* for 2G and *Anoxybacillus rupiensis* for 3A, 4A, and 6A as shown in Figure (3-6), the phylogenetic trees were constructed using the program MEGA5.

16S rDNA based phylogenetic suggested that strain 2G (Ir1) was *G. thermoleovorans* (JQ 912239) and strains 3A (Ir2) and 4A (Ir3) were *A. rupienses* (JQ912240) and (JQ 912241) respectively.

The identification based on 16S rDNA gene sequencing has a higher accuracy than conventional testing (Rahman *et al*., 2003). Techniques such as the polymerase chain reaction (PCR) and 16S rRNA sequencing have made it possible to specifically detect and identify microorganisms with high level of precision (Olusesan *et al*., 2009).

Some bacteria are difficult to identify with phenotypic identification schemes commonly used outside reference laboratories. Therefore 16S ribosomal DNA-based identification of bacteria potentially a useful alternative when phenotypic characterization method fail (Drancourt *et al*., 2000).

Janda and Sharon (2007) mentioned that the use of 16S rRNA gene sequence to study bacterial phylogeny and taxonomy has been by far the most

common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1500bp) is large enough for informatics purposes.

The genus *Anoxybacillus* was firstly separated from *Bacillus* by Pikuta *et al*. (2000) based on the phenotypic properties and 16S rDNA sequence. Many microorganism belonging to *Anoxybacillus* have been isolated, including *A. pushchinoensis* (Pikuta *et al*., 2003), *A. gonesis* (Belduz *et al*., 2003), *A. contaminans* (Declerck *et al*., 2004), *A. voinovskiensis* (Yumoto *et al*., 2004), and *A. rupiensis (*Derekova *et al*., 2007), and most of them were thermoalkaliphilic.

The results showed that the ten thermophilic isolates (9SM, 2G, 3A, 4A, 12SM, 6A, 13SM, 14SM, 21SM and 34SM) may be formed a novel group of aromatic hydrocarbons degrading extreme thermopilic bacteria in oil-contaminated soils in Iraq.

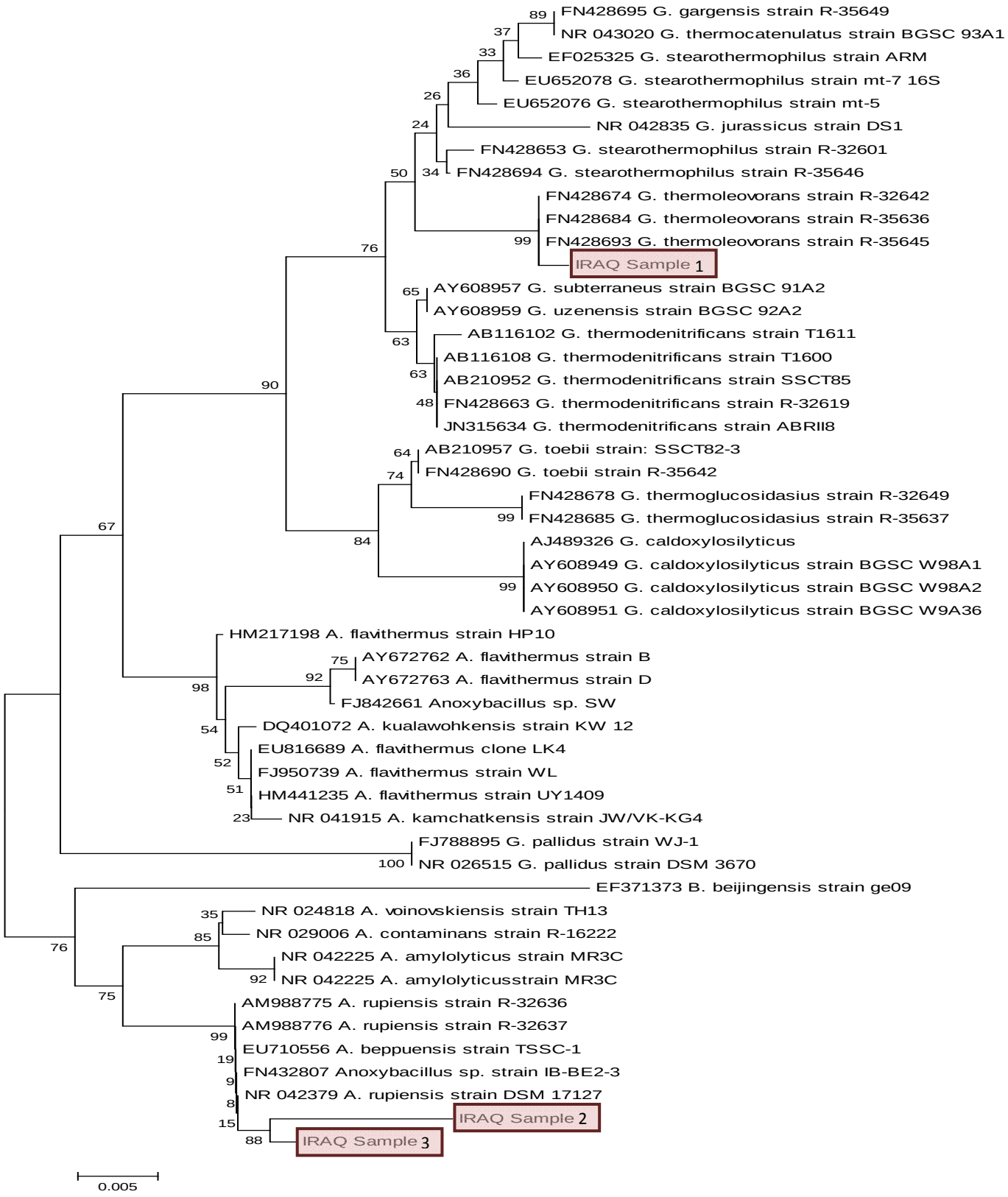

Figure (3-6) Phylogenetic tree showing the position of *A. rupiensis* strain Ir3, other representative of the genus *Anoxybacillus* and some *Geobacillus*. Based on a comparsion of 16S rDNA sequences.

3.4- Description of *A. rupiensis* strain Ir3 (JQ912241).

Anoxybacillus rupiensis sp. Nov. isolated from hydrocarbons contaminated soils in Iraq, it was the most efficient isolate for utilizing aromatic compounds (Figure 3-3) and the identity of the carbazole-degrading culture. It was investigated using biochemical tests, microscopic observation, and a determination of its 16s rDNA gene. The culture is a Gram positive or (gram variable) long rod that forms medium sized, smooth, round colonies with cream color, regular and complete margins on LB agar plate. Cell of this strain is appeared as motile, strictly aerobic, thermophile. Most cells occur in exponential growth phase singly or in chain. Terminal endospores are observed. Obligate thermophilic growing between 40 and 70◦C optimum 55-65◦C and in pH range from 5.0-9.0 (optimum 7.0), indole is not produced, the voges-proskauer reaction is negative, catalase and oxidase reaction are positive and methyl red test is negative as shown in Table (3-4). The 16S rRNA gene sequence of this bacterium compared with database of NCBI with BLAST program has 1500bp and 97% similarity to *A. rupiensis* (HQ 696615.1).These data indicated that this carbazole-degrading bacterium can be identified to the genus and species level as *A. rupiensis* (JQ 912241), with comparison to other species in that genus as shown in Table (3-5).

According to the biochemical and molecular identification, the isolate may represent a new CAR- degrading bacterium. Also the results of growth in different hydrocarbon sources by this strain have been shown that this strain was able to grow in the presence of these hydrocarbons (Figure 3-3) as a carbon and energy source for naphthalene and as a carbon, nitrogen and energy source for carbazole, nitrobenzene and ρ-nitrophenole. Carbazole is one of the most predominant polyaromatic N-heterocyclic compounds in oil (Loh and Yu, 2000), Naphthalene belongs to PAHs, and Nitrobenzene and ρ-nitrophenole

belong to nitroaromatic compounds whose reduction alters petroleum quality. Therefore, further research on this novel species may develop bioremediation strategies for polluted environments, and improvements of biorefining processes.

Table (3-4) Biochemical characteristics of hydrocarbon degradable thermophilic bacteria *A. rupiensis* strain Ir3 (JQ912241):

Test	Result
Colony color	Cream
Gram staining	Gram positive(or gram variable) rods
Indole test	-
Methyl red (MR)	-
Voges-proskauer (VP)	-
Citrate	-
Triple sugar iron agar	-
Motility	+
Urea hydrolysis	-
Nitrate reduction to nitrite	+
Catalase	+
Oxidase	+
Co_2 req.	-
Growth in pepton	+
Growth in xylose	+
Gelatin	-
Casein hydrolysis	-

(+) Positive result

(-) Negative result

Table (3-5) Comparison of the phenotypic and biochemical characteristics of *A. rupiensis* Ir3 (JQ912241) and other *Anoxybacillus* species.

Characteristics		*Anoxybacillus* species						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Colony color		Creamy	White	Creamy	Creamy	Creamy	Creamy	yellow
Growth conditions		Aerobic	Anaerobic	Facultative	Facultative	Facultative	Facultative	Facultative
Temperature range (◦C)		40-70	37-66	40-70	30-70	40-70	45-65	37-69
Optimum temperature		55-65	62	55-60	50	50-55	61	60
pH range		5-9	8-10.5	6-10	6-11	6-10.5	5-6.5	5.5-9.5
Optimum pH		7	9.5-9.7	7.5-8	7.5-8.5	7.5-8.5	5.6	8-9
Tolerance to NaCl (w/v)%		4	3	4	2.5	4	<3	4.5
Tetracycline hydrochloride		-	ND	-	-	-	-	ND
Ampicillin		-	+	-	-	-	-	ND
Oxidase		+	ND	+	+	+	-	+
Catalase		+	-	+	+	+	+	+
Motility		+	-	+	+	+	+	+
Nitrate reduction		+	+	+	+	+	-	+
Utilization of	**Starch**	+	+	+	+	+	+	+
	Gelatin	-	-	+	+	-	+	+
	Glucose	+	+	+	+	+	-	+

1. A. rupiensis Ir3 (JQ912241) 2.*A.pushchinensis* 3. *A.gonensis* 4.*A. aydernsis*

5. *A. kestanbolensis* 6. *A. amylolyticus* 7.*A.salavatliensis*

+: positive - : negative ND: not determined

3.5- Antibiotic susceptibility test:

The standard disk diffusion method was used to determine the sensitivity of *A. rupiensis* strain Ir3 (JQ912241) to twenty antibiotic. The results showed that the growth of this strain was inhibited by these antibiotics, and it was resistant to ceftazidine only as shown in Table (3-6). It resembles to *A. amylolyticus* in its sensitivity to penicillin (Poli *et al*., 2006).

Table (3-6) Antibiotic susceptibility of *A. rupiensis* Ir3 (JQ912241).

Antibiotics	Abbrevia-tion	Sensitivity	Antibiotics	Abbrevia-tion	Sensitivity
Ceftazidine	CAZ	R	Naldixic acid	NA	S
Clarithromycin	CLR	S	Amikacin	AK	S
Ciprofloxacin	Cip	S	Aztreonam	ATM	S
Bacitracin	BA	S	Penicillin	P	S
Cefotaxime	CTX	S	Vancomycin	VA	S
Cephalxin	CL	S	Tobramycin	ToB	S
Gentamycin	CN	S	Chloramphenicol	C	S
Oxacillin	OX	S	Tetracyclin	TE	S
Nitrofurontion	F	S	Erythromycin	E	S
Pipracillin	PRl	S	Carbenicillin	CAR	S

R: Resistant

S: Sensitive

3.6- Plasmid profile:

In order to determine the plasmid profile of *A. rupiensis* strain Ir3 (JQ912241), salting out method (Kieser, 1995) and two types of plasmid isolation miniprep kits Promega and Fermentas were used.

