تحضير مزيج بكتيري محلي كنموذج للتحلل الحيوي للمخلفات النفطية في مصفى الدورة

أطروحة مقدمة إلى
مجلس كلية العلوم – جامعة بابل
وهي جزء من متطلبات نيل درجة دكتوراه فلسفة علوم
في علوم الحياة / الأحياء المجهرية
من قبل

إيمان محمد جار الله

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صفر - 1429 هـ شباط - 2008 م

Preparation of a local bacterial consortium as a model of biodegradation of oily waste products of AL- Dora Refinery

A Thesis
Submitted to the Council of College of Sciences /University of Babylon in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in
Biology / Microbiology

By
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Supervised by
Dr. Hassan Fadhil Naji **Dr. Wesam Abd-Alzahra Hindy**

February – 2008 **Safar – 1429**

Certification

We certify that this thesis was entitted ((***Preparation of a local bacterial consortium as a model of biodegradation of oily waste products of AL-Dora Refinery***)) prepared under our supervision at the Department of biology , College of Science , University of Babylon as a partial of fulfillment for the requirements of the degree of Doctor of philosophy in Biology (Microbiology) and this work has never been published anywhere .

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e

إِنَّا فَتَحْنَا لَكَ فَتْحاً مُّبِيناً ۝ لِيَغْفِرَ لَكَ اللَّهُ مَا تَقَدَّمَ مِن ذَنبِكَ
وَمَا تَأَخَّرَ وَيُتِمَّ نِعْمَتَهُ عَلَيْكَ وَيَهْدِيَكَ صِرَاطاً مُّسْتَقِيماً ۝
وَيَنصُرَكَ اللَّهُ نَصْراً عَزِيزاً ۝

صدق الله العلي العظيم

سورة الفتح الاية (1-3)

Examination committee

We certify that we have read this thesis entitled " ***Preparation of a local bacterial consortium as a model of biodegradation of oily waste products of AL-Dora Refinery*** " and as an examining committee , examined the student " ***Eman Mohammed Jaralla*** " in its content and , in our opinion it meets the standards requirement as a thesis in fulfillment for the degree of Ph. D. in Biology / Microbiology, and the thesis has been accepted with (***Excellent***) estimation.

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Acknowledgments

I would like to express my gratitude to my supervisors Dr. Wesam Abd-Alzahra Hindy Al-Taher and Dr. Hassan F.Naji for help , support and guidness throughout this work.

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Finally , I would like to thank Dr. Khalid Abdalla , The chairperson of water treatment unit in Ministry of science and Technology for his experimental assistance in HPLC analysis .

E. M. jaralla

Dedications

To my husband , my children with my great love .
To my parents , my brothers and sisters with my deep grateful.
To the memory of my dear brother ((Haider))

Conclusions

1. Wastewater of Al-Dora Oil Refinery was contained of both low and high molecular weight aromatic hydrocarbons

2. The Gram negative bacterial isolates were construct 63.3 % of the total isolates were construct 63.3 % of these were able to grow in hydrocarbon substrate as a sole carbon and energy source 36.6% of the total isolates were Gram positive , all of these were failed to grow in hydrocarbon substrate

3. Gram negative isolates **P. Putida , S. spiritivorum , S. liqifacienm , A. media , F. breve . , K. oxytoca , A. baumanni , A. eutrophus** were able to degrade hydrocarbon substrate .

4. bacterial isolates **S.aurens , S.agalactiae , P.vulgaris . M.leutus , C.amycolatum , E.coli , B. Subtilis and M.catarrhalis .**

5. **P. Putida** was the most efficient hydrocarbon degrading isolate , while **A.eutrophus** was the less one.

6. **P. Putida , S. spiritivorum , S. liqifacienm , A. media ,** were consumed C6 and C7 completely .

All isolates were exhibited less ability to consume a high molecular weight poly aromatic hydrocarbons .

7. Isolated bacterial consortium composed of the eight hydrocarbon – degrading isolates exhibited a good efficincy to degrade C6 , C7 , C8 and C10 completely , while it consume 92 % of C14 , But utilized about half amount of HMW PAHs .

8- isolated consortium was removed 61.1 % of the total hydrocarbon mixture components , and this percentage represented 73% of the imported consortium efficiency .

9. Imported consortium composed of several types of microorganisms (Fungi , Gram positive and negative bacteria , candida , protozoa , and actinomycetes).

Recommendations

1- screening for another types of microorganisms that have hydrocarbon-degradation ability such as fungi , algae , atcinomycetes , cyanobacteria and protozoa

2- prepare a microbial consortium mimic to that imported one to be use din the biological treatment of petroleum contaminated in addition to genetic studies may be done as a further researches to improve an increase the potency of the isolated strains for hydrocarbon degradation .

3- in addition to carbon & energy sources , other mineral nutrients such as nitrogen in the from of ammonia , sulfur in the from of sulfide or sulfate and phosphorus which may limit microbial growth an activity , may be studied to determine their role in biodegradation .

4- petroleum – containing wastes generally fall into the hazardous – wastes category as identified and limited by the (EPA) , but the potential health risk is difficult to asses and evaluation of data , the excursion of preventive and remedial measures is still a matter of opinion and likely to be so for many years , so it is a good to concentrate some efforts on health aspects and potential risks .

5 most of the studies of the microbial metabolism of PAHs have been conducted using pure cultures and / or purified enzymes , however , little is known , if these reactions occur under environmental conditions at future research should be designed to study these mechanisms in the natural microbial habitats

6 HC- degrading isolates maybe genetically engineering to become more efficient to degrade HMW PAHs

الخلاصة

تم عزل 22 عزلة بكتيرية هوائية تعود إلى 16 جنسا مختلفا من خزانات تجميع المياه الملوثة بالهيدروكاربونات الناتجة عن تكرير النفط في مصفى الدورة ببغداد ، بدأت الدراسة من الفترة الممتدة من شهر أيار / 2006 ولغاية كانون الاول / 2007

شخصت جميع العزلات بالاعتماد على الصفات المظهرية والفحوصات الكيميوحياتية، وكان مجموع العزلات السالبة لصبغة كرام 14 نوعا تعود الى 11 جنسا ، اما الموجبة لصبغة كرام فقد كان 8 انواع تعود الى خمسة اجناس . درست قدرة هذه العزلات على النمو في وسط الاملاح المعدنية السائل الحاوي على 1 % من المسحوق الهايدروكاربونات الخام كمصدر وحيد للكاربون وللطاقة وقد اظهرت الدراسة ان ثمانية عزلات سالبة لصبغة كرام كانت لها القدرة على استهلاك الهايدروكاربونات وهي :

Pseudomonas putida , Sphingomonas spritivorum , Flavobacterium breve , Aeromonas media , Serratia liquifaciens , Acinetobacter baumanni , Klebseilla oxytoca , Alcaligenes eutrophus .

وقد درست كفاءتها في تكسير الهايدروكاربونات في الوسط الزرعي اذ تم تعيين العدد الحي الكلي والاس الهيدروجيني والشد السطحي للوسط الزرعي وانتاج المستحلبات الحياتية وخفض محتوى الوسط الزرعي من الزيوت والشحوم واستهلاك الاوكسجين المذاب والنسبة المئوية لازالة الهيدروكاربونات من الوسط وقد وجد ان اكثر العزلات كفاءة هي ***P.putida*** اذ استهلك 52,2 % من وزن الهيدروكاربونات المستخدم في الوسط ، اما اقلها كفاءة فكانت ***A.eutrophus*** اذ استهلكت 24,6 % منه . اظهرت قراءات جهاز كروماتوغرافيا السائل العالي الكفاءة وجود اختلافات معنوية في تراكيز المركبات الهايدرو كاربونية الحلقية بعد المهاجمة بالعزلات البكتيرية مقارنة بنموذج السيطرة . وقت استهلكت المركبات ذات الست والسبع ذرات كاربون بشكل كامل من قبل ***P.putida , S.spritivorum A.media, S.liquifaciens ,*** بالإضافة إلى المركبين C8 و C10 اللذان استهلكا أيضا بشكل كامل من قبل **P.putida,A.baumanni** على التوالي .

لم تكن جميع هذه العزلات كفوءة باستهلاك الهايدروكاربونات الحلقية ذات الاوزان الجزيئية العالية (المكونة من اربع حلقات بنزين او اكثر) ، وقد كانت اقل العزلات كفاءة هي ***K.oxytoca*** حيث استهلكت 3.4% من المركبات C22 .

اظهرت العزلة ***K.oxytoca*** زيادة في المركبات C8 بنسبة 6.6% ، في حين اظهرت العزلتين ***A.media*** و ***A.eutrophus*** زيادة في المركبات C8 بنسبة 12.3% و 30.7% على التوالي.

من ناحية اخرى جمعت العزلات البكتيرية المستهلكة للهايدروكاربونات مع بعضها لغرض تحضير خليط بكتيري وقد اختبرت قابليتها على تكسير المركبات الهايدروكاربونية وقد اظهر كفاءة في استهلاك المركباتC10وC8 وC7وC6 بشكل كامل , كما انه استهلك 92% من المركبات C14 ولكنه لم يتمكن من استهلاك المركبات الهايدروكاربونية الحلقية ذات الاوزان الجزيئية العالية بنفس الكفاءة ، حيث استهلك 57.8% و 55.8% و 40.8% من المركبات C18 و C20 و C22 على التوالي ، مقارنة بنسبة 80.2% و 80.8% و 75.1% على التوالي من قبل الخليط المايكروبي المستورد لنفس المركبات .

اظهر الخليط البكتيري المحضر في هذه الدراسة قدرته على تحطيم الهايدروكاربونات بنسبة شكلت 73% من كفاءة الخليط المستورد حيث ازال 61.1 % من المجموع الكلي للهيدروكاربونات مقارنة مع الخليط المستورد والذي شكل نسبة ازالة 83.3% منه.

Summery

Twenty two aerobic bacterial isolates belonged to sixteen different genera were isolated from the industrial effluent of Al-Dora Oil refinery in Baghdad through the period of May , 2004 to December / 2006 .

These isolates were characterized. The Gram negative isolates were 14 species , belong to 11 genera , while the gram positive isolates were 8 species belong to 5 genera.

The abilities of these isolates to grow in miniral salts medium (MSM) containing 1 % of hydrocarbon mixture as a sole source of carbon and energy were studied. The results showed that only eight isolates, were able to degrade hydrocarbons, all of them were gram negative bacteria.

These isolates were *Pseudomonas putida* , *Sphingomonas* , *spritivorum* , *Aeromonas media* , *Serratia liquifaciens* , *Acinetobacter baumanni* , *Klebseilla oxytoca* , *Flavobacterium breve* , *Alcaligenes eutrophus.*

The activity of these isolates in hydrocarbon degradation were assessed using different parameters which include the total viable count , pH , surface tension values , biosurfactant production , crease & oil content , dissolved oxygen uptake and hydrocarbon loss percentage . The results showed that *P.putida* was the most efficient bacteria which removed 52.2% of the initial hydrocarbons , while the less efficient one was *A.eutrophus* which removed 24.6% .

High performance liquid chromatography spectral data showed that there are significant differences ($p \leq 0.05$) in hydrocarbon composition after attacking by different bacterial isolates compared with the control sample .

C_6 and C_7 compounds were consumed completely by *P.putida*, *S.spritivorum*, *S.liquifaciens* and *A.media*, C8 was consumed by mainly *P.putida*, and C10 was consumed by *A.baumanni*.

K.oxytoca growth lead to an increasing in C8 compounds at 6.6% , while *A.media* and *A.eutrophus* increased C10 compounds at 12.3% and 30.7%, respectively .

The most notable finding was that all isolates were less favor the high molecular weight polyaromatic hydrocarbons (composed of four benzene rings or more) .The less efficient isolate was *K.oxytoca* which consumed 3.4 % of C22 compounds .

Moreover , the local consortium prepared exhibited a high efficiency to consume aromatic hydrocarbons C6 , C7 , C8 , C10 , at 100% and 92 % of C14 compounds , but it showed less efficiency to consume C18 , C20 and C22 at 57.8%, 55.8% , and 40.8% , respectively , compared with 80.2% , 80,8% and 75.1% , respectively by imported microbial consortium for the same compounds

The efficiency of the isolated consortium represented 73 % of the imported microbial consortium efficiency , since it consumed 61.1% of the hydrocarbon substrate , compared with 83.3% of the imported one .

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List of Abbreviations

LPS	Lipopolysaccharide
HCs	Hydrocarbon
O.D	Optical density
D.O	Dissolved oxygen
CFU	Colony forming unit
hr	Hour
min	Minute
nm	Nanometer
ml	Millimeter
rpm	Round per minute
MSM	Mineral salts medium
Vol	Volume
PAH	Poly aromatic hydrocarbon
HMW PAH	High molecular weight polyaromatic hydrocarbon
TVC	Total viable count
MR-VP	Methyl red – Vogas proskour
D.W	Distilled water
Conc.	Concentration
R.T	Retention time
P.A	Peak area .

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بسم الله الرحمن الرحيم

إنا فتحنا لك فتحا مبينا ۝ ليغفر لك الله ما تقدم
من ذنبك وما تأخر ويتم نعمته عليك ويهديك صراطا
مستقيما ۝ وينصرك الله نصرا عزيزا ۝

صدق الله العظيم

سورة الفتح الاية (1-3)

Acknowledgments

I would like to express my gratitude to my supervisors Dr.Wessam A. Z. H. Al-Taher and Dr.Hassan F.Naji for help , support and guidness through this work .

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Detections

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List of Abbreviations

Api	A standard Profile Identification
CFU	Colony forming unit
Conc.	Concentration
D.O	Dissolved oxygen
D.W	Distilled water
HCs	Hydrocarbons
HMW PAH	High Molecular Weight Polyaromatic hydrocarbon
HPLC	High performance liquid chromatography
hr.	Hour
LPS	Lipopolysaccharide
min	Minute
ml	Millimeter
MR-VP	Methyl red – Vogas proskour
MSM	Mineral salts medium
nm	Nanometer
O.D	Optical density
P.A	Peak area.
PAH	Poly aromatic hydrocarbon
R.T	Retention time
rpm	Round per minute
TVC	Total viable count
Vol	Volume

1. Introduction

Petroleum is a mixture of many thousands of hydrocorbonic compounds including large and diverse groups of organic compounds. By definition, HCs comprise solely those compounds containing carbon and hydrogen. However the term HCs has expanded to include related heteroatom organic compounds derived from petroleum and it's products (Ojumu *et al*., 2005).