The results showed that this strain harbored small and large plasmid DNA bands. Small plasmid DNA bands were shown when salting out method was used. Whereas large plasmid DNA bands were shown when plasmid miniprep kits were used (Figures 3-7, 3-8 and 3-9).

Velder (2009) reported that microbial biodegradation pathways are often, either fully or partially, encoded on mobile genetic elements, including catabolic plasmids. As these plasmids frequently contain beside catabolic genes/ operons the full set of determinants necessary for conjugative transfer, they are relatively large from approximately 50kb up to more than 1Mb, for example *Pseudomonas putida* EST1020 harbored pEST1026 (109Kb) responsible for phenol degradation and *Arthrobacter keyseri* 12B harbored pREI (130Kb) for phthalate degradation.

Previous studies showed that biodegradation of some oil derivatives depend on plasmids. Plasmids pWWO, pTOM, pNAH, and pOCT are involved in degradation of benzene, toluene, naphthalene and alkane (Pemberton, 1983).

Mirdamadia *et al*. (2010) elucidated that certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation, including the *alk* (C5 to C12 n-alkanes), *nah* (naphthalene) and *xyl* (toluene) pathways have been extensively characterized and are generally located on large catabolic plasmids.

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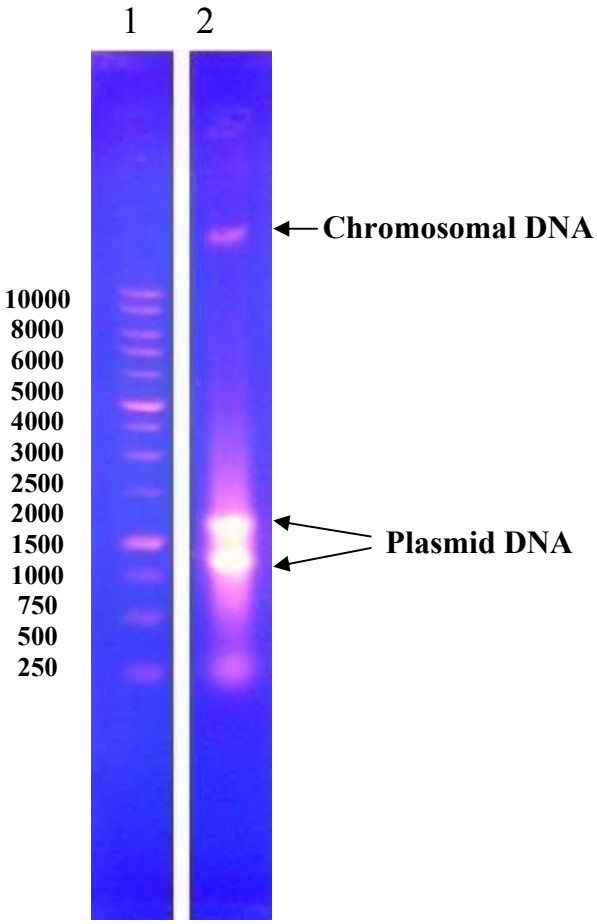

Figure (3-7): Gel electrophoresis for plasmid isolation from *Anoxybacillus rupiensis* strain Ir3 (JQ912241) by salting out method. Electrophoresis was performed on (0.7%) agarose gel with 5V/cm for 1.5 hours. 1: marker DNA ladder 10Kb RTU, 2:A. *rupiensis* Ir3 (JQ912241)

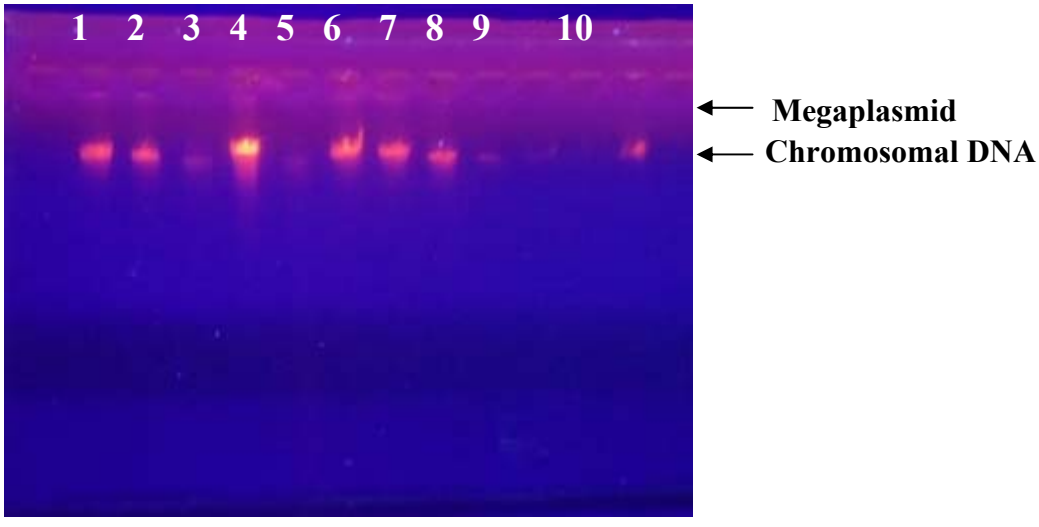

Figure (3-8) Gel electrophoresis for plasmid isolation from *Anoxybacillus rupiensis* strain Ir3 (JQ912241) and the other nine thermophilc bacterial isolates by using promega plasmid miniprep kit. Electrophoresis was performed on (0.7%) agarose gel and run with 5V/cm for 50 minutes.
1:9SM, 2:2G, 3:3A, 4: 4A, 5:12SM, 6:6A, 7:13SM, 8:14SM, 9:21SM, 10:34SM

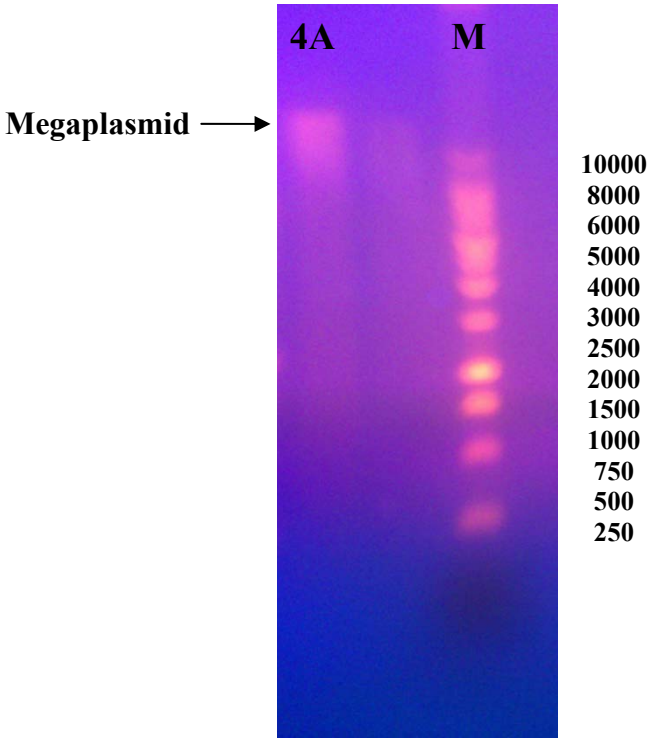

Figure (3-9): Gel electrophoresis for plasmid isolation from *Anoxybacillus rupiensis* strain Ir3 (JQ912241) by using Fermentas miniprep kit. Electrophoresis was performed on (0.7%) agarose gel and run with 5V/cm for 1.5 hours. M: marker DNA ladder 10Kb RTU. 4A: ***Anoxybacillus rupienses* **Ir3 (JQ912241).**

Urata *et al*., (2006) elucidated that the carbazole degradative car-/gene cluster (carAalBalBblClACl) of *Sphingomonas* sp. strain KA1 is located on the 254kb circular plasmid pCAR3. Carbazole conversion to anthranilate is catalyzed by carbazole 1, 9a-dioxigenase (CARDO;carAalAcl), meta- cleavage enzyme (carBalBbl), and hydrolase (carCl). Also Shintani *et al*. (2005) stated that the carbazole-catabolic plasmid pCAR1 isolated from *Pseudomonas resinovorans* strain CA10 was sequenced in its entirety to elucidate the mechanism by which the car operon may have been assembled and distributed in nature. pCAR1 is a 199,035-bp circular plasmid, and carries 190 open reading frames, and it was found that pCAR1 carries the class II transposon Tn4676 containing carbazole-degradative genes, also a new plasmid designated pCAR2 was isolated from *P. putida* strain HSO1.

3.7- Role of plasmid in aromatic hydrocarbons degradation:

In order to study the role of plasmid of *A. rupiensis* strain Ir3 (JQ912241) in aromatic hydrocarbon degradation (for example carbazole), curing experiments were done.

3.7.1- Curing:

Plasmid curing of *A. rupiensis* strain Ir3 (JQ912241) was used to determine whether the genes responsible for hydrocarbon degradation are located on the plasmid or not. For this purpose, many attempts were made in order to cure *A. rupiensis* strain Ir3 (JQ912241) plasmid(s) using SDS and Et.Br. No colonies appeared after treatment with different concentrations of SDS (0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, and 5%), which indicated that this bacterium is sensitive for SDS. The results in Table (3-7) indicated that the highest concentration of EtBr (225µg/ml) allowed the bacterial growth. From this treatment, an appropriate dilutions were made and spread on LB agar plates, (incubated at 70◦C in addition to 55◦C, in order to determine the role of plasmid in temperature resistance), and on selective media, mineral salt agar (CDM) containing carbazole (1mM), in order to determine the cured colonies that lost their ability to utilize carbazole.

Table (3-7) Effect of EtBr (µg/ml) on the growth of *A. rupiensis* strain Ir3 (JQ912241)

EtBr Concentration (µg/ml)	70	80	90	100	125	150	175	200	225	250
Bacterial growth	+++	+++	+	+	+	±	±	±	±	-

(+++): Very good growth
(++): Good growth
(+): Moderate growth
(-): No growth
(±): Slight growth

The result indicated that all the tested colonies of *A. rupiensis* strain Ir3 (JQ912241) (50 colonies were selected randomly) were still able to grow on the presence of carbazole at 70◦C. Plasmid isolation from one of these colonies showed that they are still harboring plasmid (Figure 3-10). No cured cell from *A. rupiensis* strain Ir3 (JQ912241) was obtained, and this may be related to megaplasmid that cannot be cured easily. Therefore, other molecular techniques to confirm the role of plasmid may be needed.

Catabolic pathways, which encode degradative routes of different aromatic and aliphatic hydrocarbon, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid (Coral and Karagoz, 2005).

Chauhan *et al*. (2000) mentioned that *Arthrobacter protophormia* strain RkJ100 is capable of utilizing ρ-nitrophenol as the sole source of carbon, nitrogen, and energy. A large plasmid of approximate 65kb was found to be responsible for harboring genes for ρ-nitrophenol degradation in this strain. This was based on the fact that ρ-nitrophenol (-) derivative was devoid of plasmid and had simultaneously lost their capability to grow on this nitroaromatic compound. Also the plasmid mediation of ability to degrade phenanthrene was demonstrated by Coral Karagoz (2005).

Shimizu *et al*. (2001) found that many aromatic degradation genes are known to be encoded on plasmid DNAs. In particular, several *Rhodococcus* strains harbor aromatic degradation genes on large linear plasmids, for example *Rhodococcus erythropolis* strain BD2 harbors a linear 210Kb plasmid pBD2 carrying the *ipb* genes for isopropylbenzene degradation (Stecker *et al*., 2003).

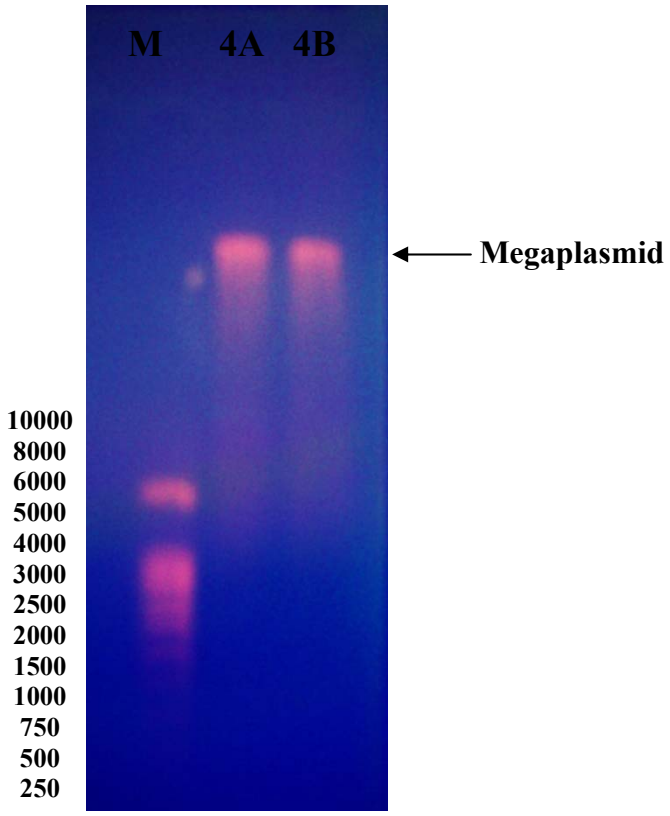

Figure (3-10): Gel electrophoresis of plasmid content of *Anoxybacillus rupiensis* strain Ir3 (JQ912241). Electrophoresis was performed on 1% agarose gel and run with 5V/cm for 1.5 hours.

M: marker DNA ladder 10Kb RTU

4A: plasmid content of the *A. rupiensis* strain Ir3 (JQ912241).

4B: plasmid content of the *A. rupiensis* strain Ir3 (JQ912241) (after treatment with Et.Br).

3.8- Optimization of growth conditions of *Anoxybacillus rupiensis* strain Ir3 (JQ912241):

3.8.1- Determination of the optimum pH:

The growth pH of *A. rupiensis* strain Ir3 (JQ912241) was studied in the range between 5- 9 and at optimum temperature, 55◦C. The result showed that this strain could grow well between pH 5-9, which indicated that *A. rupiensis* strain Ir3 (JQ912241) was a neutrophilic bacterium, like some other members of the *Anoxybacillus* genus, *A. gonensis* CTISari, *A. Kestanbolensis* AC26, *A. voinovskiensis* B9.3 (Inan *et al*., 2011) and *A. flavithermus* DSM 2641^{T} (Pikuta *et al*., 2000) .and the optimum peak for growth was at pH 7 (Figure 3-11),

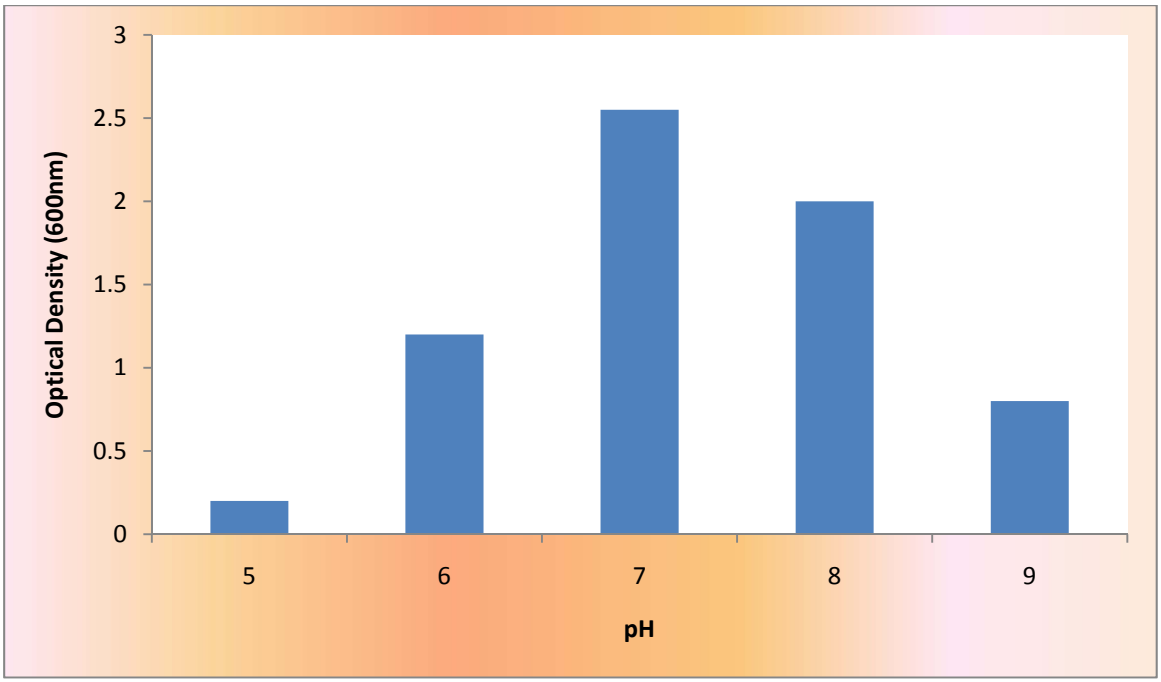

Figure (3-11): Effect of pH on the growth of *A. rupiensis* strain Ir3 (JQ912241), at 55◦C in a shaker incubator (150 rpm) for 24hrs.

which resemble to *A. contaminans* DSM15866T (De Clerck *et al*., 2004) and *Geobacillus tepidamans* GS5-97 (Derekova *et al*., 2007). Bioremediation of oil contamination can only be accomplished by using indigenous bacteria capable of degrading petroleum compounds.

3.8.2- Determination of the optimum temperature:

The growth temperature of *A. rupiensis* strain Ir3 (JQ912241) was studied between 35- 70◦C on LB broth medium. The optimum temperature for growth was 55-65◦C (Figure 3-12). *A. rupiensis* strain Ir3 (JQ912241) grows best by incubating it in different temperatures. One plate was incubated at either: 35, 40, 45, 50, 55, 60, 65 and 70◦ C.. *A. rupiensis* strain Ir3 (JQ912241) grown at 40 ◦C to 70◦C; therefore it is thermophilic, and it may have good potential for application in microbial oil recovery.

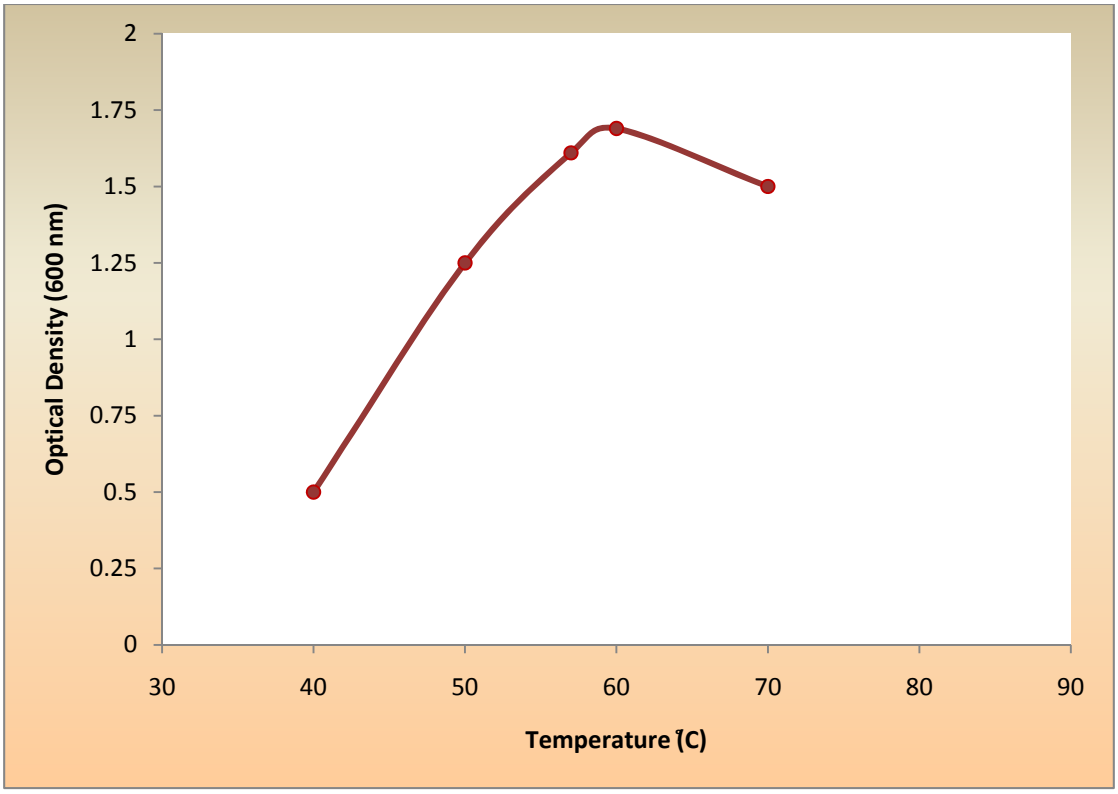

Figure (3-12): Effect of temperature on the growth of *A. rupiensis* strain Ir3 (JQ912241), at pH 7, in a shaker incubator (150 rpm) for 24hrs.

Anoxybacillus.rupiensis strain Ir3 (JQ912241) grew in wide ranges of temperatures and pH. It was similar to other *Anoxybacillus* spp. that also grow in the wide ranges of temperatures and pH such as *A. rupiensis* DSM 17956 sp. nov.which grow at pH 5.5- 8.5 and 35 -67 ◦C (Derekova *et al*., 2007), *A. amylolyticus* sp. nov.which grow at pH 5.0- 6.5 and 45- 65◦C (Poli *et al*., 2006), *A. flavithermus* comb. nov.which grow at pH 5.5- 9.5 and 30- 72 ◦C (Pikuta *et al*., 2000), and *A. contaminans* strain JT-12, which grow at pH 5.0- 10.0 and 45-75◦C (Thitikorn *et al*., 2012). The results indicated that Iraqi soils are rich source of many thermophilic bacteria which could be a good source of interested enzymes from the industrial point of view and further studies are recommended on these thermal soils including study of microbial biodiversity and the biotechnological potent of the isolated strains.

3.8.3- Growth of *A. rupiensis* strain Ir3 (JQ912241) at different sodium chloride concentrations.