Petroleum was first obtained in prechristion times by the Chinese. The modern petroleum industry has its beginning in Romania and Pennsylvania in 1859. The early principal use of the petroleum industry was for lighting. But today it is utilize as fuel and its dominance in the world market as a source of chemicals has diversified tremendously (Alloway and Ayres, 1993),

The high demand for petroleum and its derivitives during the last ten decades has made petroleum spills inevitable consequences of oil production and refining despite fluctuations in its prices. Oil will remain the major source of energy in the next several decades because a reliable alternative has not yet been found. The problem of pollution during production and transportation of oil would remain the major issue, and recognized as the most significant contamination problem in the world (Kasai *et al*., 2001).

Microbial degradation appears to be the most environmentally safe methods of removal of oil pollution since other methods such as surfactant washing and incineration lead to introduction of more toxic compounds to the environment. The potential of various organisms to catabolize and metabolize organic compounds has been recognized as potentially important and effective means of disposing of hazardous wastes such as hydrocarbons . HC-degrading microorganisms are widely distributed in marin, freshwater and ecosystems (Challiant *et al.*, 2004).

On structural basis, the hydrocarbons in crude oil are classified as alkanes (normal or iso), cyclohexanes, and aromatics, while alkanes are rare in crude oil but occur in many refined petroleum products as a consequence of the cracking process. The smalle amounts of oxygen- (phenols, naphthenic acids), nitrogen- (pyridine, pyrrole, indole), and sulfur – (alkylthiol, thiophens) containing compounds, collectively designated as a "resins" and partially oxygenated highly condensed asphaltic fraction occur also in crude but not in refined petroleum (Atlas & Bartha, 1973).

Crude oil could be classified according to its respective distillation residues as paraffin, naphthenes, or aromatics and based on the relative proportions of the heavy molecular weight constituents as light, medium or heavy. Also, the composition of crudes may vary with the location and age of an oil field, and may even be depth dependent within an individual well. About 85% of the components of all types of crude oil can be classified as either asphalt base, paraffin base, or mixed base; asphalt base contains little paraffin wax and asphaltic residues. The sulfur and nitrogen contents are often relatively higher in asphaltic base in comparison with paraffin base crudes which contain little or no asphaltic material. Mixed crude oil contains considerable amount of nitrogen oxides and asphalt (Atlas, 1981).

In general the petroleum hydrocarbons have been known to belong to a family of carcinogenic and neurotoxic organopollutants, so it must be treated before reaching the environment. There are three kinds of treatment lead to the eventual removal of hydrocarbon from refinery waste water which are: physical, chemical , and biological treatments, and the biodegradation of oil pollutant has acquired a significance as an increasingly effective and potentially inexpensive clean-up technology (Spence *et al.,* 2005).

The ability to isolate high numbers of certain oil-degrading microorganisms from an environment is commonly taken as an evidence that these organisms are the active degraders of the environment. Most of HC-degrading microorganisms are bacteria which have evolved regulatory systems that enable them to synthesis the required enzymes at the initial attack on these compound (Ojo, 2006).

In general, the alkanes fraction is the most biodegradable fraction, while the polar fraction (i.e. resins and asphaltenes) is the more resistant. The aromatic compounds especially polycyclic aromatic HC (PAHs) are of intermediate biodegradability, and this is of most concern owing of their toxicity and tendency to bioaccumulate (Pheleps and Stewart, 1974).

The PAHs constitute a large and diverse class of organic compounds and are generally described as molecules which consist of three or more fused aromatic rings. In various structural configuration, PAHs concentration in the environment vary widely depending on many factors such as proximity of the contaminated site to the production source and the level of industrial development (Cerniglia *et al*., 1990).

It has increasingly evident that a wide range of microbial species (bacteria, fungi, algae, and cyanobacteria) have used both low (three rings or fewer) and high-molecular weight (four rings or more) PAHs such as naphthalene and anthracene and flouranthene as a sole carbon source, while few species found to utilize PAHs with more than four rings such as pyrene (Juhasz and Naidu, 2000).

The low water solubility and high sorption capacity of PAHs are often found to greatly influence biodegradation, but other factors, such as production of toxic or dead-end metabolites, metabolite repression , presence of preffered substrates, and the lack of metabolic or inducer substrate must be considered when PAHs persistence is evident (Junson *et al.,* 2002).

Petroleum refining and transport activity are major contributors to localized loading of PAHs into the environment. Such loading may occur through discharge of industrial effluents and through accidental release of raw and refined products. However HC released into the environment may originate from sources including gasoline and diesel fuel combustion (Lim *et al.,* 1999).

Environmental impacts from the petroleum industry derive from recovery, transport, refining, various operations of productions, processing leads to large volumes of waste which is generated as oily sludges. HC bind strongly to solid surface, including soils, and remediation of these materials represents significant challenge. The lighter and often toxic HC components tends to volatilization into the atmosphere, reducing air quality and threatining human and animal health. High level of sulfur compounds are also emitted in petrochemical waste stream, which require treatment. Howeverabiotic losses due to evaporation, dispersion , and photooxidation also play a major role in decontamination of oil spill (Garreat *et al.*, 1998).

There are many hydrocarbonic compounds still as a chemical residuals during crude oil refining processes. These residuals undergo different steps of physical , chemical and biological treatments in order to reduce their toxicity before throwning them to the environment (water and soil). The types and concentration of degrading contaminants and the efficiency of using treatment methods determine the degree of ecological pollution resulting from reaching these contaminants to the water bodies and concomitant negative effects on living organisms (Jimenez *et al*., 1991).

The aim of the present study is to isolate local aerobic bacterial species that have ability to degrade hydrocarbon compounds in order to construct an experimental consortium to be use in biological treatment instead of the imported mixture which have expensive cost .

Considering the above facts , the objectives of the present study were as follow:-

1. To isolate aerobic bacteria from environmental site which are normally contaminated with petroleum wastes .
2. Study of the ability of these isolates to degrade the HCs compounds and select those which have a high rate of potency in biodegradation of HCs.
3. Study the changes in the composition of the HC waste before and after treatment with bacteria.
4. Making an experimental consortium of the isolates and compare it's biodegradation activity with the imported one.

2. Literatures Review

2.1. Hydrocarbon Biodegradation

Biodegradation means the breakdown of complex chemical through biological processes that can result into a minor loss of functional groups fragmentation number of constituents or complete breakdown to CO_2 and water. The imprical observation is no natural organic compound is totally resistant to biodegradation when conditions for that are favorable (Huesemann, 1995).

A wide range of studies have dealt with bio-remediation, biotrans formation and biodegradation of HCs, and exploiting petroleum – degrading organisms for environmental clean-up become central to petroleum microbiology (Margesin, 1998; Premuzic *et al.*, 1999).

Much of the experimental conclusions of petroleum microbiology are a result of the pioneering work of Claude ZoBell who began his researches in the 1930s and extended through the late of 1970s, and established that bacteria are important in a number of petroleum related processes, and defined the petroleum microbiology as the branch of microbiology that is concern with the activity of microorganisms in the formation, recovery and uses of petroleum (ZoBell, 1972).

Metabolic studies were implemented on the aerobic biodegradation of alkanes, cycloalkane , aromatic and (PAH) biodegradation. Recently the anaerobic microbial biodegration of HCs as well as the biochemical mechanisms involved in HC degradation and uptake have been conducted (Pheleps & Young, 2001).

There is a vast range of substrates and metabolites present in HC-impacted soils providing an environment for development of quite complex microbial community, culture-based methods being developed and implemented to improve the understanding of these microbial community (Magot *et al.,* 2000).

A refinery is a factory takes crude oil and turns it into petrol and hundreds of other useful products . It breaks crude oil down into its various components , which then are selectively reconFigure d in to three basic steps : separation (fractional distillation) , conversion (cracking and rearranging the molecules) , and treatment (finishing touches) include blending , purifying , fine – tuning and improving products to meet specific requirements . The technicians also add performance additives and dyes that distinguish the various grades of fuel to suit the weather . So in winter , they put in more volatile hydrocarbons (with short carbon chain) and in summer they add less volatile hydrocarbons to compensate for the higher temperatures (Nazirudin *et al.* , 1995) .

On the other hand , wastewater pass through three treatment steps : physical , chemical and biological treatments before disposal to the surface water bodies in order to minimize the hazardous effects of its pollutants (Grady and Lim,1996).

Heavy crude oil recovery , facilitated by microorganisms was suggested in the 1920s and received growing interest in the 1980s as microbial enhanced oil recovery. Bacteria with selected petroleum – metabolizing enzymes amenable to be linked to electronic interfaces are being engineered and developed as biosensors. These systems have been applied in monitoring environmental contaminant concentration and toxicities during implementation of remedial processes and also have potential application in control of environmental pollutions (Daunert *et al.,* 2000).

Davies (1967) and Atlas (1984) have discussed the oxidation of HC by bacteria. In addition to many varities of aerobic bacteria that oxidize HC, important anerobes also exist (e.g. sulfate-reducing bacteria and denitrifying bacteria). Also, certain fungi and actinomycetes readily oxidize HC in soil, although bacterial activity is most pronounced in surface soil, it can occur at all depths.

The chemical properties and hence the environmental fate of HC molecule are dependent in part upon molecular size (i.e, the number of aromatic rings). Molecule topology or the pattern of ring linkage which may occur such as that tertiary carbon atoms which are centers of two or three interlined ring. In general an increase in the size and angularity of PAH molecules results in a concomitant increase in hydrophobicity and electrochemical stability. PAH molecule stability and hydrophobicity are two primary factors which contribute to the persistence of high molecular weight poly aromatic hydrocarbons in the environment, due to their lipophilic nature PAHs have potential for biomagnification through trophic transfer (Twiss *et al*., 1999).

There are few number of bacterial species (such as *Mycobacterium spp.*) known to utilize four rings PAHs as a sole carbon source even in the absence of a cofactor or surfactants (Kampfer *et al.*,1993).

HC biodegradation capacity was observed in species of fungi such as *Ascomycetes*. These species attacked compounds within these aliphatic fraction of crude oil (n-12 to n-26). These results showed that the aliphatic compounds were being mineralized into simply transformed intermediate metabolites (April *et al.,* 2000).

2.2. Microorganisms Involved in Hydrocarbon Biodegradation

Hydrocarbons degrading bacteria and fungi are widely distributed in marine, freshwater , and soil habitates. Similarly, hydrocarbon degrading cyanobacteria have been reported. Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Pseudomonas ,Marinobacter, Alcanivorax, Sphingomonas, Micrococcus, Cellumonas*, and *Gordonia* groups (Brito *et al.*, 2006).

Molds belonging to the genera *Aspergillus, Penicilliums, Fusarium, Amorphoteca, Neosartorya, Paecilomyces Talaromyces, Graphium,* and the yeast *Candida yarrowia* and *Pichia* have been involved in hydrocarbon degradation too (Chaillan *et al.*, 2006).

Atlas (1981) recognized that many microorganisms have the ability to utilize hydrocarbon compounds as a sole carbon and energy source, and such microorganisms are widely distributed in nature. He further demonstrated that microbial utilization of hydrocarbons was highly dependent on the chemical nature of the components of the petroleum mixture, and the environmental determinants.

Kappeli *et al.*, (1991) reported that *P.aeruginosa* was able to grow on minimal media containing individual alkanes with chain lengths ranging from C_{12}-C_{19} as a sole carbon and energy source. The ability of *P.stutzeri, P.mullei* and *Alcaligenes sp.* to degrade naphthalene, Kerosene and diesel was studied by Oboh, *et al.*, (2006). They reported that these species are able to utilize hydrocarbon as a sole carbon and energy sources, Okoh (2006) demonstrated that the degradation rates of different strains of *P.aeruginosa* on crude oil were observed with evidence of significant reduction of major peaks components of the oil.

Bacterial species of *B.subtilis* and *Citrobacter* sp. were found to be able to degrade the hydrocarbons in the crude oil as the sole carbon and energy source (Ojo, 2006).

Dutta and Harayama, (2001) noted the degradation of the long side chains of n-alkyl benzene and n-alkylcyclo hexane by *Alcanivorax* sp.

Different bacterial genera such as *Rhodococcus, Mycobacterium and Nocardia* are capable of using high molecular weight PAHs like pyrene , flourene as a sole carbon source (Menni *et al.*, 1993).

Schippers *et al.,* (2005) isolated the species *Mycobacterium oleivorans* and *M.hydrocarbonoxydans* which are involved in crude hydrocarbon degradation, another bacterial species *Gordonia polyisoprenivorans* has been shown to enhance mineralization of aliphatic hydrocarbon and recently PAHs (Mutnuri *et al.*, 2005).

The consortium composed of *Desulfosporsinus* sp. and *Desulfobacterium aniline* are capable of benzene degradation, and the latter species initiated benzene degradation in this consortium, another consortium composed of *Methanosaeta* sp., *Methanospirillum* sp.and *Desulfotomaculum* sp. are capable of anerobic toluene degradation (Beller *et al.*, 2000).

Romine *et al.*, (1999) showed that the species *Sphingomonas yanoikuyae* is capable of degrading aromatic compounds like toluene, xylenv, biphyl, dibenzothiophene, flourene and benzoate.

Grund *et al.*, (1992) noted that *Rhodococcus* sp. was naphthalene degraders, while *Sphingomonas aromaticivorans* and *P.saccharophila* are phenanthrene and Flourene degrading microorganisms.

The two denitrifying bacterial species *Azoarcus* sp. and *Thauera aromatico* have benzylsuccinate synthase which convert toluene to benzyl succinate, and this reaction is followed by a series of modified B-oxidation reaction that convert benzylsuccinate to benzyl C*o*A which considered the central intermediate in the anaerobic degradation of aromatic compounds (Harwood and Gibson, 1997).