The results showed that the growth occurred at 0.5% to 4% of sodium chloride concentrations. The optimum concentration for growth was 0.5% to 1% as shown in Figure (3-13). Growth of *A. rupiensis* strain Ir3 (JQ912241) was inhibited gradually in the presence of NaCl concentration above 4%. It was similar to other *Anoxybacillus* spp. such as *A. kestanbolensis* strain $K4^T$, *A. ayderensis*, and *A. pushchinensis* (Table 3-5).

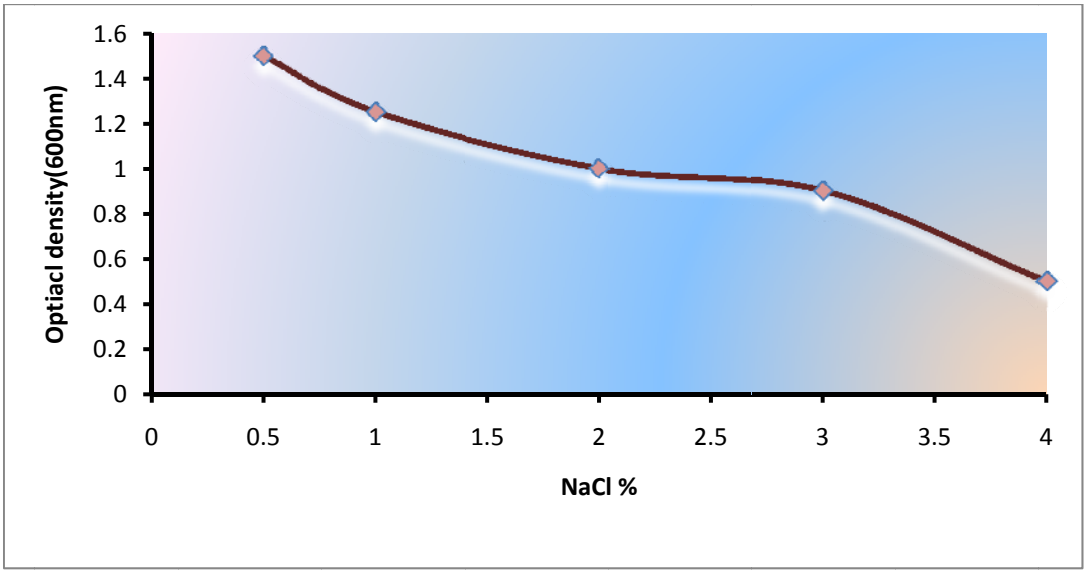

Figure (3-13): Growth of *A. rupiensis* strain Ir3 (JQ912241) at different concentrations of NaCl, at pH 7, 55◦C in a shaker incubator (150 rpm) for 24hrs.

3.8.4- Studying the thermal death curve for *A. rupiensis* strains Ir3 (JQ912241):

The thermal death curve of strain *A. rupiensis* strain Ir3 (JQ912241) after exposure to 80 degree Celsius in water bath for ninety minutes was investigated (Figure 3-14).

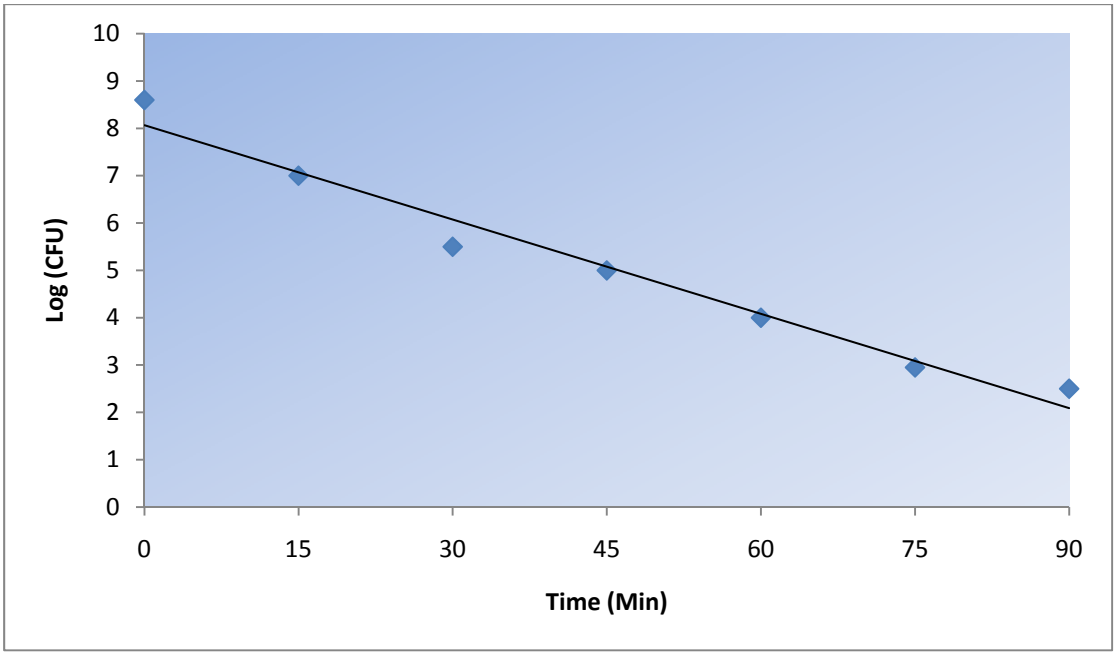

Figure (3-14): Thermal death curve for *A. rupiensis* strain Ir3 (JQ912241) at (80◦C).

Common practices to determine how temperature affects microorganisms are thermal death time (TDT), which is the shortest time it takes to kill specimens, so temperature is kept constant. Most organisms have an optimal temperature ranges for their best growth (K. S. Al Dleamy, personal communication).

After analyzing the results, *A. rupiensis* strain Ir3 (JQ912241) withstands 80◦C for at least 90 minutes. No growth was evident over 90 minutes. *A. rupiensis* Ir3 (JQ912241) could claim to be a thermophilic bacterium, as it grew well at 45 to 75◦C but could not grow at 37◦ C.

Since petroleum is often produced, transported, and processed at elevated temperatures, thermophilic microorganisms/ enzymes that can function at temperatures ranging from 60 to 100◦C may be the most appropriates microorganisms to examine for biorefining applications. Performing biorefining processes at higher temperatures is compatible with existing industry practices and may also result in higher catalytic rates. The reduced viscosity of petroleum

at higher temperatures will allow lower processing costs. Microorganisms which function at thermophilic temperatures can also function at mesophilic / ambient temperatures; whereas, mesophilic microorganisms almost never function at thermophilic temperatures. Therefore, the isolation of thermophilic cultures capable of selectively removing organic nitrogen from petroleum should result in a highly flexible biorefining process that can be used at a wide range of temperatures. Thermophilic microorganisms have not been well studied and no systematic examination of thermophilic cultures for possible use in biorefining has been reported. Moreover studies seek to identify or develop cultures, thermophilic or otherwise, for the removal of nitrogen or metals from petroleum are rare (Kilbane, 2002).

3.8.5- Growth of *Anoxybacillus rupiensis* strain Ir3 (JQ912241) at different carbazole concentrations:

A. rupiensis strain Ir3 (JQ912241) is a representative of carbazole-utilizing Gram-positive bacterium, which utilizes CAR as a sole nitrogen and energy source, was tested at 0.2, 1, 5, and 10mM in the presence of glucose as carbon source. The obtained result revealed higher bacterial growth at 10mM than 0.2mM, i.e. the observed bacterial growth with CAR as an N-source was concentration-dependents (Figure 3-15). An attempt was made to study the biodenitrogenation ability of *A. rupiensis* strain Ir3 (JQ912241**).** The results showed better growth of the bacterium in the minimal medium in presence of carbazole as a nitrogen source and glucose as a carbon source than in presence of carbazole as the only source of N and C, and also better than using NH_4CL (N-source) and glucose (C- source)**.** This indicated that *A. rupiensis* strain Ir3 (JQ912241) might be utilizing organonitrogen compound as a nitrogen source only via the specific cleavage of C-N bond (denitrogenation of carbazole) (data not show).

To the researcher's best knowledge, this is the first study showing that the *A. rupiensis* belong to CAR-degrading bacteria. Various terrestrial bacteria belonging to *Pseudomonas* and *Sphingomonas* show CAR- degrading ability (Ouchiyama *et al*., 1993; Hisatsuka and sato, 1994; Gieg *et al*., 1996; Shepherd and Lloyd- Jones, 1998; Habe *et al*., 2002; Inoue *et al*., 2004, 2005).

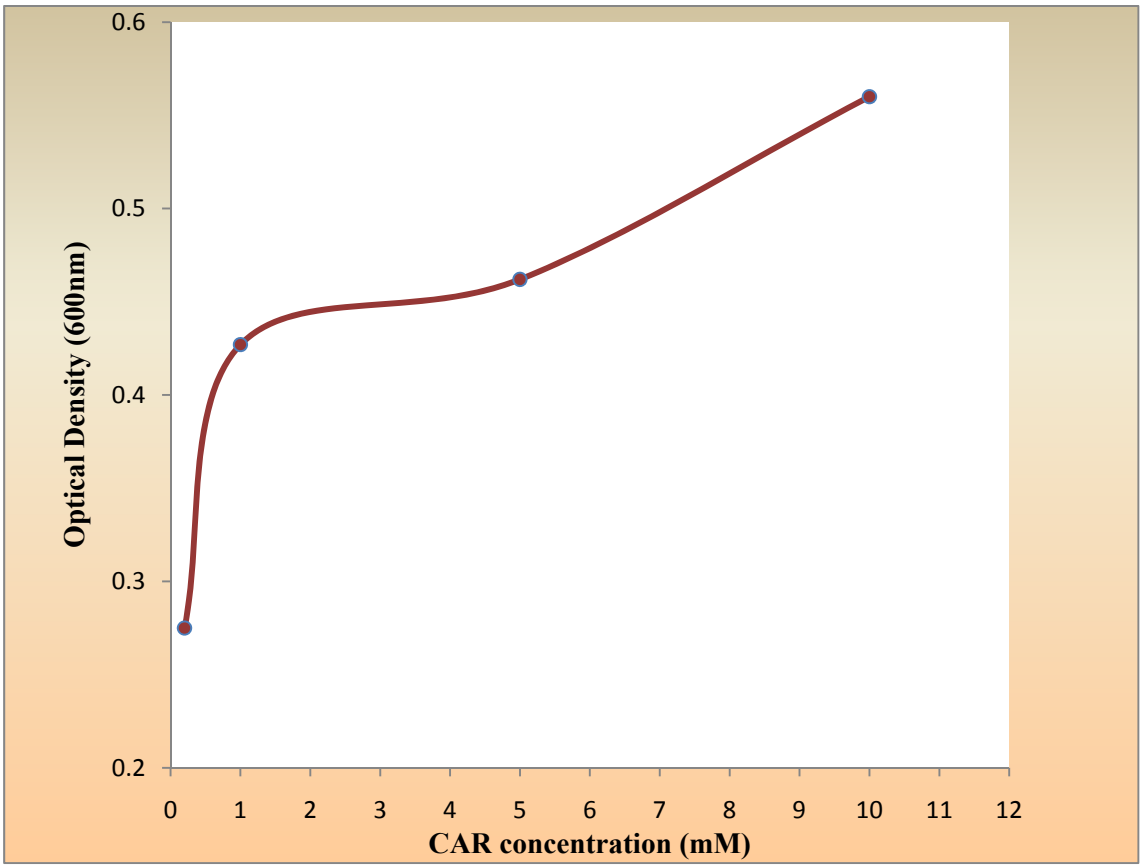

Figure (3-15): Effect of carbazole concentration on the growth of *A. rupiensis* strain Ir3 (JQ912241), at pH 7, 55◦C in a shaker incubator (150 rpm) for 48hrs.