There are several bacterial genera which are able to utilize aromatic hydrocarbons including *Beijerinckia*, *Burkholderia*, *Serratia*, *Xanthomonas*, *Comamonas* by using oxygenase enzymatic systems (Aslabie *et al.,* 1998).

The phototrophic bacterial species of *Chromatium* and *Amoebacter* have been shown their ability to accumulate polyaromatic hydrocarbons in natural conitions (Guerrero *et al.,* 1985).

Fungi generally could play an important role in the degradation of petroleum hydrocarbons in the soil, so the white rot fungus *Cladophialophora* sp. exhibited an ability to degrade the aromatic hydrocarbons, benzene , ethylbenzene, toluene, and Xylenes, and can mineralized these compounds (Prenafeta *et al.,* 2002).

Alcalde *et al.,* 2002 showed that the fungi *Trametes vesicolor* and *Myceliophthora thermophia* are able to grow on media containing (12) types of PAHs as a sole carbon and energy source.

2.3. Hydro carbon Uptake by Microoganisms

Microbial HC oxidation occurrsd via one of two main metabolic pathways. First , aerobic bacteria oxidize HC to form carbon dioxide or bicarbonate that eventually precipitates as carbonat. Second, once oxygen is deplated within the HC compound, other bacteria reduce sulfate to produce hydrogen sulfide, these changes can significantly alter the oxidation reduction potential (Eh) of the environment and can affect the pH of the system , such pH/ Eh changes can result in new mineral stability fields in which some minerals become unstable and are dissolved and mobilized while others are precipitated from solution (Al-Shaieb *et al.,* 1984).

The aerobic fate of HC compounds is to activate the molecule by oxygenases enzymes (Mono-and dioxygenases) and molecular oxygen has to participate in these reactions. Initial transformation leads to catechol or protochtechuate , or degrades the compounds that can enter central metabolic cycles (Harrison *et al.,* 2000).

The biodegradation aspect of petroleum microbiology has grown on importance in the latter decades of the twentieth century. In the 1980s, the massive spill of unprocessed (crude) oil of coast of Alaska demonstrated the usefulness of bacteria in the degradation of oil that contaminating both water and soil. Since then, researchers have identified many species of bacteria and fungi that are capable of utilizing the HCs compounds that comprise oil. The hydrocarbons can be brokendown by bacteria to yeiled carbon dioxide and water. Furthermore, the bacteria often acts as a consortium with the degradation waste products generated by one microorganism being used as a substrate by another bacteria, and so on (Ojumu, 2005).

The inherent biodegradability of hydrocarbons components is a reflection of their chemical structure and influenced strongly by the physical state and toxicity of the compounds. So n-alkanes (the C_5-C_{10} homologous) have been shown to be inhibitory to the majority of hydrocarbon degraders, because these compounds tend to distrupt lipid membrane structures of microorganisms. Similarly, alkanes in the C_{20} – C_{40} range, often referred to as "waxes" are hydrophobic solids at physiological temperature and this physical state that strongly influence their biodegradability (Atlas and Bratha, 1973).

Although the actual uptake of alkane by bacteria is thought to be by passive transport, microorganisms possess a number of adaptive mechanisms for accumulating and transporting HC molecule into the cell for initial enzymatic catabolism, bacteria transport and assimilates soluble alkanes that are dissolved in the aqueous phase. Indeed, it was initially though that bacteria could utilize only solubilized HC, however alkanes are degraded at rates which exceed the rates of dissolution of HC in the aqueous phase, indicating that other uptake mechanisms are also utilized by HC-degrading microorganism (Lonon *et al*., 1999).

Many bacteria are capable of producing emulsifing agents known as biosurfactants. These amphipathic compounds reduce surface tension by accumulating at the interface of immicible fluids or of a fluid and solid and increasing the surface areas of insoluble compounds which lead to increased bioavailablity and subsequent biodegradion of the HC (Hommel, 1990). One of the significant problem in biodegradation of HC contaminants is the very low solubility of the compounds in the aqueous phase. This is especially true at lower temperature, at which longer chain alkanes (> 10) are generally more insoluble or exist as a solids. This hinder the biodegradative activity of HC-biodegrading bacteria (Whyte *et al.,* 1998).

Microorganisms may also utilize insoluble HC by adhering hydrocarbon molecule at the water- HC liquid or solid interface, to facilitate adhesion of hydrophobic substrate hydrocarbon – degrading bacteria may increase cell surface hydrophobicity by modifying components of cell surface, in addition, microbial cell may produce extracellular polymeric substances in the form of capsules or mucoid secretions that may interact with hydrophobic substrates such as hydrocarbon compound (Wolfardt *et al.,* 1998).

Bacteria are also known to adapt to changes in environmental conditions, such as temperature or growth in the presence of HC substrate by altering the lipid composition of the cytoplasmic membrane in order to maintain or adjust membrane bilayer fluidity and this alterations to the fatty acids moieties of membrane lipids are thought to be the most effective means of maintaining the liquid crystalline state in membrane function (Herbert, 1986).

Primary attack on intact hydrocarbon always requires the action of oxygenases, and therefore requires the presence of oxygen. In the case of alkanes, monooxygenases attack results in the production of alchol. Most microorganisms attack alkanes terminally, whereas some of them perform sub-terminal oxidation. The alchol produced is oxidized finally to the fatty acid which is further degraded by B-oxidation (Okoh, 2006). The extensive methyl branching interferes with the B-oxidation process and necessitate diterminal attack or other bypass mechanisms, therefore, n-alkanes are degraded more readily than iso-alkanes. Cyclohexanes are transformed to acorrosponding cyclic alchol, which is dehydrated to ketone, then monooxygenase system lactonize the ring, which is subsequently opend by a lactone hydrolase. These two oxygenase systems usually never occur in the same organism and hence, the frustrated attempts to isolate pure culture that grow on cycloalkanes. However, synergistic actions of microbial communities are capable of dealing with degradation of various cycloalkanes quite effectively. As in case of alkanes the monocyclic compounds, cyclohexane, and cycloheptane have strong solvent effect on lipid membranes, and are toxic to the majority of hydrocarbon degrading microorganism, highly condensed cycloalkane compounds resist biodegradation due to their structure and physical state (Bartha, 1986 a).

Prokaryotes convert aromatic hydrocarbon by an initial dioxygenase attack, to cis-dihydrodiols that are further oxidized to dihydroxy products , e.g. catechol. Eucaryotic microorganisms use monooxygenase, producing trans – dihydrodiols which oxidized in turn to catechol, a key intermediate in biodegration of aromatics, which is then opened by ortho-or meta-cleavage, yielding muconic acid or 2-hydroxymuconic semialdehyde, respectively, condensed polycyclic aromatics are degraded one ring at a time, by a similar mechanism, but biodegradability tend to decline with the increasing number of rings and degree of condensation (Asia, 2000). Aromatics with more than four condensed rings are generally not suitable as substrates for microbial growth, though they may undergo metabolic transformation. Biodegradation process also declines with the increasing number of alkyl substituents on the aromatic nucleuse. Asphaltics tend to increase during biodegradation in relative and sometimes, absolute amounts, this would suggest that they not only tend to resist biodegradation but may also be formed de novo by condensation reactions of biodegradation and photodegradation intermediates.

In crude petroleum as well as in refined product, petroleum hydrocarbons occur in complex mixtures and influence each others in biodegradation. The effects may go in negative as well as in positive directions. Some iso-alkanes are apparently spared as long as n-alkanes are available as substrates, while some condensed aromatics are metabolized only in the presence of more easily utilizable petroleum hydrocarbons a process referred to as co-metabolism (Wackett, 1996). Biodegradability is inherently influenced by the composition of oil pollutant, e.g. , kerosene which consist of almost of medium chain alkanes is totally biodegradable under suitable conditions. Similarily, crude oil is biodegradable quantitatively, but for heavy asphaltic-naphthenic crude oils, only about 11% may be biodegrade with a reasonable time period , even if the conditions are favorable (Bartha, 1986).

The problem of biodegradation has been reported to be mostly enhanced in presence of a consortium of bacterial species compared to monospecies activities (Ghazali, *et al.*, 2004).

The sorption of nonpolar contaminant such as hydrocarbons by microorganisms is often characterized by rapid step involving association with labile domains near the surface, followed by a slower step controlled by diffusion through intraparticle pores to hydrophobic region within the solid phase matrix. Many different HC-degrading isolates have cultured in media contain contaminants as a sole carbon source often in crystalline form at concentration greater than the aqueous solubility limit showing the importance of sorption in controlling HC bioavailability (Guerin & Boyd, 1997).

In addition to the intractions among chemical components of a mixture undergoing biodegradation, the intractions among microbial species in a mixed culture may be important too. Murkami & Alexender (1989) showed that interspecies intraction beyond pure and simple competition including some effects harmfull to one species while the other was unaffected occurring between membranes of a binary culture isolated from the same environment.

Jacques *et al.*, (2007) used a microbial consortium that produces HC emulsification and degraded a variety of hydrocarbon substrates indicating the possibility of using a consortium for bioremediation of sites contaminated with mixtures of aliphatic and polynuclear aromatic hydrocarbons.

2.4. Factors Affecting HC-Biodegradation

The efficiency of HC biodegradation process depends on the nature of HC- contaminated material and the environmental conditions (such as pH, temperature, and permeability of the soil rocks.) and the characteristics of microbial population that is present, nutrient availability, especially of nitrogen and phosphorous which appears to be the most common limiting factors . In simple bioremediation systems, which require little or no microbial expertise, process-limiting factors often related to nutrient especially (N, P) or oxygen availability or the lake of relatively homogenous condition throughout the contaminated medium . Microbial growth and degradation processes operating under such conditions are typically variable and suboptimal , leading at best to prolonged degradation (Mulder *et al.*, 1998).

When contaminants are degraded by cometabolism early eliminants of the co-substrate necessary for degradation of these contaminants can halt the degradation process, the non-homologous and unpredictable nature of these processes makes them intensive in terms of contaminant and analytical activities, as patterns of contaminant removal have to be monitored throughout three dimensional grids. Several laboratories and field investigations have indicated that the addition of commercial microbial cultures not significantly enhance rates of HC biodegradation over that achieved by nutrient enrichment of the natural microbial population (Fayad *et al.*, 1992).

The low water solubilities of the majority of petroleum HC compounds have the potential to limit the capacity of microbes, which generly exist in aqueous phase , to access and degrade these substrate. HC-degrading microbes produce avariety of biosurfactants as apart of their cell surface or as molecules released extracellularly (Makkar & Gamcotra, 2002).

The biosurfactants, and added chemical surfactants enhanced removal of potroteum HC from soil or solid surfaces, however both enhancement and inhibition of biodegradation of hydrocarbon have been observed (Jennings and Tanner , 2000).

2.5. Production of Biosurfactant by microorganisms

Bushnell and Hass (1941) were among the first that demonstrated bacterial production of biosurfactant by isolating *Corynebacterium simplex* and strains of *Pseudomonas* in a mineral media containing either kerosene, mineral oil, or paraffin.

Several species of microorganisms (bacteria, fungi, yeast) produced surface active agents when grown on HC substrate. Biosurfactants are polymers (partially or totally extracellular) with amphipathic structure that allow them to form micelles that accumulate at the interface between liquids of different polarities such as water and oil. This process is based upon the ability of biosurfactant to reduce surface tension, blocking the formation of hydrogen bridges and certain hydrophobic and hydrophilic interactions (Zajic, 1976).

The surface activity causes substantial lowring of the culture medium surface tension. There are several different types of biosurfactants have been isolated and characterized such as glycolipids, lipo polysaccharides, lipopeptides, phospholipids, and natural lipids (Makula et al. , 1975).

All complex lipids contain fatty acids and often these fatty acids have hydroxyl function on the Beta carbon atom to the carboxyl group or further along the chain (Itoth and Suzuki, 1972).

Biosurfactants are directly involved in the process of oil and oil-related products removel from the environment. So there are several apparent advantages to the use of these biomolecules rather than synthetic ones. They are biodegradable, and may be cost – effective and it may be possible to produce them in situ at contaminated sites (Raina, 1995).

At low concentration surfactants are present as individual molecules. However, as the concentration increased to reach the critical micelle concentration (CMC) where no further change in the interfacial properties takes place, the surfactant molecule, aggregate and form structures of bilayers vesicle, or micelles, the type and size of aggregate formed depends on the surfactant structure and the pH of the solution (Cantor and Schimmel , 1980).

Micelles are smallest basic structure formed, generally lesser than (5nm) in diameter. A micelle is composed of monolayer of surfactant molecules where the polar heads are oriented towards the surrouding aqueous solution and the non polar tails that are oriented towards the hydrophobic center of the micelle. Vesicle structures are next in size and range from (10 nm) to more than (50 nm) in diameter, vesicles are composed of surfactant bilayers, which are similar in structure to biological membranes. Jolivant *et al.,* (1990) showed that the ability of these aggregates to solubilize water varies widely with the molecular structure of the surfactant and the nature of the solvent.

The properties of the water present in the micellar core differ from those of the bulk waters in aqueous solution. The polar surfactant heads of the bilayer face the outside position while the non polar tails are sandwiched between the heads, thus the environment both inside and outside vesicle is hydrophilic (aqueous) while the environment within the bilayer composed of non polar surfactant tails , is hydrophobic (Battistel *et al.,* 1988).

Bilayer can also exist as flexible sheets or planner bilayer which are the largest of the basic surfactant structure, while the bilayer sheet is essentially unlimited in size. So if the solution on both sides of the bilayer is the same, the properties and behavior of the two bilayer surfaces will be identical (Lang and Wagner, 1987).

The yield and composition of biosurfactant are affected by growth conditions including carbon source, culture medium nutrients, temperature, pH and agitation (Hommel and Ratledge , 1993).

In addition , there are species level difference in the chemical structure of biosurfactants for instance the rhamnolipids produced by varios *Psendomonas spp*. differ both in the number of rhamnose molecule (1 to 2) and in the length of lipid moiety, and the range of biosurfactant molecular weight is from 500 to 1500 dalton.