3.9- Tracing the *Anoxybacillus rupiensis* strain Ir3 (JQ912241) consumption of pure aromatic compounds:

The consumption of 1mM carbazol, ρ-nitrophenole, nitrobenzene as the sole of carbon and nitrogen source and naphthalene as the sole carbon source was traced by HPLC in cell-free supernatants of cultures. The *A. rupiensis* strain Ir3 (JQ 912241) showed clear growth with the four aromatic compounds.

The HPLC analysis indicated that carbazole showed as much as 99.62% consumption depending on the area of peaks eluted at 3min (Figure 3-16 a, b). ρ-nitrophenole showed as much as 99.4% consumption depending on the area of peaks eluted at 3min (Figure 3-17 a, b) whereas nitrobenzene showed as much as 97.73% consumption depending on the peaks eluted at 3min (Figure 3-18 a, b), and naphthalene showed as much as 98.89% consumption depending on the peaks eluted at 3.5min (Figure 3-19 a, b).

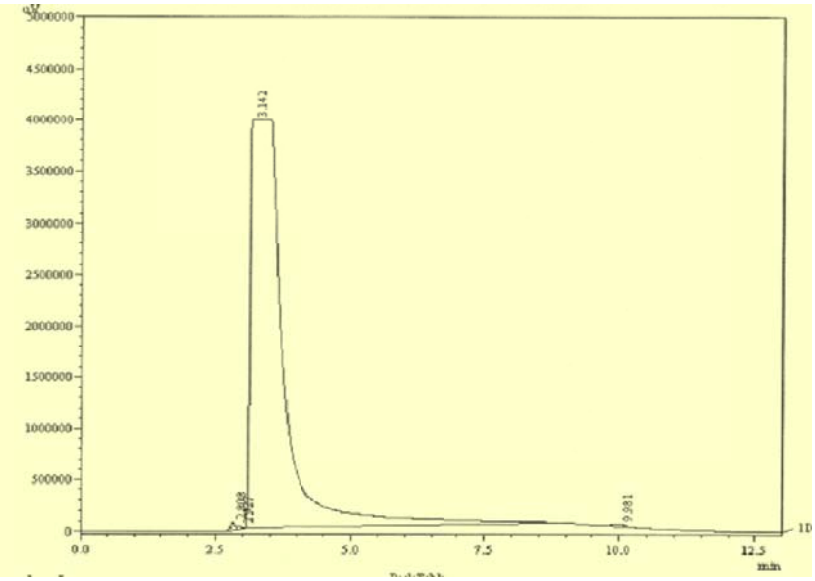

Figure (3-16a) HPLC chromatogram showing retention time (3min) for authentic sample of carbazole. Absorbance was followed at 295nm.

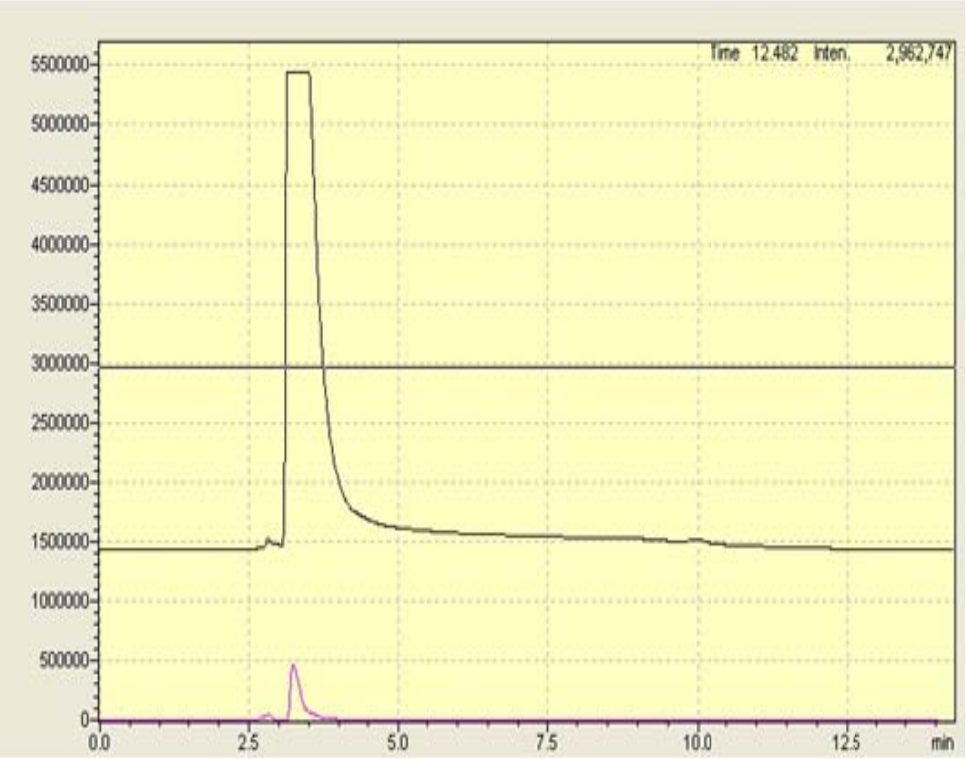

Figure (3-16b): Tracing the consumption of carbazole by HPLC in cell-free supernatant of bacterial culture.

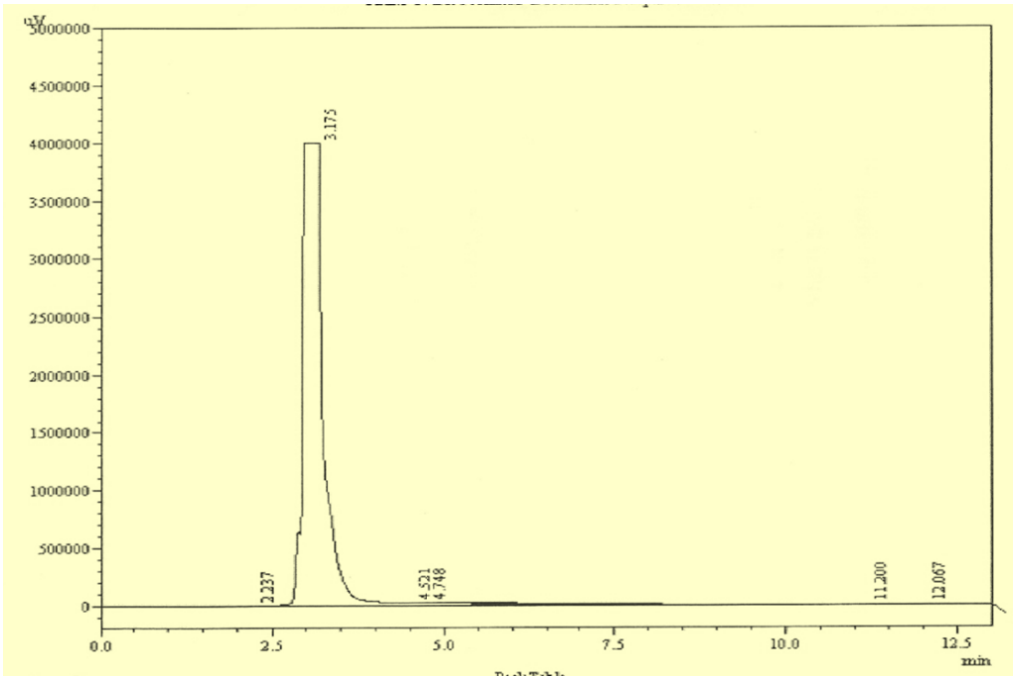

Figure (3-17a): HPLC chromatogram showing retention time (3min) for authentic sample of ρ-nitrophenol. Absorbance was followed at 295nm.

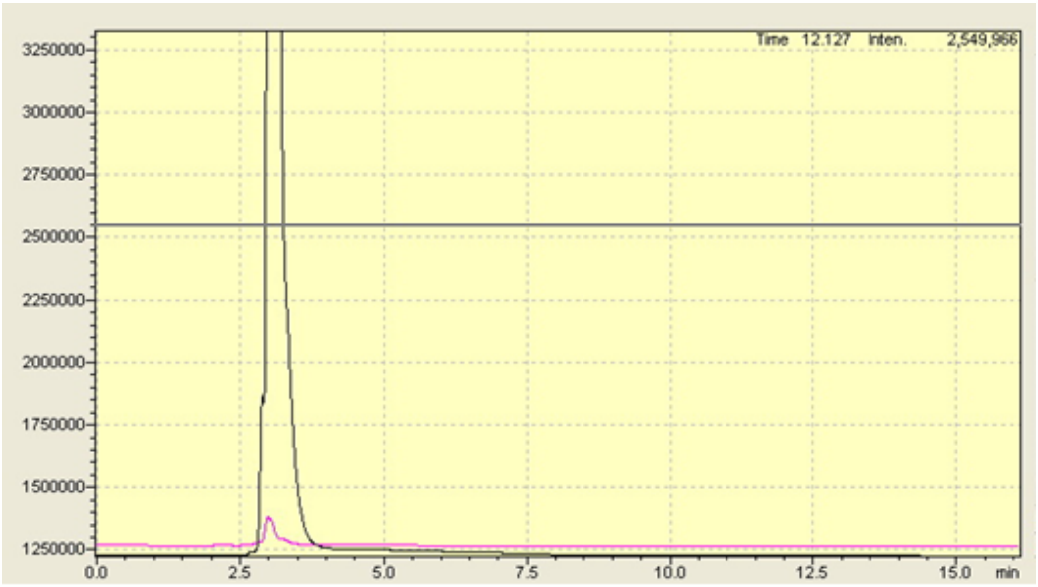

Figure (3-17b): Tracing the consumption of ρ-nitrophenol by HPLC in cell-free supernatant of bacterial culture.

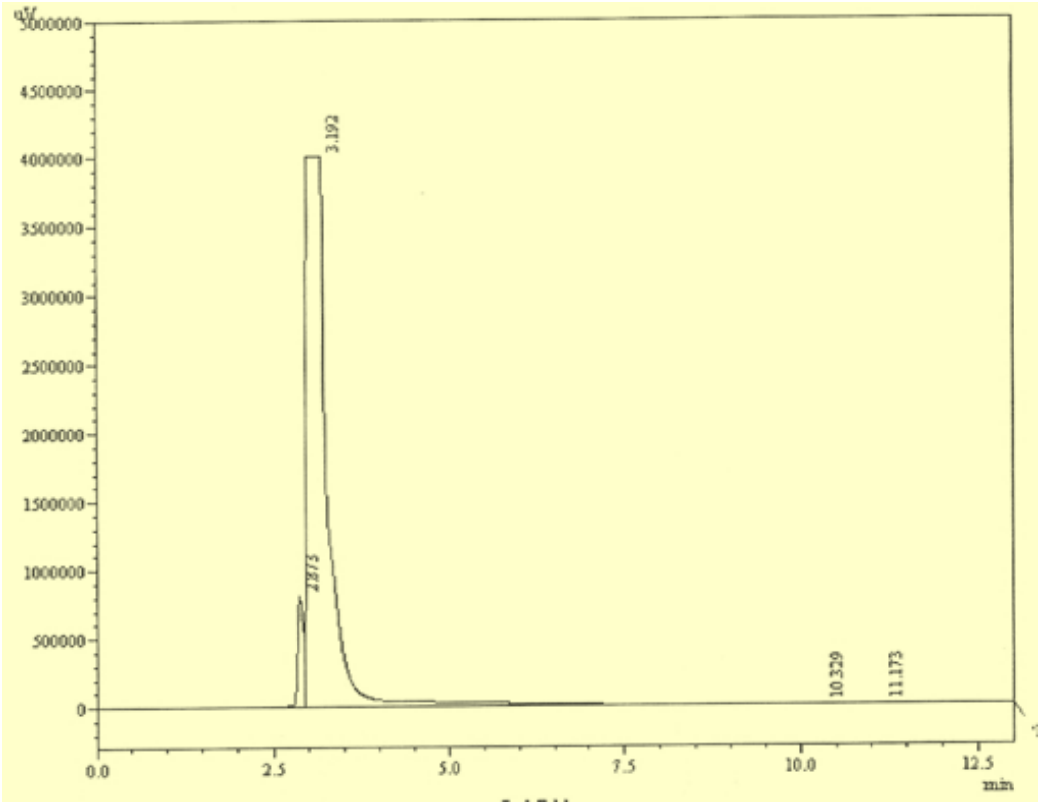

Figure (3-18a): HPLC chromatogram showing retention time (3min) for authentic sample of nitrobenzene. Absorbance was followed at 295nm.

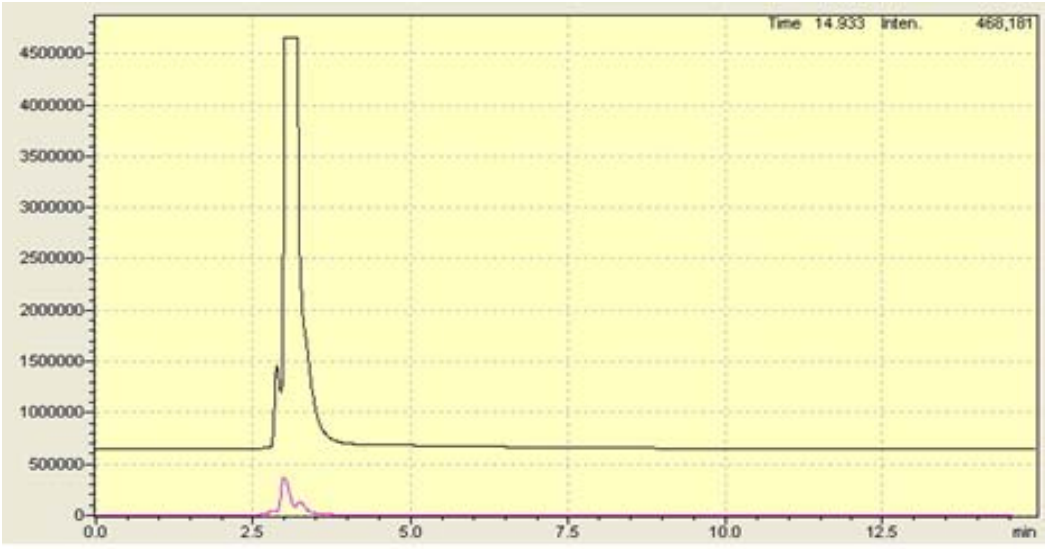

Figure (3-18b):Tracing the consumption of nitrobenzene by HPLC in cell-free supernatant of bacterial culture.

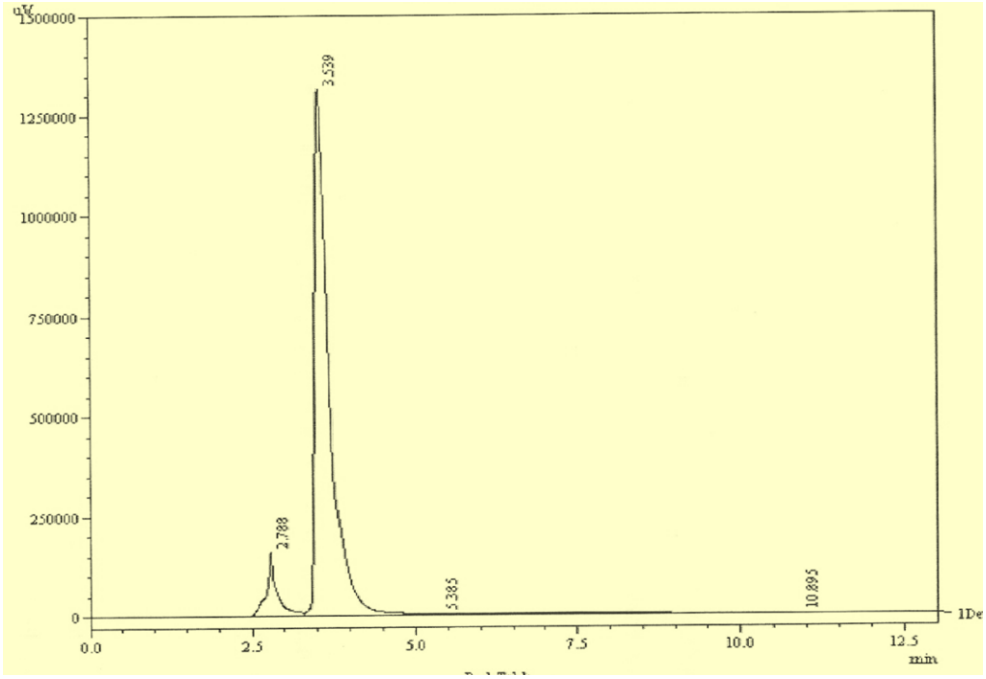

Figure (3- 19a): HPLC chromatogram showing retention time (3.5 min) for authentic sample of naphthalene. Absorbance was followed at 295nm.

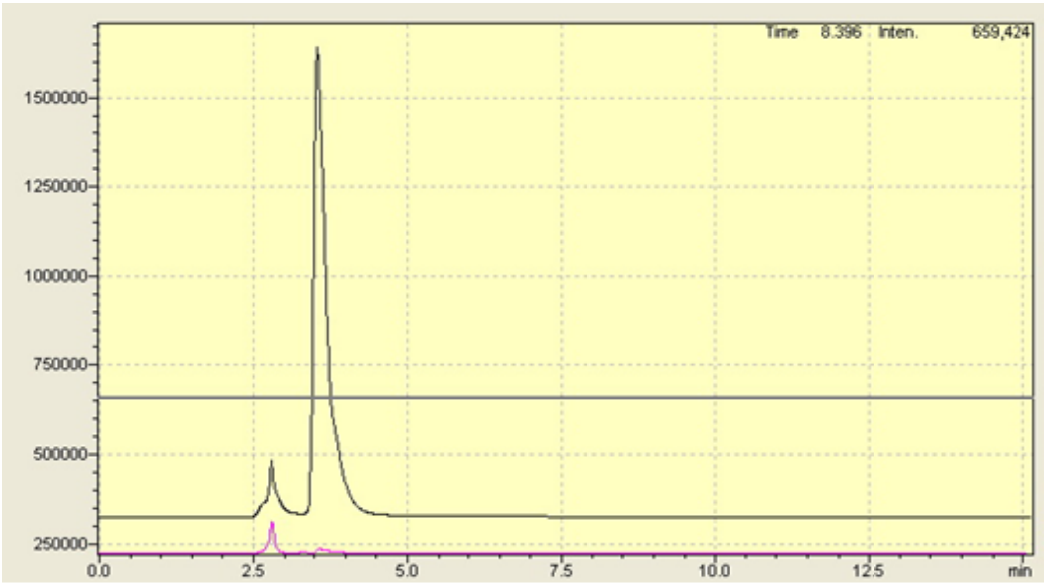

Figure (3- 19b) Tracing the consumption of naphthalene byHPLC in cell-free supernatant of bacterial culture.

It was considered the potential of *A. rupiensis* strain Ir3 (JQ912241) for degradation of carbazol, ρ-nitrophenole, nitrobenzene, and naphthalene. Carbazole and other nitrogen compounds are oxidized to nitrogen oxides throughout petroleum combustion and causes acid rain. During petroleum upgrading, carbazole, as other nitrogen- containing compounds, reduces the efficiency of hydrodesulfurization (HDS) process of diesel feed stocks because of the inactivation of the catalyst (Laredo *et al*., 2004). Therefore, the elimination of nitrogen containing molecules from petroleum distillates enhances the quality of the final fuels (Laredo *et al*., 2004; Choi *et al*., 2004).

Several methods to eliminate nitrogen from fuels have been devised and the most widely used is the hydrodenitrogenation (HDN) at high temperatures and pressure, carried out simultaneously with HDS. An alternative to HDN for carbazole elimination is the bio-treatment of diesel feed stocks. Some bacterial strains have been reported to be able to metabolize carbazole (Kilbane, 2002; Inoue *et al*., 2005) and the most studied strain is *Pseudomonas resinovorans* CA10 (Nam *et al*., 2003). This strain carries the carbazole degrading enzyme coded in a gene cluster (car) that resides in a large plasmid, pCAR1 (Meada *et al*., 2009).

3.10-FTIR analysis

Anoxybacillus rupiensis strain Ir3 (JQ912241) was grown in 100ml CDM supplemented with crude oil (0%, 1%, 2%, 5%, 10%, 20%, and 30%), in addition to negative control (crude oil only). The bacterium showed high resistance and still life until the time of analysis and there were great differences among the control and the test flasks appeared through the FTIR analysis (data are not shown).

It was also possible to verify alteration in superficial film oil which indicates possible bio-degradation process. Some observed alteration suggest the capability of bioemulsifer production by this strain through the formation of oil drops coalescence. In another words, during the incubation, the crude oil

morphology went through three obvious changes. In the first, crude oil appeared as either a large stretch covering the upper part of the aqueous medium or attached on glass wall. Later, the large stretch or attachment of petroleum turned gradually in to disperse oil drops. Immediately after that, the oil drops disappeared even without shaking. This phenomenon is closely related to the emulsification.

3.10.1- Utilization of carbazole in oil mixture:

Since *A. rupiensis* strain Ir3 (JQ912241) degrades carbazole in experiments employing model compounds, it was of interest to determine if similar results could be obtained in experiments using petroleum where a complex of chemicals is present and exposure to petroleum could be potentially damaging to biocatalysts. To test the ability of *A. rupiensis* strain Ir3 (JQ912241) to degrade carbazole (nitrogen compound) in a complex oil mixture, the FTIR analyses were done to the same flasks containing 30% crude oil and negative control.

Sample with 30 % crude oil and control was characterized by FTIR as shown in Figure (3-20b). The following characteristic absorption bands (KBr disc cm^{-1}): 3549, 3475 and 3417 for O-H stretching of anthranilic acid and catechol which produce from the CAR degradation. The spectrum also showed bands at 2954, 2924 and 2854 which attributed to C-H aliphatic stretching due to the presence of different hydrocarbons in crude oil. The three bands are as follow: bands at 1620 due to C=C stretching for benzene ring, bands at 1458, 1377 due to C-H aliphatic banding, while the band at 1118 which could be attributed to C-O stretching of phenol group.

The results of FTIR analysis of biotreated crude oil sample illustrate that *A. rupiensis* strain Ir3 is capable of removing carbazole from crude oil.