Studies of organic contaminants have shown that both biological and synthetic surfactants can enhance either the chemical removel or the biodegradative removal of organic contaminants from soil. The goal of the use of surfactant is to increase the apparent water solubility of the contaminant of interest to facilitate removal by biodegradtion, since contaminant sorption depends on the chemical properties of both the soil and the contaminant. So the choice of surfactant used will be important (B anat *et al.,* 2000).

The low-molecular weight biosurfactants (glycolipids, lipopeptides) are more effective in lowring the interfacial and surface tension, wherease the high- molecular weight biosurfactants (amphipathic, polysaccharides, proteins, lipo polysaccharides, and lipoproteins) are effective stabilizers of oil-in-water emulsion (Mulligan *et al.,* 2001).

Microbial species including *Nocardia amarae, Corynebacterium petrophilum, Rhodococcus auraticus, Bacillus subtilis, Micrococcus spp.*, *Torulopsis bombicola*, *Pseudomonas* and *Acinetobacter* exhibited deemulsification capabilities. Microorganisms, generally exploit petroleum hydrocarbons that induced hydrophobic cell surface or hydrophobic/ hydrophilic properties of biosurfactant to displace or alter the emulsifiers that are present at the oil- water interface. Although some organisms grown on non- petroleum hydrocarbons substrate deemulsified petroleum hydrocarbons substrate, some biologically produced agents such as acetoin, polysaccharide, glycolipids, glycoprotein, phospholipids, and rhamnolipids destabilized petroleum emulsions (Janiyani *et al.,* 1994).

There are two mechanisms by which biosurfactant enhance the biodegradation of slightly soluble organic compounds. First, biosurfactant can solubilize hydrophobic compounds within micelles structures effectively increasing the apparent aqueous solubility of the organic compounds and it's avaiability for uptake by a cell. Second, biosurfactants can cause the cell to become more hydrophobic thereby increasing the direct physical contact between the cell and the slightly soluble substrate (Shreve *et al.,* 1995).

2.6. Methods for Determining Biodegrability

Biodegradation methodologies are designed to confirm, demonstrate and explore both the net chemical changes and the associated intracellular details pertinent to how microorganisms influence the fate of organic contaminants (Kleinsteub *et al.,* 2006). Biodegradation is routinely measured by applying chemical and physiological assays to laboratory incubated flasks containing pure cultures of microorganisms, mixed cultures, or environmental samples (e.g. soil, waters, sediment, or industrial sludges) then measuring the cell growth, substrate loss, consumption of final electron acceptors and production of both intermediary metabolites and final metabolic and products. All biodegradation equipments have the same general goal to contain both microorganisms and the test organic compounds in ways that allow physiological modification of the organic compounds to be measured and this may be gauged by indirect metabolic activity assay. Oxygen consumption, this and other indirect physiological biodegradation tests indicats chemical loss through a related microbiological parameter (cell growth, respiration and colorimetry) (Alexender, 1982).

In contrast, methods which focus directly on demonstrating diminished mass of contaminant and / or production of contaminant specific metabolites are considered more vigorous. These direct procedures utilize specific chemical purification and/ or detection instrument including gas chromatography, mass spectrometry, high-per formance liquid chromatography, spectrophotometry and radioisotopic tracers to monitor the abundance of test chemical in experimental vessels (Liang *et al.,* 2006). Furthermore, genetic methods include the use of engineered reporter microorganisms that serve as in situ biosensers for target contaminants. Gene probe and hybridization procedures that target specific gene sequences or enzymes in a microbial community in addition to DNA sequences of gene that code for metabolic pathways become increasingly available. Molecular procedures will continue to gain prominence in biodegradation protocols. But it is important to note that although the measurement of microbial activity and number affords only an indirect measure of overall degradation activity. However, due to their broad applicability, relative simplicity and low cost, these methods are often common measures for the assessment and prediction of biodegradation (Wang *et al.,* 1996).

3.Materials & Methods

3-1: Materials

3-1-1: Apparatus

The following apparatuses being used in this study are presented below.

Apparatus	Model & Company	Source
1. Shaker incubator	Memmert	Germany
2. Oven	Gallenkamp	England
3.Digital pH-meter	Beckman model 3500	England
4.Light microscope	Olympus	Japan
5.Centrifuge	Griffin	England
6.Autoclave	Memmert	Germany
7.Water bath	Memmert	Germany
8. Spectrophotometer	Spectronic-20 Bausch & Lomb Company	Germany
9.Sensitive balance	Sartorius	Germany
10.Incubator	Gallenkamp	England
11. High Performance Liquid Chromatography	KNAUER ASI- smartline with software Chrom Gate V.3.1.7	Germany

3-1-2: Media

3-1-2-1: Blood agar

This medium was prepared according to company manufacturing procedure (Difco).After autoclaving the sterile blood then added, as 5% of basal medium this medium was used in primary isolation of bacteria (Benson,1998).

3-1-2-2: Mineral salts medium

This medium was prepared as described by Bushnell & Hass (1941) as abase medium, , and used to determine the ability of bacterial isolates to consume the hydrocarbons as a sole source of carbon and energy. This medium composed of the following salts:

Mg SWO_4	0.2g
$CaCl_2$	0.02 g
K H_2 PO_4	1 g
$(NH_4)2SO_4$	1 g
$FeCl_3$	2 drops
D.W	1 L

The pH was adjusted to 7.2 and sterilized by autoclave at 121 C° for 15 min.

3-1-2-3: MacConkey's agar

This medium was prepared according to manufacturing procedure(Difco), and used to differentiate lactose fermenter bacteria (Benson,1998).

3-1-2-4: Sugars assimilation media

Ten gram of peptone, (1 g) of beef extract, (5 g) of NaCl, and (0.018 g) phenol red, were dissolved in one liter of D.W., sugars (glucose, sucrose, lactose, arabinose, xylose, raffinose, sorbitol, and mannitol) at a final concentration of 1% were added. The pH was adjusted to 7.2. The medium was distributed in test tubes (5 ml) for each, and sterilized by autoclave for 10 min. at 121 C° (Benson, 1998).This medium was used to determine the ability of bacteria to utilize acertain sugar.

3-1-2-5: Amino acids consuming media

Five grams of tryptone, (5 g) yeast extract, (0.25 g) glucose, (0.1 g) bromocresol purple were dissolved in 1 liter of D.W. One of the following amino acid (arginine and lysine) was added at a final concentration of 1%, the pH was adjusted to 7.2, and the medium was distributed in test tubes (5 ml), and sterilized by autoclave (121 ºC for 15 min.) (Benson, 1998).This medium was used to determine the ability of bacteria to utilize acertain amino acid.

3-1-2-6: Urea agar medium

Urea agar was prepared by adding 40% of urea which was sterilized by filtration through 0.2μ Millipore filter membrane (Sartorius membrane filter GmbH, W. Germany) to sterilize basic media after autoclaving and distributed in test tubes (5 ml) for each (Benson, 1998).This mediun was used to determine urease producer bacteria.

3-1-2-7: Nitrate utilization medium

This medium was prepared by dissolving 3 g of beef extract, 5 g peptone, and 1 g of KNO_3, in one liter of D.W. The pH was adjusted to 7.2, then distributed in test tubes and sterilized by autoclave (121 C° for 15 min.) (Benson, 1998).This medium was used to determine the ability of bacteria to reduce nitrate to nitrite.

3-1-2-8: Starch agar medium

It was prepared by adding 2 % of starch to nutrient agar and sterilized by autoclave (121 C° for 15 min.) (Benson, 1998).This medium was used to determine amylase producer bacteria.

3-1-2-9: Citrate consuming medium

Simmon citrate media was used to determine the citrate consuming bacteria as a sole carbon source. This medium was prepared according to the company manufacturing procedure (Oxoid), and sterilized by autoclave (121 C° for 15 min.).

3-1-2-10: Gelatin liquefaction medium

It was prepared by dissolving 3 g of beef extract, 5 g of peptone, 120 g of gelatin in 1 liter of D.W., then distributed in test tubes and sterilized by autoclave (121 C° for 15 min.) (Benson, 1998).This medium was used to determine ability of bacteria to liquefied gelation.

3-1-2-11: MR-VP media

It was prepared as described by the company (Oxoid) constructions and distributed in test tubes (5 ml) for each, then sterilized by autoclave (121 C° for 15 min.) .

3-1-2-12: Kligler iron agar

It was prepared by dissolving the following chemicals:-

Yeast extract	1 g
$(NH_4)_2\ SO_4$	2 g
K_2HPO_4	0.6 g
KH_2PO_4	0.4 g
NaCl	2 g
glucose	0.25 g
Lactose	0.25 g
Na-mabanate	3 g
Bromothymol blue	0.25 g
D.W	1L

The pH was adjusted to 6.7 and distributed in test tubes (5ml for each), then sterilized by autoclave (121 C° for 15 min.) (Benson, 1998).This medium used to determine the hydrogen sulfide producer bacteria .

3-1-3: Reagents

The following reagents were used in the present study:-

3-1-3-1: Oxidase reagent

It was prepared by dissolving 1g of N,N,N,N-tetramethyl-*P*-Phenylene diamine dihydrochloride (BDH) in 100 ml of D.W. (Benson, 1998).This reagent was used to determine.

3-1-3-2: Catalase reagent

This reagent was prepared by adding 3ml of hydrogen peroxide (H_2O_2) to 97 ml of D.W. to reach the final concentration 3% (Benson, 1998).This reagent was used to determine catalase producer bacteria.

3-1-3-3: Barritt's reagent

This reagent was composed of two solutions:- (Benson , 1998).

Solution A:- α-naphthol 5% in absolute ethanol.

Solution B:- 40% KOH in D.W.This reagent was used in MR-vp test.

3-1-3-4: Starch hydrolysis reagent

Ten grams of KI was dissolved in 25 ml of D.W. then 5g of Iodine was slowly added with continuous stirring until complete dissolving then the volume was completed to 100 ml with D.W. (Benson, 1998).This reagent was used to determine the amylase producer bacterta.

3-1-3-5: Kovac's reagent

Ten grams of P-dimethyl amino benzaldehyde in 150 ml of ethyl alcohol, then 50 ml of absolute hydrochloric acid (HCl) was added (Benson, 1998).This reagent was used to setermine indol producer bacteria.

3-1-3-6:Methyl red reagent

It was prepared by dissolving 0.1g of methyl red stain in 300 ml of ethyl alcohol 95% then the volume was completed to 500 ml of D.W. (Benson, 1998).This reagent was used in MR-vp test.

3-1-3-7: Nitrate reduction reagent

This reagent was composed of the following solutions according to Benson (1998):- This reagent was used in nitrate reduction test.

a-Solution A:- 0.6 g of N.N-dimethyl-α-naphthyl amine dissolved in 100 ml of glacial acetic acid (5 N).

b- Solution B:- 0.8g of sulfuric acid dissolved in 100 ml of 3% glacial acetic acid.

3-1-3-8: Alkali-iodide azide reagent

It was prepared by dissolving 700 g of KOH and 150 g of KI in D.W. and diluted to 1 liter, then 10 g of sodium azide (NaN_3) was dissolved in 40ml of D.W. This reagent should not give a color with starch solution when diluted and acidified (APHA, 1998).This reagent was used to determine the level of dissolued oxygen.

3-1-3-9: Stock solution of sodium thiosulfate

Tow hundred forty eight gram of $Na_2S_2O_3.5H_2O$ were dissolved then in a boiled cooled distilled water and diluted to 1 liter. It was preserved by adding 1g of NaOH per liter. 125 ml of stock solution was diluted in 1 liter of D.W. to be used in dissolved oxygen measured experiment (APHA, 1998).

3-2: Methods

3-2-1: Collection of samples

Two hundred liters of industrial waste water were brought from the effluent tank of Al-Dora Oil Baghdad Refinery before undergoing any treatments as shown below. The samples were brought in a clean dry plastic containers to be used in the experiments.

3-2-2: Separation of hydrocarbons from the waste water:-

Crude hydrocarbon mixture from industrial waste water was separated by (1L) separatory funnel by mixing one volume of it with three volumes of absolute diethyl ether (EMscience, > 99. 8%) with shaking for several times, and left it for 10 min. at room temperature to settle down. The organic layer above the water layer which was withdrawn from the down screw . The organic layer was washed again by another 50 ml of diethyl ether, and the filterate was collected in a clean dry conical flask, and let it to dry at room temperature (Nadalig *et al*.,2000).

3-2-3: Isolation of bacteria from the waste water

Pour plate technique was used 0.1 ml of waste water was transferred to an empty sterile plate, then 20ml of blood agar at 45C° was poured
on it. The contents were mixed slightly. The plates were incubated at 35 C° for 24-48 hr (Kiyohara *et al*., 1982).

3-2-4: Purification of bacterial isolates

A small part of each colony on blood agar plates was transferred to another sterile blood agar plate by streaking technique. The plates were incubated at 35C° for 24-48 hr, this purification step was repeated 3 times to obtain a pure isolated colonies (Benson , 1998).

3-2-5: Hydrocarbon biodegradation of bacterial isolates

Each pure colony that was obtained from previous step (3-2-4) were transferred to 100 ml of sterile mineral salts medium containing 1% of crude hydrocarbon mixture to determine the ability of these isolates to utilize the hydrocarbon compounds as a sole carbon and energy sources and incubated at 37 C° for 7 days (Randall & Hemmingsin, 1994).

3-2-6: Standard curve for determination of bacterial density of inoculum

The relationship between the dilution factors against their optical density (O.D.) at 600 nm measured by spectrophotometer (Fig.1) was drawn in order to unify the bacterial inocula densities of the bacterial isolates that be used in this study Macfaddm cwrre.

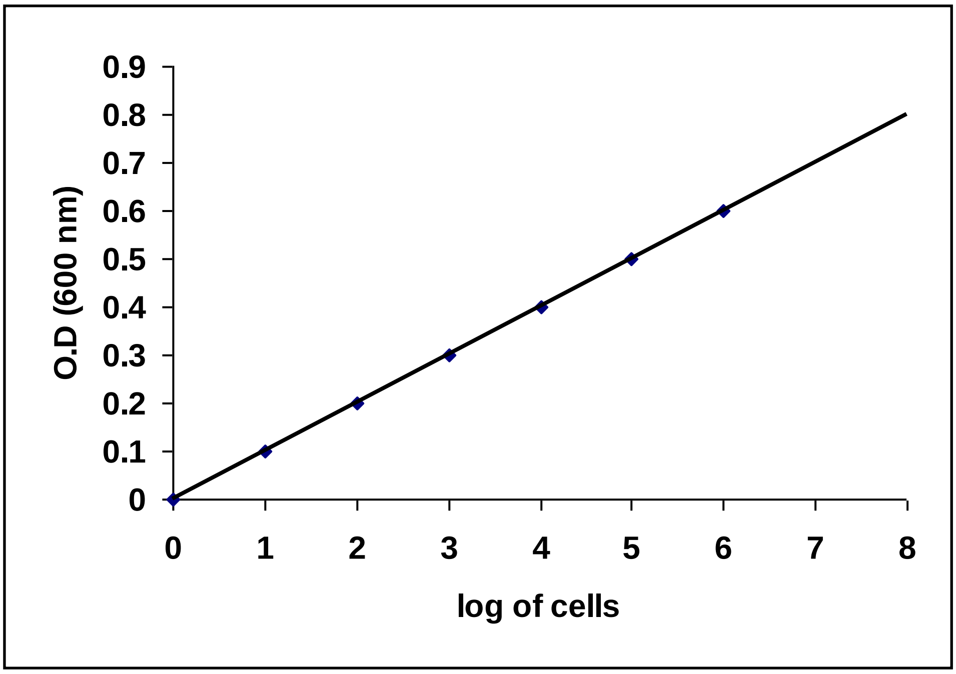


Fig.1.Standard curve of cells number

3-2-7: Inoculum preparation

The different bacterial isolates that isolated and purified from waste water samples were inoculated in 250 ml of mineral salts medium. The Budziniski *et al.*, (2000) method was used for this purpose. This method was summarized as follows: neutrient broth medium canbe inoculated with 0.2 ml of each one of the eight bacterial isolates and incubated at 35 Cº for 14-18 hr The culture medium was centrifuged at 6000 rpm for 10 min., the precipitate was resuspended in mineral salts medium, 0.2 ml that gave 0.6 at 600nm was used to inoculate 250 ml of mineral salts medium containing 5 g of dried hydrocarbon mixture as a sole source of corbon and energy .

3-2-8: Determination of biodegradation efficiency of the isolates

The experiment was designed to evaluate the hydrocarbon biodegradability efficiency of bacterial isolates as follows:

13.500 liters of mineral salts medium were prepared and sterilized by autoclaving (121 C° for 15 min.), then distributed into 54 sterile Erlenmeyer flasks (volume 500ml). Each flask was contained 250ml of mineral salts medium and 5g of which prepared in (3-2-2). These flasks were incubated at 37 C° for six weeks in orbital shaker (200 rpm).

The cultivation batch was withdrawn weekly to carry out the following parameters:-

3-2-8-1-A:Measurement of residual hydrocarbons in the culture medium

The culture medium was filtered by a previously weighed Wattman filter paper, then dried at room temperature. The difference between the two weights was represented the residual hydrocarbon weight in the culture medium compared to control sample which was free from bacterial culture (Dibble & Bartha, 1979).

3-2-8-1-B: Measurement of HC within the bacterial cells

Bacterial culture was filtred through Wattman filter paper as mentioned in the previous step (3-2-8-1-A), The filterate was used to measure the HC loss which was still inside bacterial cells by adding acetone: hexane (3:1 V/V) to release the adhering HCs, then the pH was adjusted to 2 by adding drops of concentrated HCl. Dichloromethane was added (1/2 volume:1volume of suspension) and shaked well, then separated by separatory funnel, the solvent layer was collected in previously weighted flask and evaporated at 45 C°. The difference in weights represented the dissolve HC in bacterial cells (Copper & Goldenberg, 1987).

3-2-8-2: Total viable count

Total bacterial count was performed on nutrient agar using spread plate technique. Total viable counts of culturable aerobic bacteria were obtained by preparing serial dilution of bacteria and surface plating on nutrient agar in triplicate plates, and incubated at 35 C° for 48 hr. colonies numbers were assessed (Benson, 1998).

3-2-8-3: pH measurement

The digital pH-meter Bechman model-3500/England was used for measurement of pH of culture filtrates weekly.

3-2-8-4: Crease and oil measurement

Weight method was used to calculate the crease and oil concentration, 10 ml chloroform was used as a solvent. 100 ml of culture medium after filtration was transferred to separatory funnel (1L). 10 ml of chloroform was added and 0.5 ml of HCl (1/1) with shaking well for two minutes, and let it to settle at room temperature for 10 min. The water layer was eluted from the down screw while the organic layer was filtrated on filter paper contains unhydrous sodium sulfate , the water layer rewashed by 10-20 ml of solvent and added to organic layer , then collected in a previously weighted Erlenmyer flask. The filtrate was heated in water path at 60-65 C° until drying, then reweighted the flask after cooling (APHA, 1992).

Calculations:-

$$\text{Crease and oil (mg/L)} = \frac{\text{A - B} \times 100}{\text{Sample volume (ml)}}$$

A= The weight of flask after drying.
B= The weight of empty flask.

3-2-8-5: Surface tension measurement

Capillary method was used for evaluating the changes in surface tension (dyns/cm units) of culture medium weekly according to Cooper *et al.*, (1981). After filtration of culture medium, the filterate was used for measuring its density which mean (W/V) in units of dyns/cm^3. Then the surface tension (γ) was calculated according to the following equation:

$$\gamma = \frac{1}{2}\text{rh}\rho\text{g}$$

γ = surface tension
r = capillary radius
h = height of rise
ρ = density of liquid
g = accelerartion due to gravity

3-2-8-6: Measurement of biosurfactant amount

One volume of bacterial culture filtrate was treated with 3 volumes of acetone in previously weighted container for 10 hours at 4 C°. The precipitate will be form and then collected by centrifugation (if it a lot) or by evaporation of acetone (if it a few), and reweighed the container. The differences between the two weights represented the biosurfactant amount in units of mg/L (Cooper & Paddock, 1983).

3-2-8-7: Dissolved Oxygen levels

Two ml of $MnCl_2$ was added to 200 ml of sample with continuous shaking then 2 ml of alkali-iodide azide was added. After that 2 ml of concentrated H_2SO_4 was added which change the colour to yellow. 2 ml of starch solution was added. This mixture was titrated against sodium thiosulfate ($Na_2S_2O_3.5H_2O$) which was mensioned in (3-1-3-9) until the blue colour was disappeared. The volume of sodium thiosulfate represents the dissolved oxygen level (APHA, 1992).

3-2-9 Prepartion of isolated bacterial consortium :-

0.2 ml of each HC- degrading bacterial isolates were grown together in 5g ml of previousely sterilized mineral salts medium as aconfrol sample , 0.5 of hydrocarbon mixture was added and the pH was adjusted at 7.2 (Cerniglig , 1992).

At the same time , suspension of imported consortium was inoculated in the same medium . Eighteen flask of each consortium were incubated in orbital shaker (200 rpm) at 37C° . Three flasks of each consortium were withdrawn weekly to study the following parameters :-

1. pH value
2. Optical density (O.D 600)
3. Surface tension (dyns/cm)
4. Biosurfactants production (mg/L)
5. Crease and oil concentration (mg/L)
6. Dissolved oxygen concentration (mg/L)
7. Hydrocarbon loss percentage (%)

3-2-10: Diagnosis of bacterial isolates

A. Gram's stain

Gram's stain of bacterial isolates were performed to show the reaction of isolated cells with Gram's stain, and their morphological properties which include shape, size etc. (Benson, 1998).

B. Morphological properties

Morphological properties of bacterial isolates (cells and colonies) were studied according to Holt *et al*., (1994).

C. Biochemical tests

Several biochemical tests were carried out for identification of bacterial isolates as follows:-

3-2-10-1: Sugar assimilation

Culture media tubes that mentioned previously in (3-1-2-4) were inoculated with each bacterial isolate and incubated at 37 C° for 24-48 hr.

The changing of colour from red to yellow is the positive result (Benson, 1998).

3-2-10-2: Amino acids consumption

The tubes of culture media mentioned in (3-1-2-5) were inoculated with bacterial isolate, and incubated at 37 C° for 24-48 hr. The positive result is the changing of colour from yellow to purple. The formation of orange precipitation (when adding three drops of Nessler's reagent) confirmed the positive result (Benson, 1998).

3-2-10-3: Urease enzyme production

The slants of urea agar tubes were inoculated with each bacterial isolate and incubated at 37 C° for 24-48 hr. The appearance of pink colour represented the positive result (Benson, 1998).

3-2-10-4: Nitrate reduction test

The nitrate media tubes were inoculated with each bacterial isolate and incubated at 37 C° for 24-48 hr. The appearance of red colour after adding several drops of α-naphthol and several drops of sulfanilic acid represented the positive result (Benson, 1998).

3-2-10-5: Starch hydrdysis hydrolysis

The starch agar plates were inoculated with bacterial isolates and then inocubated at 37 C° for 48 hr. After incubation period a few drops of iodine solution were added. The appearance of clear zone around the colonies represented the positive result (Benson, 1998).

3-2-10-6: Citrate utilization

The slants of Simmon citrate agar were inoculated with each bacterial isolate and incubated at 37 C° for 24-48 hr. The change of colour from green to blue represented the positive result (Benson, 1998).

3-2-10-7: Gelatin liquefaction

The tubes of gelatin agar were stabbed with each bacterial isolate and incubated at 37 C° for 24-48 hr. Then the tubes were transferred to the refrigerator at 4 C° for 10 min. If the gelatin remains liquefied, the result is positive (Benson, 1998).

3-2-10-8: MR-VP test

The tubes of MR-VP medium were inoculated with bacterial isolates (2 tubes for each isolate). Then added to the first tube 5 drops of methyl red and several drops of Barritt's reagent to the second tube. The appearance of red colour in the first tube and pink colour in the second tube was represented the positive result, respectively (Benson, 1998).

3-2-10-9: H_2S production

Kligler iron agar tubes were stabbed with each bacterial isolate and incubated at 37 C° for 24-48 hr. Appearance of black precipitation in the bottom was represented the positive result (Benson, 1998).

3-2-10-10: Indole production

The tubes of peptone water were inoculated with each bacterial isolate and incubated at 37 C° for 24 hr. The appearance of red ring on the surface after adding of 0.5 ml of Kovac's reagent was represented the positive result (Benson, 1998).

3-2-10-11: Api 20 system

Api 20 E (BioMerienx /Germany) was used to accomplish the following tests :

1- β- galactosidase .
2- Inositol assimilation
3- Ramnose oxidation
4- Melibiose oxidation
5- Mannose oxidation
6- Galactose oxidation
7- Fructose oxidation
8- Ribose oxidation

3-2-11:Maintenance of bacterial cultures

All isolates were maintained in mineral salts medium (MSM) containing 1% glucose, 0.4% Na2HPO4, 0.15% KH2PO4, 0.1% NH4Cl, 0.02% MgSO4.7H2O, 0.0015% CaCl2. These stocks were preserved in glycerol at -20 C°. For each experiment, a fresh nutrient agar (Difco) plates was inoculated from a glycerol stock and incubated at 37 C° for 24 hr. Colonies from these plates were used to inoculate an experiment within 3 days (AL-Tahhan *et al.*, 2000).

2-3-12: HPLC analysis

The samples of hydrocarbon mixture before and after treatment with each bacterial isolates , in in addition to isolated and imported consortium were diluted with one volume of acetone (EM. Science > 99.8%) and analysed by HPLC (Knauer-ASI-Smartline/Germany) using C_{18} colon (2.1×150 mm) at room temperature. Acetone-water (50%) was used as the mobile phase at flow rate 0.2 ml/min. The determination was performed using UV-detector at 254 nm.

Aromatic hydrocarbons used as standards for HPLC analysis .

chemical	**No. of carbon atoms**	**Source**	**Purity**	**Structure**
Benzene	6	Sigma	99%	
Toluene	7	Sigma	99%	
Xylene	8	Sigma	99%	
Naphthalene	10	Sigma	99%	
Acenaphthene	12	Sigma	99%	
Phenanthrene	14	Sigma	99%	
Pyrene	16	Sigma	99%	
Benzo (a) anthracene	18	Sigma	99%	
Benzo (b) Fluoranthene	20	Sigma	99%	
Benzo (a,h) anthracene	22	Sigma	99%	

3-2-13: Statistical analysis

The statistical significance of the differences was calculated using student's test. Each test was repeated three times for each variable and differences was considered statically significant for $P \leq 0.05$ (Allan , 1998).

4.The Results

4.1. Isolation and identification of bacteria isolated from industrial wastewater

Twenty two different bacterial isolates were obtained from the industrial wastewater sampley.

The results revealed that the isolates were belonged to 16 different genera. 11 genera (68.75%) were Gram negative and 5 genera (31.25%) were Gram positive (Table 1). Table 2 and 3. represent the biochemical tests.

The distribution of the bacterial isolates , based on their morphology and Gram staining reaction , indicated that 36.4% of these isolates were gram positive, while 36.3% were Gram negative that have no ability to degrade hydrocarbons , and only 27.33% were gram ngative and have such ability (Fig.2).