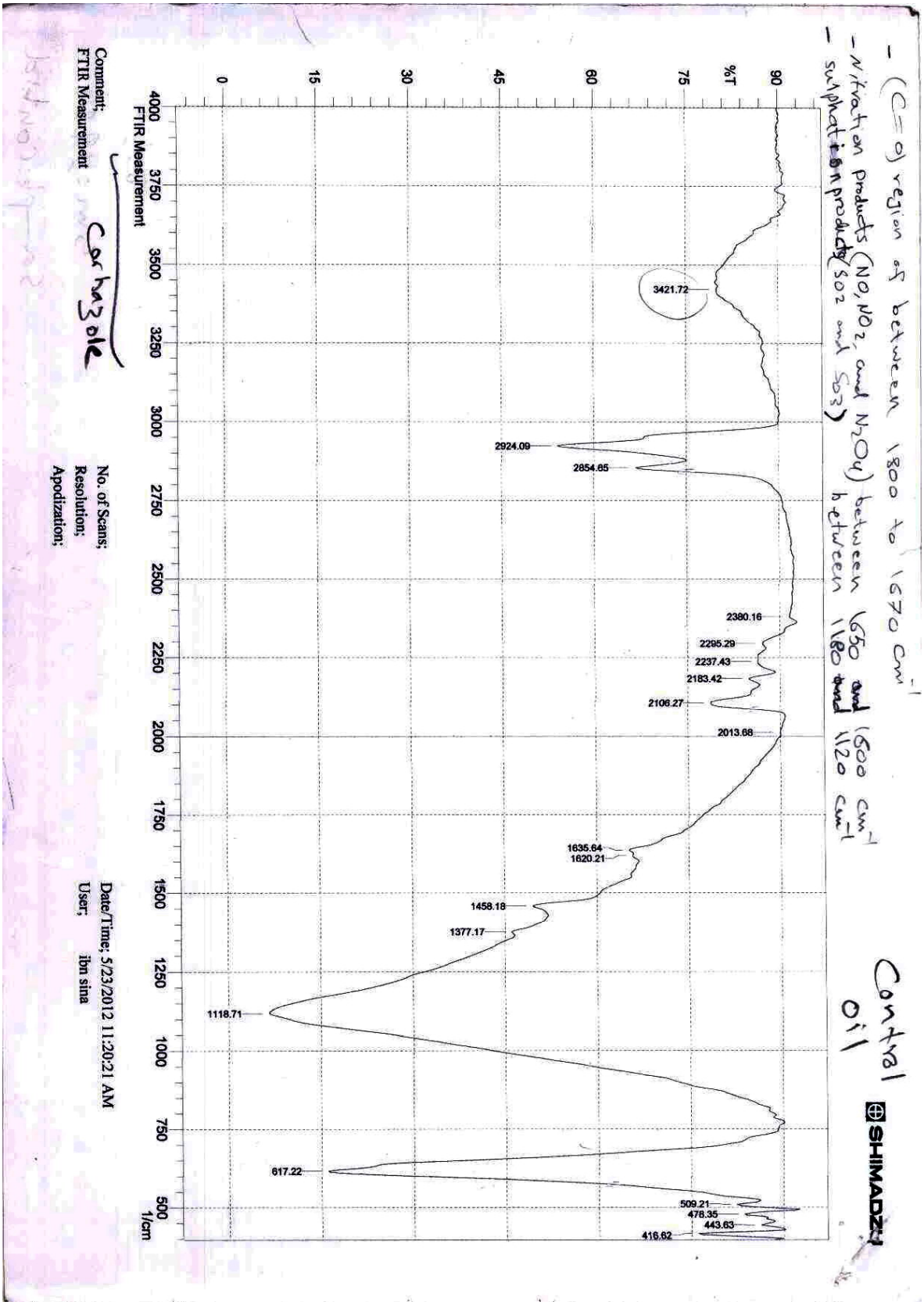

Figure (3-20 a): FTIR spectroscopy for crude oil (control)

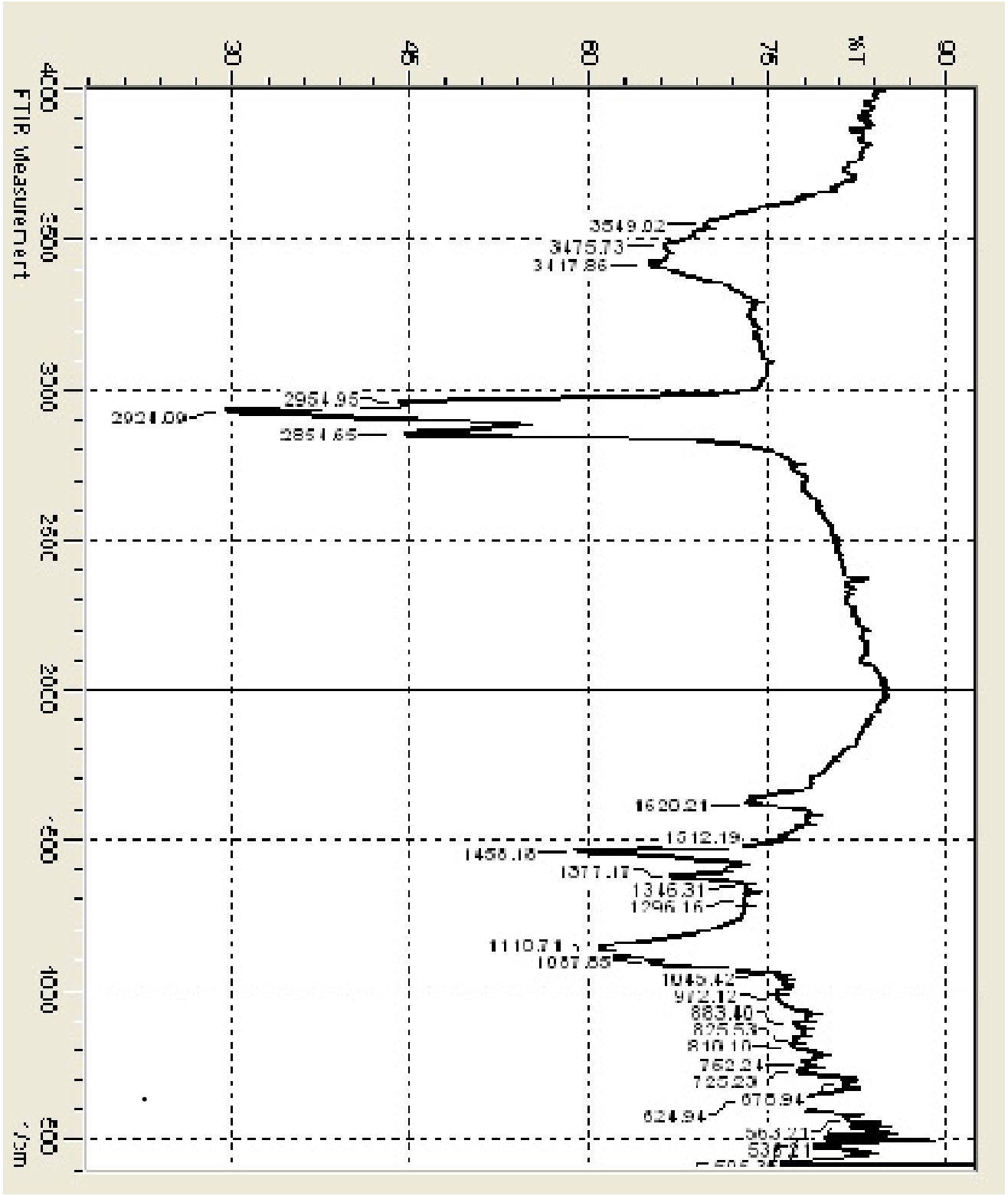

Figure (3-20b) FTIR spectroscopy for 30% crude oil in presence of *A. rupiensis* strain Ir3 (JQ912241), at 55◦C, (150 rpm) shaking for 30 days.

Cleavage of the aromatic ring is a key reaction in the oxidation of aromatic compounds. One of the most frequently encountered key dihydroxy aromatic pieces before ring cleavage occur is catechol. These results suggest that *A. rupiensis* strain Ir3 has genes encoding a cotechol catabolic enzyme.

A. rupiensis strain Ir3 (JQ912241) colonies grown on LB (containing 1mM) and CDM agar plates were examined for the presence of meta-cleavage enzymes activity for monitoring the conversion of 2, 3-dihydroxybiphenyl to brown metabolite, 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoic acid (HOPDA) (oxidation products of the aromatic substrate). *A. rupiensis* strain Ir3 (JQ912241) may produce HOPDA on these media may also suggest a constitutive expression of the carbazole degradation trait. Hamzah and Al-Baharna (1994) showed that *P. cepacia* ATCC29351 possesses the genetic capacity for enzyme of both ortho and meta cleavage pathway of benzoate degradation also, Cunha *et al*. (2005) found that *Bacillus* strains contain genetic capacity for enzymes involved in cleavage pathways of aromatic degradation.

Hence, to the researcher's best knowledge, this is the first study of representative of this genus being capable of mineralizing carbazole. Moreover, it is noteworthy that although all of the known carbazole-utilizing bacteria are Gram-negative bacteria, strain Ir3 was identified as a Gram-positive *A. rupiensis*, in addition to its ability to degrade a variety of aromatic compounds, including ρ-nitrophenol, nitrobenzene (nitroaromatic compounds) and naphthalene (polyaromatic hydrocarbon). Such results are similar to those of Inoue *et al*. (2005) who found that a Gram positive *Nocardioides* sp strain IC177 was capable of mineralizing carbazole. *Nocardioides* species have previously been shown to degrade a variety of aromatic compounds including 2, 4, 6- trinitrophenol, 2, 4, 5- trichlorophenxyacetic acid, ρ-nitrophenol, phenanthrene, and dibenofuran. Also their results indicated that the degradation

pathway for carbazole to anthranilic acid in the strain IC117 was similar to that of the Gram-negative *Pseudomonas resinovoran* CA10.

The results suggest that the degradation pathway for CAR to anthranilic acid in this strain may be similar to strain CA10.The capability of bacterial strain to degrade CAR to anthranilic acid is one of the most important characteristics since anthranilic acid is easily degradable which regarded as harmless substance. The anthranilic acid is assimilated for the tryptophan biosynthesis pathway by various organisms (Gibson and Pittard, 1968). Zaki (2006) found that the typical pathway for metabolizing an aromatic compound like phenol is to dihydroxylate the benzene ring to form a catechol derivative and then open the ring through ortho and meta oxidation. Also Omokoko *et al*. (2008) indicated that *Geobacillus sterothermophilus* is able to utilize phenol as a sole carbon source. A DNA fragment encoding a phenol hydroxylase catalyzing the first step in the *meta*-pathway has been isolated previously. Catechol is either oxidized in a reaction catalyzed by catechol-1, 2-oxygenase which is described as an *ortho* pathway, or is oxidized in reaction catalyzed bycatechol-2, 3-dioxygenase (C23O) the *meta* pathway to 2-hydroxymuconic semialdehyde. The final products of both the pathways are molecules that can enter the tricarboxylic acid cycle.

Lean and Manoj (2005) reported that the heavy crudes (bitumen) are extremely viscous and contain high concentration of asphaltene, resins, nitrogen and sulfur containing heteroaromatics and several metals, particularly nickel and vanadium. These properties of heavy crude oil present serious operational problems in heavy oil production and downstream processing. There are vast deposits of heavy crude oils in many parts of the world. In fact these reserves are estimated at more than seven times the known remaining reserves of conventional crude oils. It has been proven that reserves of conventional crude oil are being depleted, thus there is a growing interest in the utilization of these

vast resources of unconventional oils to produce refined fuels and petrochemicals by upgrading. Presently, the methods used for reducing viscosity and up-gradations are costy, less selective and environmentally reactive. Biological processing of heavy crudes may provide an eco-friendly alternative or complementary process with less severe process conditions and higher selectivity to specific reactions to upgrade heavy crude oil.