Table 1. Distribution of the isolates according to Gram stain, genera, numbers, & Percentages

Genus	Number	Percentage (%)
1. Gram negative isolate	**11**	**68.75**
Pseudomonas	4	18.18
Escherichia	1	4.54
Klebseilla	1	4.54
Serratia	1	4.54
Flavobacteriun	1	4.54
Moraxella	1	4.54
Proteus	1	4.54
Acinetobacter	1	4.54
Aeromonas	1	4.54
Alcaligenes	1	4.54
Sphingomonas	1	4.54
Total Gram^{-ve} isolates	**14**	**63.63**
2. Gram positive genera	**5**	**31.25**
Staphyllococcus	3	13.64
Bacillus	2	9.09
Streptococcus	1	4.54
Corynebacterium	1	4.54
Micrococcus	1	4.54
Total Gram^{+ve} isolates	**8**	**36.36**
All isolates	**22**	**100.0**

Table 2. Biochemical tests of bacterial isolates that have the ability to grow in 1% of HC

Bacterial Isolate tests	*Alcaligenes eutrophus*	*Klebseilla oxytoca*	*Acinetobacter baumanni*	*Sphingomonas spiritivorum*	*Flavobacterim breve*	*Pseudomonas .putida*	*Aerononas ,media*	*Serratia liquifaciens*
B-galactosidase	-	-	-	-	-	-	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	-
Lysine decarboxylase	+	+	-	-	+	-	-	+
Ornithine decarboxylase	+	+	-	-	-	-	-	+
Citrate utilization	-	+	+	-	+	+	+	+
H_2S production	-	-	+	+	-	-	-	-
Urea hydrolysis	+	-	-	-	+	-	-	-
Indol production	-	+	-	+	-	-	-	+
MR	+	+	-	-	+	-	-	-
VP test	-	+	+	+	-	+	+	+
Gelatine liquification	+	-	-	-	+	-	+	+
Glucose oxidation	+	+	+	+	-	-	+	+
Mannitol oxidation	-	-	-	-	+	-	+	+
Inositol oxidation	-	+	+	+	+	+	-	-
Sorbitol oxidation	-	+	+	+	+	+	-	+
Rhamnose oxidation	-	+	-	+	-	-	+	+
Sucrose oxidation	-	+	-	+	-	-	+	-
Melibiose oxidation	+	+	+	+	+	+	-	+
Maltose oxidation	+	-	-	-	-	+	+	+
Arabinose oxidation	+	+	-	-	-	-	+	-
Mannose oxidation	-	-	+	-	+	-	+	-
Lactose oxidation	-	+	+	-	-	-	+	+
Raffinose oxidation	+	-	-	+	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	+	+
Oxidase test	+	-	-	+	+	+	+	-
Catalase test	+	+	+	-	+	+	+	+

Symbols : + : postive result , - : negative result

Table 3. Biochemical tests for bacterial isolates which failed to grow on (1%) hydrocarbon as a sole source of carbon and energy.

Bacterial Isolate tests	*Micrococcus luteus*	*Staphyllococcus aureus*	*Stroptococcus agalactiae*	*Bacillus subtilis*	*Corynebacterium amycolatum*	*Escherichia coli*	*Proteus vulgaris*	*Moraxella catarrhalis*
Gram stain	+	+	+	+	+	-	-	-
Oxidase test	+	-	-	-	+	-	-	-
Catalse test	+	+	-	+	+	-	+	+
Gelatin hydrolysis	-	+	+	-	+	-	+	+
Citrate hydrolysis	-	-	-	-	-	-	+	-
Nitrate reduction	-	+	-	-	-	-	+	+
Glucose oxidation	+	+	-	+	+	+	+	+
Mannose oxidation	+	+	-	+	-	+	-	+
Lactose oxidation	-	+	+	-	-	+	-	+
Xylose oxidation	+	-	-	-	-	-	+	-
Arabinose oxidation	+	-	-	-	-	+	-	+
Raffinose oxidation	-	-	+	-	+	-	-	-
Sucrose oxidation	-	+	-	+	+	+	-	-
Maltose oxidation	-	+	+	-	+	+	-	+
Mannitol oxidation	+	+	+	-	-	+	-	+
Galactose oxidation	-	+	+	-	-	+	-	+
Fructose oxidation	+	+	-	-	+	+	-	+
Ribose oxidation	+	+	+	+	+	-	+	-
Urea hydrolysis	-	+	-	-	-	-	+	-
Arginine dihydrolase	-	-	-	-	-	-	-	+
Lysine decarboxylase	-	-	-	+	+	-	+	-
Ornithine decarboxylase	-	+	-	+	-	-	+	-
Starch hydrolysis	-	-	-	+	+	-	-	-

Symbols : + : postive result , - : negative result

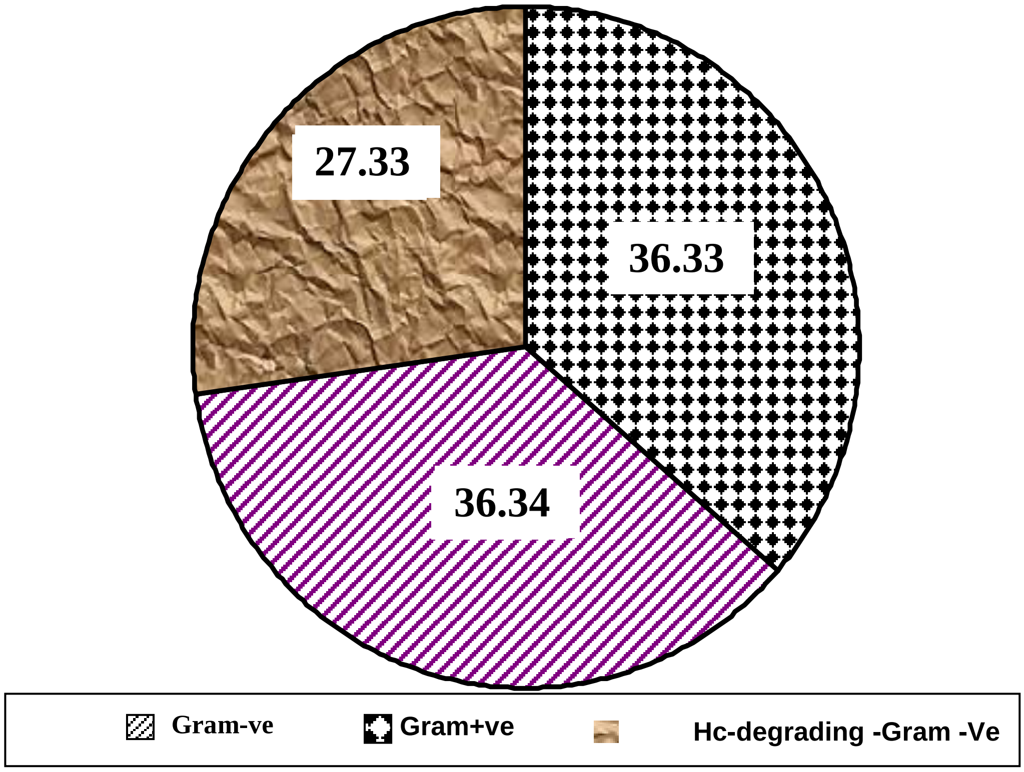


Fig.2: Distribution of bacterial isolates based on their Gram staining, and HC-degradation ability.

4.2. Ability of baeteria to degrade hydrocarbon substrate as a sole source of carbon and energy

The primary results showed that eight isolates of the total twenty two isolates were able to grow in mineral salts medium containing 1% of a mixture of crude hydrocarbon and used as a sole source of carbon and energy. All of these isolates were Gram negative, included the genera of *Pseudomonas, Klebseilla, Sphingomonas, Aeromonas, Acinetobacter, Serratia, Alcaligenes* and *Flavobacterium.* Eight genera were unable to grow in mineral salts medium contained 1% hydrocarbon substrate when incubated at 37°C for 7 days. These genera were *Proteus, Moraxella* and *Escherichia* which are Gram negative, in addition to the five Gram positive genera which were *Staphyllococcus, Streptococcus, Bacillus, Micrococcus* and *Corynebacterium.*

Figure (3) showed the growth of HC-degrading isolates after incubation for seven days. It demonstrates that some of the isolates exhibited lag phase during first and second day of growth which were *A.eutrophus, A.baumanni* and *K.oxytoca,* while the others began to increase in cell number since the first day of incubation.

The growth rate was continued in exponential phase to reach the maximum at the seventh day of incubation. 22.18 $\times 10^6$ CFU/ml for *P.putida* , 18.8$\times 10^6$ CFU/ml for *S.spiritivorum* 15.5$\times 10^6$ CFU/ml for *S.liquifaciens* , 12.6$\times 10^6$ CFU/ml for *A.media,* 13.8$\times 10^6$ CFU/ml for *F.breve* , 12.2$\times 10^6$ CFU/ml for each of *K.oxytoca and A.baumanni,* and finally *A.eutrophus* growth rate achieved 8.3$\times 10^6$ CFU/ml at the seventh day of incubation.

Figure (4) represents the growth rates of the isolates which were unable to use the hydrocarbons mixture as a sole source of carbon and energy. The first two declined isolates were *Staphyllococcus* and *Escherichai,* which died at the second day of incubation, followed by *Morxella , Proteus, Corynebacterium* and *Bacillus,* which disappeared from the culture medium at the third day of incubation, while *Streptococccus* and *Micrococcus* disappeared at the fifth day of incubation.

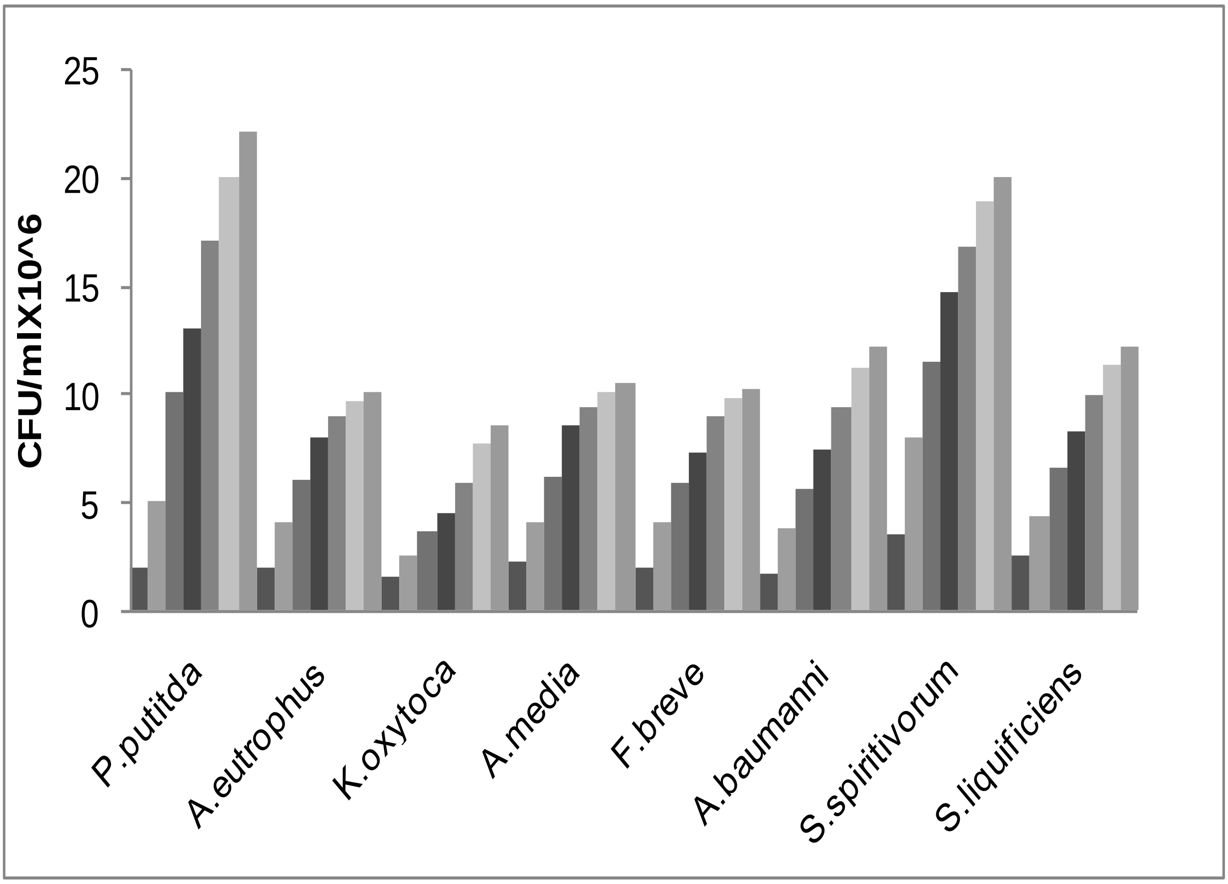


Fig. 3. Growth of HC-degrading isolates in mineral salts medium containing 1% of hydrocarbon mixture as a sole source of carbon and energy.

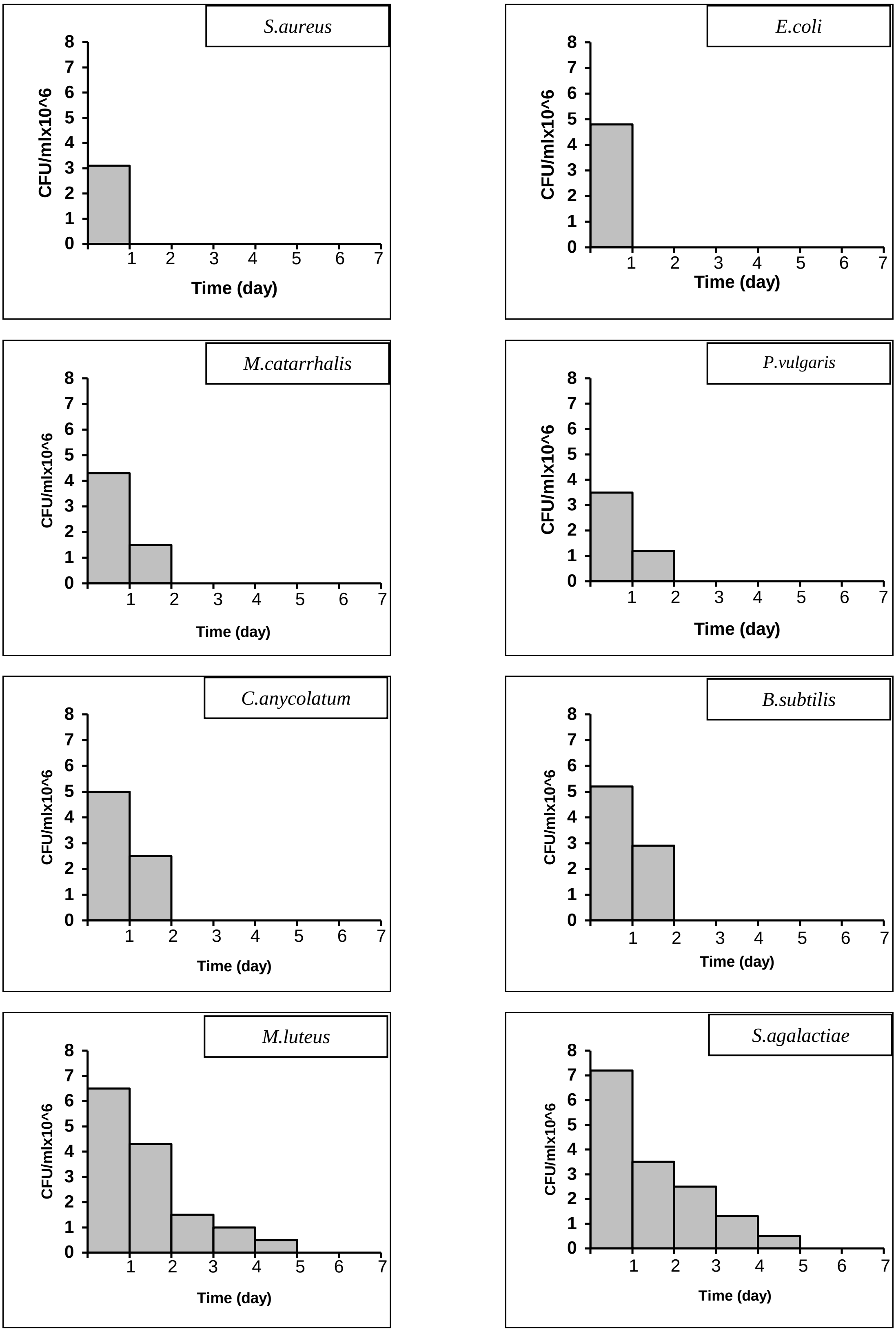


Fig. 4. Growth of bacterial isolates which are unable to utilize hydrocarbon mixture as a sole source of carbon and energy.