Chapter Four

Conclusions and Recommendations

Chapter Four

Conclusions and Recommendations

4.1: Conclusions:

- Depending on partial sequencing of 16S rDNA gene, the majority of thermophilic isolates involved in the present study is closely related to members of family *Bacillaceae*.
- The thermophilic and Gram positive *A. rupiensis* strain Ir3 (JQ912241) exhibited a unique and remarkable ability to degrade aromatic compounds at the range 55-65◦C.
- Using of ordinary morphological, physiological and biochemical tests is not applicable in this case. Based on 16S rRNA gene sequence analysis, three of these aromatic compounds degradable bacterial strains belonged to the genus *Anoxybacillus*. One strain belonged to the genus *Geobacillus*. These strains resembled the *A. rupiensis* and *G. thermoleovorance* respectively.
- Sequence analysis using 16S rDNA gene revealed that most of the genera identified in this study have not been reported previously as CAR-utilizing bacteria.
- This study may be the first one which is carried out in Iraq. It elucidates the occurrence and role of thermophilic hydrocarbon utilizing bacillus.
- Preliminary results suggest that *A. rupiensis* strain Ir3 (JQ912241) probably harbor novel metabolic capability for denitrogenation of carbazole via the specific cleavage of C-N bond.

- The degradation of carbazole might proceed via the formation of 2-aminobenzoic acid in analogy to other reported carbazole-degrading bacteria.
- Mesophilic and thermophilic aromatic compounds degraders dominate in Iraqi hydrocarbons contaminated soils.

4.2: Recommendations

- Various analytical techniques are required such as isolation the accumulated N-free aromatic end-product (s) of CAR or conducting experiments with C^{14} or C^{13} labeled CAR.
- Determination of car genes and construction of expression plasmids (to express the car genes under control of the lac promoter in *E. coli)*
- Using of nanoparticles represents a new generation of environmental- remediation technologies that could provide cost-effective solutions for both, the most challenging environmental clean-up problems and enhancement of petroleum quality.
- Further studies should be conducted to clarify the pathways probably involved in the denitrogenation of carbazole by *A. rupiensis* strain Ir3 (JQ912241).

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A

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Appendix 1

GGTACGGCTACCTTGTTACGACTTCACCCCAATCACTTGCCCCACCTTCGG
CGGCTGGCTCCCGTAAGGGTTACCTCACCGACTTCGGGTGTTGCAAGCTCT
CGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGG
CATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGC
AGCCTGCAATCCGAACTGAGAGCGGCTTTTTGGGATTCGCTCCCCCTCGCG
GGTTCGCAGCCCTTTGTACCGCCCATTGTAGCACGTGTGTAGCCCAGGTCA
TAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGACTTGTCGCC
GGCAGTCCCTCTAGAGTGCCCACCTTCGTGCTGGCAACTAGAGGCGAGGG
TTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAACCATGCACCACCTGTCACCCTGTCCCCCCAAAGGGGGAACGCCCAAT
CTCTTGGGTTGTCAGGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCT
TCAAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTT
TGAGTTTCACCCTTGCGGGTACTCCCCAGGCGGAGTGCTTATCGCGTTAGC
TGC

sample 2G (the sample is related to *Geobacillus thermoleovorans*)

ATACGGCTACCTTGTTACGACTTCACCCCAATCATCTGCCCCACCTTCGGC
GGCTGGCTCCCGTAAGGGTTACCCCACCGACTTCGGGTGTTGCAAACTCTC
GTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGC
ATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCA
GCCTGCAATCCGAACTGAGAGCGGCTTTTTGGGATTGGCTCCCCCTCGCG
GGTTCGCAACCCTTTGTACCGCCCATTGTAGCACGTGTGTAGCCCAGGTCA
TAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGATTTGTCATC
GGCAGTCACCTTAGAGTGCCCAACTCAATGCTGGCAACTAAGGTCAAGGG
TTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACAAGCTGACGA
CAACCATGCACCACCTGTCACCCTGTCCCCCGAAGGGGAACGCCCGATCT
CTCGGGGTGTCAGGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC
AAATTAAACCACATGTTCCACCTTCTT.

sample 3A (the sample is highly related to *Anoxybacillus rupiensis*)

ACGGCTACCTTGTTACGACTTCACCCCAATCATCTGCCCCACCTTCGGCGG
CTGGCTCCCGTAAGGGTTACCCCACCGACTTCGGGTGTTGCAAACTCTCGT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCAT
GCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGC
CTGCAATCCGAACTGAGAGCGGCTTTTTGGGATTGGCTCCCCCTCGCGGGT
TCGCAACCCTTTGTACCGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAA
GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGATTTGTCATCGGC
AGTCACCTTAGAGTGCCCAACTCAATGCTGGCAACTAAGGTCAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA

CCATGCACCACCTGTCACCCTGTCCCCCGAAGGGGAACGCCCGATCTCTC
GGGGTGTCAGGGGATGTCAAGACCTGGTAAGGTTCTTCCCGTTGCTTCAA
ATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTTGA
GTTTTCNTTCTTGCGAGGCGGNCT

sample 4A (the sample is highly related to *Anoxybacillus rupiensis*)

الخلاصة

الهدف الرئيسي لهذه الدراسة هو عزل وتشخيص بكتيريا محبة للحرارة قادرة على استهلاك المركبات الهيدروكاربونية الأروماتية وخصوصا المركبات الحاوية على النايتروجين والتي تعتبر من المكون الرئيسي للنفط الخام لما لها من تأثير على مواصفات النفط من حيث الجودة والنوعية، جمعت (95) عينة تربة ملوثة بالمركبات الهيدروكاربونية من مناطق مختلفة في البصرة وبغداد.

عزلت 48 عزلة بكتيرية مستهلكة للنفط الخام، منها 38 عزلة محبة للحرارة المتوسطة أظهرت قابلية جيدة للنمو على النفط الخام كمصدر وحيد للكاربون والطاقة والتي مثلت على التوالي 20 عزلة تعود الى جنس *Pseudomonas* وثلاث عزلات لكل من الأجناس *Staphylococcus, Acinetobacter* وعزلتين لكل من الأجناس *Aeromonas, Alcaligenes, Flavobacterium* وستة عزلات من جنس *Bacillus*.

بالأضافة الى هذه العزلات اظهرت النتائج وجود 10 عزلات محبة للحرارة العالية وكانت ذات قابلية ممتازة لأستهلاك النفط الخام كمصدر وحيد للكاربون والنايتروجين والطاقة، درست قابلية استهلاك هذه العزلات العشر لعدد من المركبات الأروماتية naphthalene, carbazole, nitrobenzene, ρ– nitrophenole وبينت النتائج وجود تباين في قابلية العزلات على استهلاك هذه المركبات.

يعد تشخيص العزلات المحبة للحرارة العالية بالطرق التقليدية المعتمدة على الصفات الشكلية والأختبارات الفسيولوجية والكيموحيوية امرا بالغ الصعوبة وغير قاطع لذلك خضعت العزلات (أربعة عينات مختارة فقط) للتشخيص الجزيئي بالتضخيم للدنا الرايبوزي بأستخدم سبعة بادئات (fd1, fd2, fd3, fd4, rd1, rp1, and rp2) Primers for the PCR amplification of eubacterial 16S rDNA ، ثم يتبعه دراسة التتابع. وبعد معاينة تتابع 16S rDNA gene مع نظائره في BLAST التابعه لNCBI database ، بينت النتائج تماثلا في العزلات 2G (Ir1) (*Geobacillus thermoleovoranas*), 3A (Ir2) and 4A (Ir3) (*Anoxybacillus rupiensis*) ونظرا لأصالة هذه العزلات فقد تم ضمها الى معطيات بنك الجينات National genebank database وبرقم تعريف JQ912239, JQ912240, and JQ91241 على التوالي.

أختيرت السلالة *A. rupiensis* Ir3 (JQ912241) للدراسات اللاحقة كونها أكفأ العزلات فقط أظهرت نمو اكثر من بقية العزلات عند تنميتها على المركبات الأروماتية، وعند دراسة النمط البلازميدي لها تبين أن هناك نمطين بلازميديين مختلفين، اذ أحتوت العزلة على بلازميدين أحدهما صغير والأخر كبير.

لتحديد دور البلازميدات في استهلاك المركبات الأروماتية (وعلى سبيل المثال carbazole)، اجريت عدد من تجارب التحييد بأستخدام كبريتات الصوديوم ثنائية الأسيل (SDS) وصبغة الأثيديوم بروميد (EtBr)، وأظهرت النتائج عدم الحصول على خلايا محيدة (فاقدة لقدرتها على استهلاك ال carbazole عند درجة 70◦C). جرى استخلاص بلازميدي لبعض العزلات بعد معاملتها بال Et Br، وتبين ان هذه العزلات ما زالت تحتفظ بالبلازميد الكبير الذي من الصعوبة تحييده مما يشير الى احتمالية حمله للصفة.

عينت الظروف المثلى لنمو السلالة المختارة *A. rupiensis* Ir3 (JQ912241) واظهرت النتائج ان النمو في وسط LB برقم هيدروجيني 7 والحاوي1%-0.5 من NaCl في حاضنة هزازة (150دورة/دقيقة) بدرجة حرارة 55-65◦C لمدة 24h ، كما ووجد ان هذه البكتيريا يمكنها ان تقاوم درجة حرارة تصل 80◦C ولمدة 90min . تم تضبيط الظروف المثلى لنمو السلالة *A. rupiensis* Ir3 (JQ912241) في الوسط الملحي (CDM) الرقم الهيدروجيني إلى 7 وحضن بدرجة حرارة 55-65◦C والنمو البكتيري قد اظهر زيادة طردية مع زيادة تركيز الـ carbazole وهذا يشير الى ان هذه السلالة معتمدة على التركيز.

ولغرض التاكد من قابلية السلالة *A. rupiensis* Ir3 (JQ912241) على استهلاك المركبات الأروماتية، استخدمت التجارب التحليلية والتي شملت HPLC و FTIR . فقد تم التحري كميا عن قابلية السلالة لأستهلاك المركبات الأروماتية المختلفة بأستخدام جهاز كروماتوغرافيا السائل عالي الكفاءة HPLC، أظهرت النتائج أن بأمكان السلالة استهلاك نسبا عالية جدا من المركبات الهيدروكاربونية الحلقية حيث بلغت 99.62% من carbazole ، 99.4%من ρ-nitrophenole، 97.73% من nitrobenzene ، 98.89% من naphthalene. وللتحري نوعيا عن قابلية السلالة المختارة على استهلاك المركب النايتروجيني carbazole ضمن المزيج المعقد للنفط الخام بأستخدام تقنية FTIR وأظهرت النتائج قابلية هذه العزلة على استهلاك المركب وتحويله الى مركب ابسط (anthranilic acid) والذي يمكن أن يدخل ضمن TCA cycle.

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قسم التقانة الاحيائية

دراسة جزيئية للبكتيريا المتحملة للحرارة القادرة على تحليل المركبات الحلقية النايتروجينية

أطروحة

مقدمة الى مجلس كلية العلوم – جامعة النهرين

وهي جزء من متطلبات نيل درجة دكتوراه فلسفه في التقانة الاحيائية

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