4.3. Determination of biodegradation efficiency

Seven parameters were chosen as an indicator of hydrocarbon biodegradation efficiency for the bacterial isolates. These parameters were measured weekly for each isolate . The hydrocarbon degrading isolates *P.putida, K. oxytoca, F. breve, S.spiritivornm, S.liquifaciens, A.media, A.baumanni* and *A.eutrophus* were grown in mineral salt medium containing 2% dried hydrocanbon mixture substrate as a sole source of carbon and energy. Then they were incubated in orbital shaker at 120 rpm and 37°C for six weeks. Every week a batch culture of the total isolate with a control flask was withdrawn for the measuring of the following items: total viable count (CFU/ml), pH, crease and oil (mg/L), dissolved oxygen (mg/L), surface tension (dyns/ cm), biosurfactant amount (mg/L) and hydrocarbon loss percentage (HC loss %).

The relationship between each of the previous mentioned parameters and the time was studied to determine the more efficient isolate in biodegradation of hydrocarbon among the total isolates.

4.3.1. Determination of the total viable count

The time course of hydrocarbon biodegradation in culture medium was monitored in an experiment that involved an initial cell count about (10^6 cell/ ml), then they inoculated in mineral salts medium (MSM) contains 2% of hydrocarbon mixture as a sole source of carbon and energy (5 g of hydrocarbon per 250 ml of MSM).

Figure (5) demonstrates that bacterial cells of the four isolates *P.putida, S.spiritivorum , S.liquifaciens and A.eutrophus* increase rapidly from the first and second week of incubation to reach 52.3×10^7 CFU/ml, 26.1×10^7 CFU/ml , 21.9×10^7 CFU/ml and 25.1x10^7 CFU/ml at the fifth week of incubation . This value then began to decrease at the sixth week of incubation to 35.91×10^7, 13.2×10^7 , 9.2×10^7 CFU/ml and 11.9x10^7 , respectively.

On the other hand, the three isolates *A.media*, *F.breve* and *K.oxytoca* were never increasing in cell number from the first week to the fifth week to achieve 17.3×10^7, 15.7×10^7 and 13.9×10^7 CFU/ml, respectively. then the cell number remained without changing during the last week.

A.baumanni exhibited a gradual increase in cell number during incubation period to reach 10.6×10^7 CFU/ml at the sixth week of incubation.

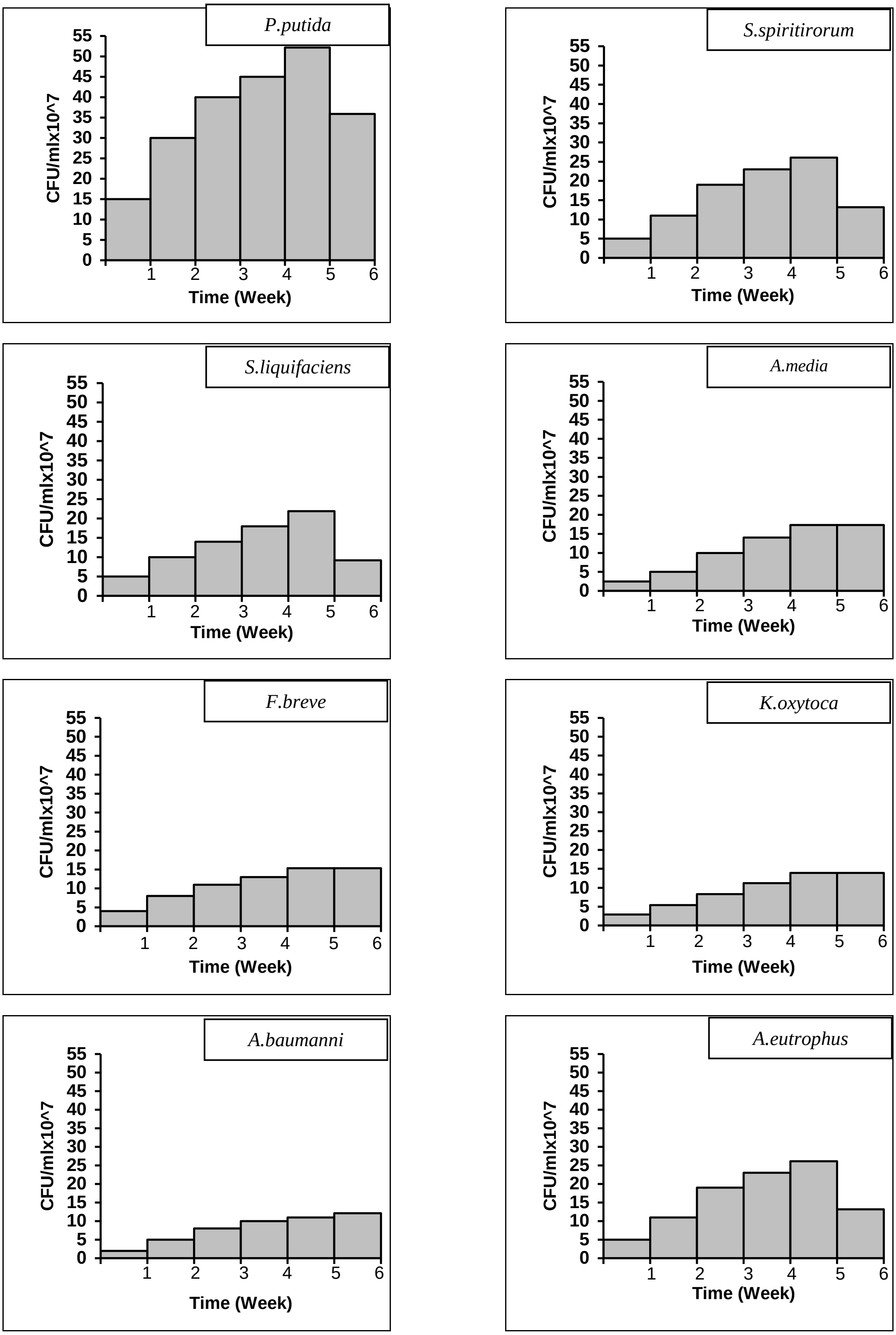


Fig. 5. Total viable count (CFU/ml) of bacterial isolates in mineral salts medium containg 2% of HC as a sole source of carbon and energy.

4.3.2. pH measurements

The hydrocarbon biodegradation by bacterial action after incubation period leads to the production of organic acids and other metabolites which probably are causative agents for the change of pH value. From this explanation the changes of pH values were studied as an indicator of bacterial activity.

Figure (6) shows that the maximum reduction of pH values happened at the first and second week of incubation , and it exhibited a slight reduction in the following period of growth to reach 5.0, 5.3 and 5.7 for *P.putida, S.spiritivornm* and *S.liquifaciens*, respectively while the growth of *A.media* and *F.breve* were reduced the pH values to 5.7 and 5.8 at the fifth week of growth. was unchanged during the last week of incubation.

The pH values were 5.9, 6.0 and 6.1 for *K.oxytoca, A.baumanni* and *A.eutrophus* at the end of incubation period, respectively .

4.3.3. Measurements of surface tension

The changes in surface tension values of bacterial isolates were measured during bacterial growth at 37°C in mineral salts medium containing 2% hydrocarbon substrate as a sole source of carbon and energy.

Bacterial isolates reduced the surface tension in a different pattern. *P.putida* reduced this value gradually to 32.2 dyns/cm at the end of growth period . *S.spiritivorum* and *S.liquifaciens* lowered the

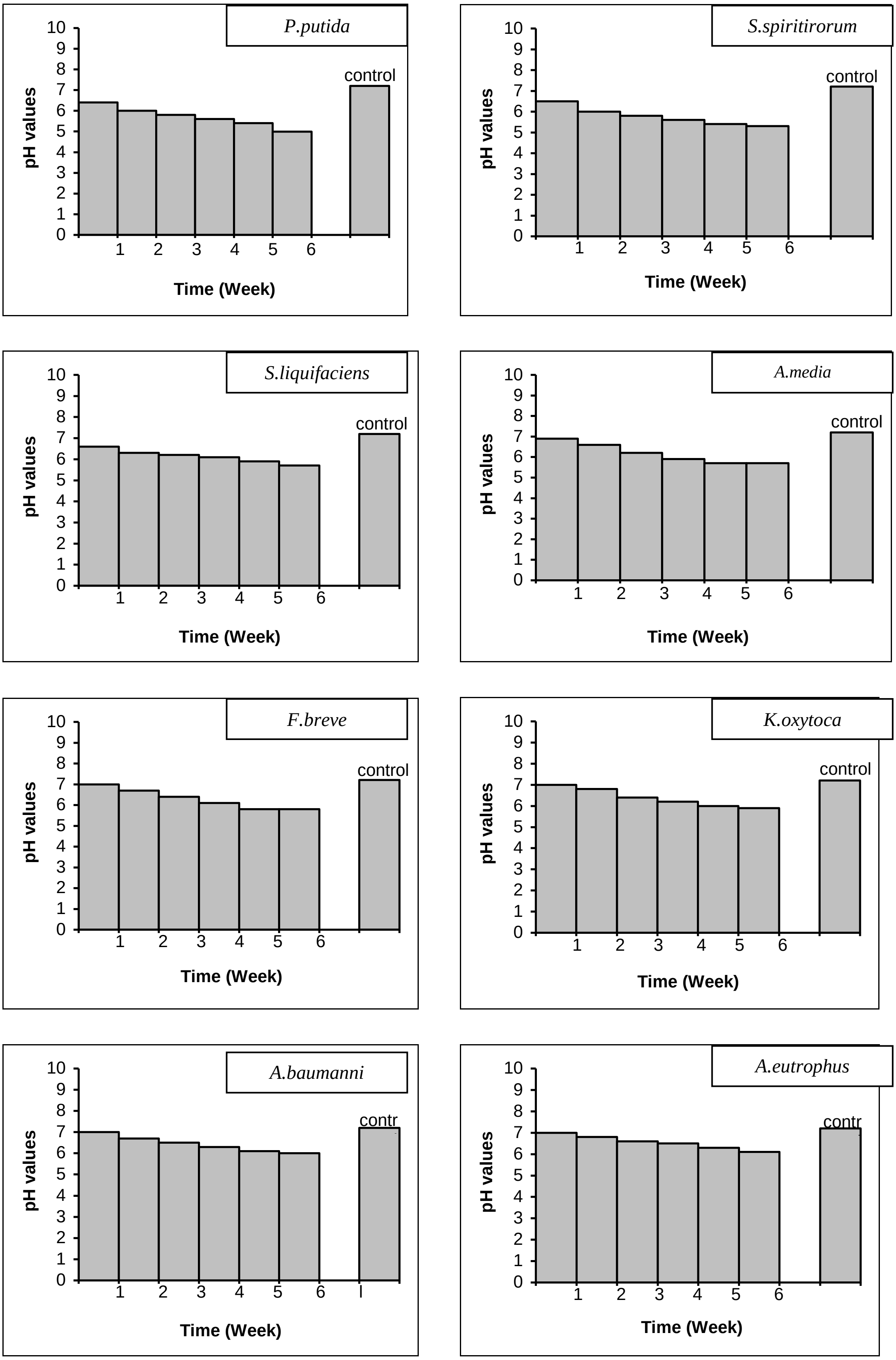


Fig. 6. pH values of isolates grown in mineral salts medium containing 2% of HC mixture as a sole source of carbon and energy.

surface tension of their culture media to 48.2 and 50.1 dyns/cm, respectively. *A.media* reduced the surface tension value from initial value 72.2 to 52.2 dyns/cm at the first four weeks then this value remained without changing during the following weeks. The culture medium surface tension of *K.oxytoca* was 51.2 dyns/cm at the end of the fifth week of incubation and this value was still constant to the end period of incubation(Figure.7.).

F.breve exhibited a reduction in surface tension during the first and second week, but it was constant at the third and fourth week at 58.8 dyns/cm. Then it returned to increase again to 60.1dyns/cm at the end of the last week.

A. baumanni growth reduced the surface tension of culture medium to 41.2dyns/cm at the third and fourth weeks then it exhibited a distinct increase at the last two weeks to reach 48.0 dyns/cm. Finally, *A.eutrophus* decreased the surface tension gradually during the growth period to 52.3 dyns/cm at the end of sixth week of incubation.

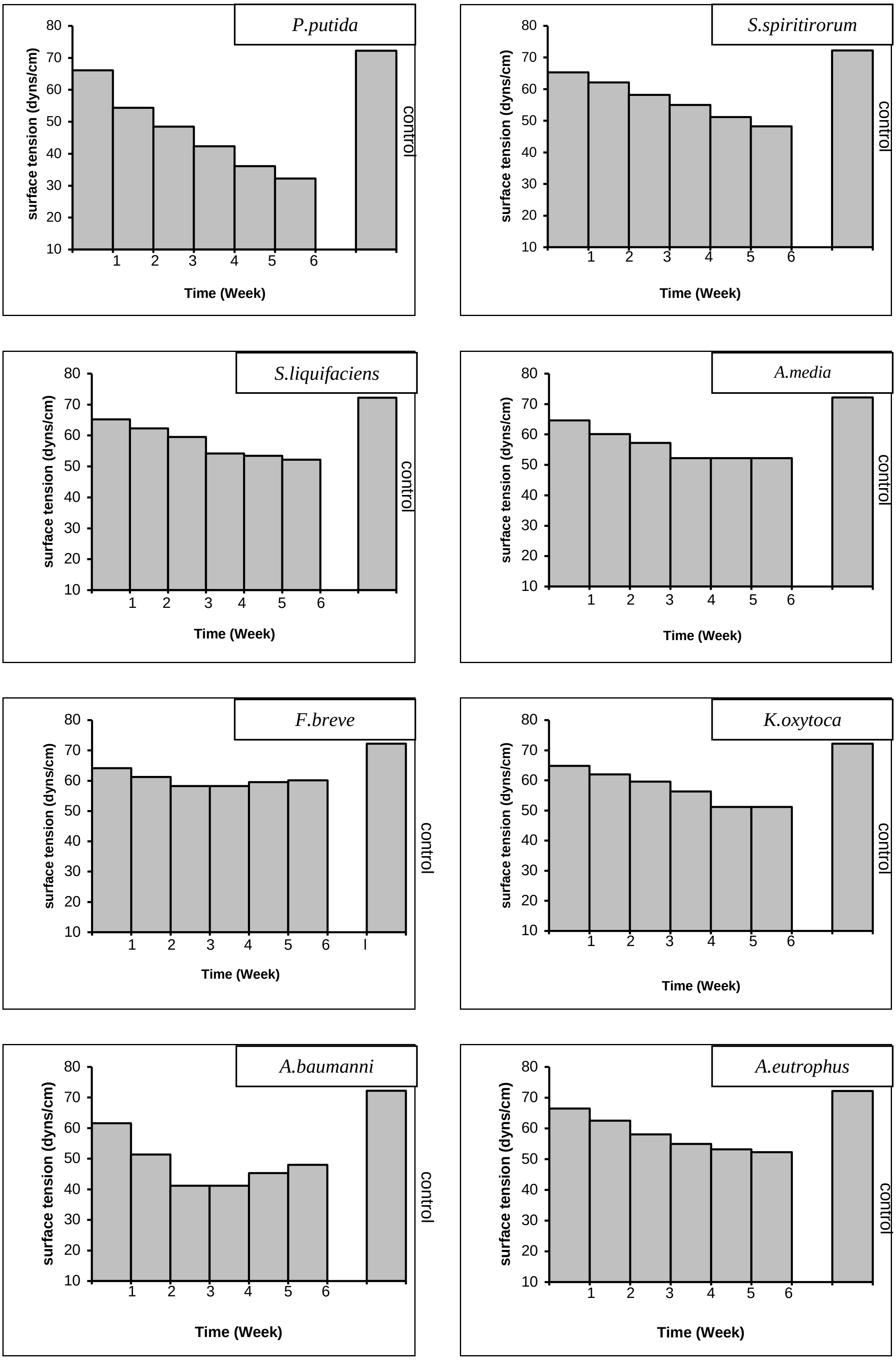


Fig.7. Changes of surface tension values in mineral salts medium containing 2% of HCs de to the growth of the bacterial isolates for 6 weeks.

4.3.4. Production of biosurfactant

The biosurfactant concentration was evaluated during bacterial growth for six weeks. All isolates *P.putida* , *K.oxytoca, S.spiritivoum, S.liquifacens, A.media, F.breve, A.baumanni,* and *A.eutrophus* produced biosurfactant but in different amounts (Figure 8).

P.putida, S.spiritivorum, S.liquifacious and *A.eutrophus were* exhibited continuous increasing in biosurfactant amount during the growth period to reach 69.7, 65.6, 60.2 and 19.6 mg/L, respectively, at the end of incubation period.

A.media reached the maximum production at the fourth week of incubation 58.8mg/L to be unchanged during the following weeks.

F.breve production was 48.2mg/L at the fourth week of incubation, and this value remained without changing at the fifth and sixth weeks of incubation.

A.baumanni was released 40.1 mg/L of biosurfactant at the fourth week of incubation and this value was still unchanged till the end of incubation.

Finally, *K.oxytoca* was produced biosurfactant at gradual increasing to reach 37.8 mg/L at fifth and sixth weeks of growth period, and this value remained constant at the last week .

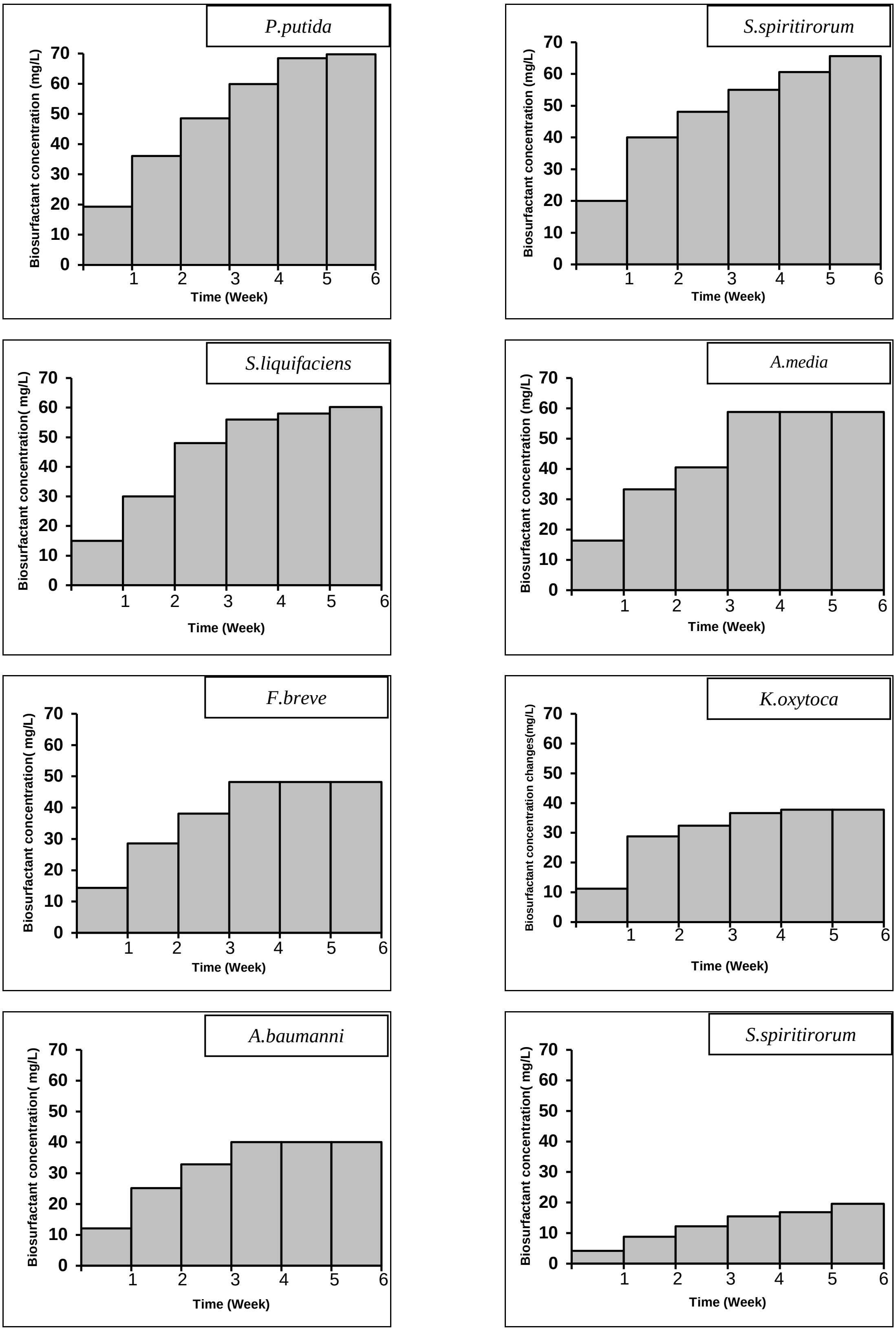


Fig.8. Biosurfactant concentration changes of isolates grown in mineral salts medium containing 2% of HC substrate.

4.3.5. Crease and oil values

The previous mensioned eight isolates exhibited differences of crease and oil content.

Figure (9) demonstrated that the crease & oil content of *P.putida* in culture medium was 22.5 mg/L at the last week of incubation compared to control flask which was 54.3 mg/L . *S.spiritivorum* reduced this value to 28.2 mg/L and *S.liquifaciens* was reduced it to 31.6mg/L at the final of incubation period. *A.media* and *F.breve* were decreased that content to 31.3 mg/L at the sixth week of growth period.

K.oxytoca and *A.baumanm* reduced crease and oil content to 35.2 mg/L at the fifth week of incubation and this value remained without changing during the sixth week. Finally *A.eutrophus* decreased the amount of crease and oil to 36.1 mg/L up to the fourth week, then this value remained constant till the end of incubation period.

4.3.6. Measurement of dissolved oxygen concentration

The results of dissolved oxygen concentrations of bacterial isolates were represented in Figure (10). From the view on this figure, it appeares that various amounts of dissolved oxygen of culture medium throughout growth period were consumed . All experimental isolates showed unsiginficant differences of oxygen content . *P.putida* consumed a dissolved oxygen during growth period of amount up to 9.5 mg/L and reduced the initial oxygen concentration to 5.2 mg/L , compared to 14.7 mg/L of control sample. *S.spiritivoram* reduced this value to 8.3 mg/L

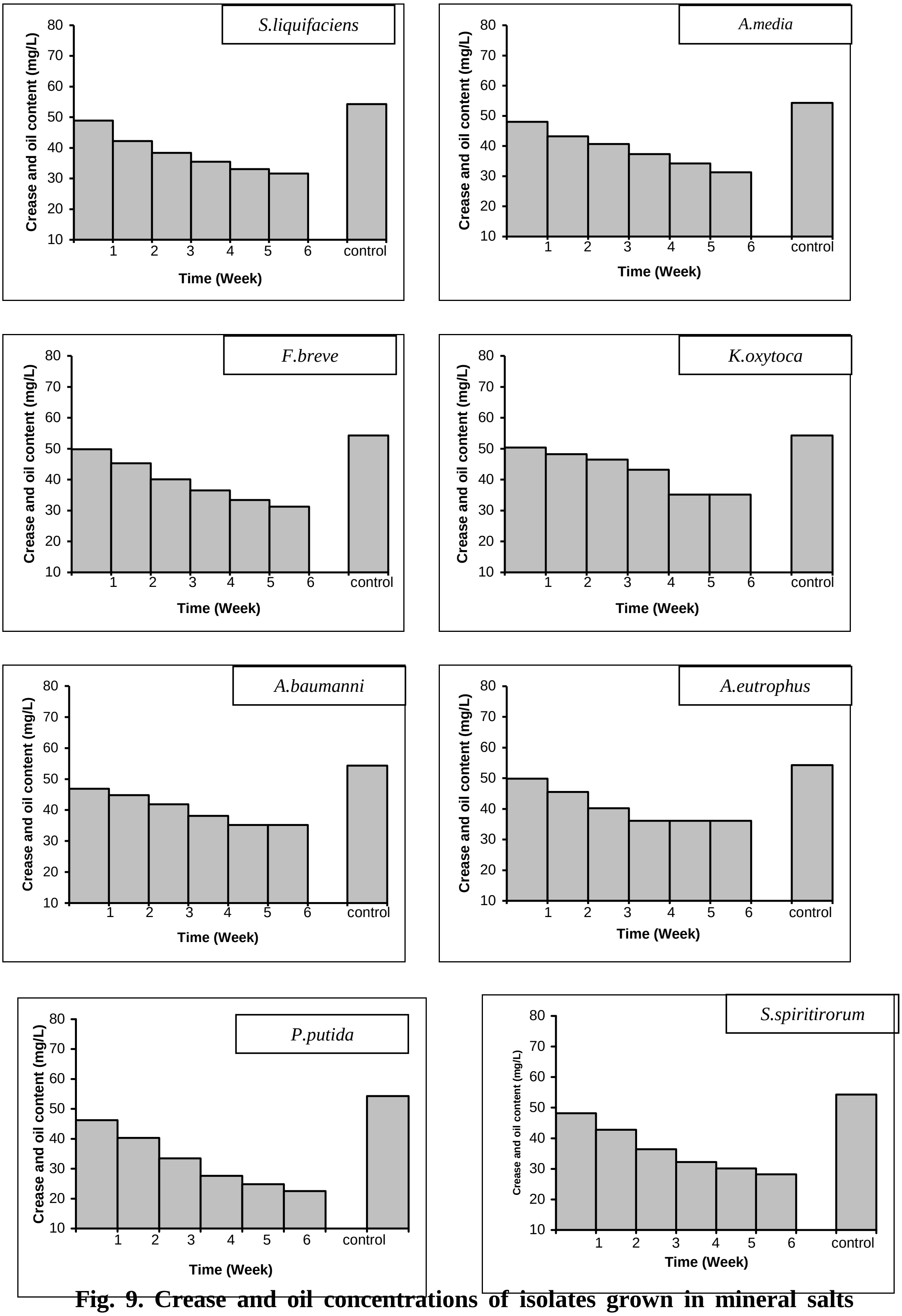


Fig. 9. Crease and oil concentrations of isolates grown in mineral salts medium containing 2% of HC as a sole source of carbon and energy

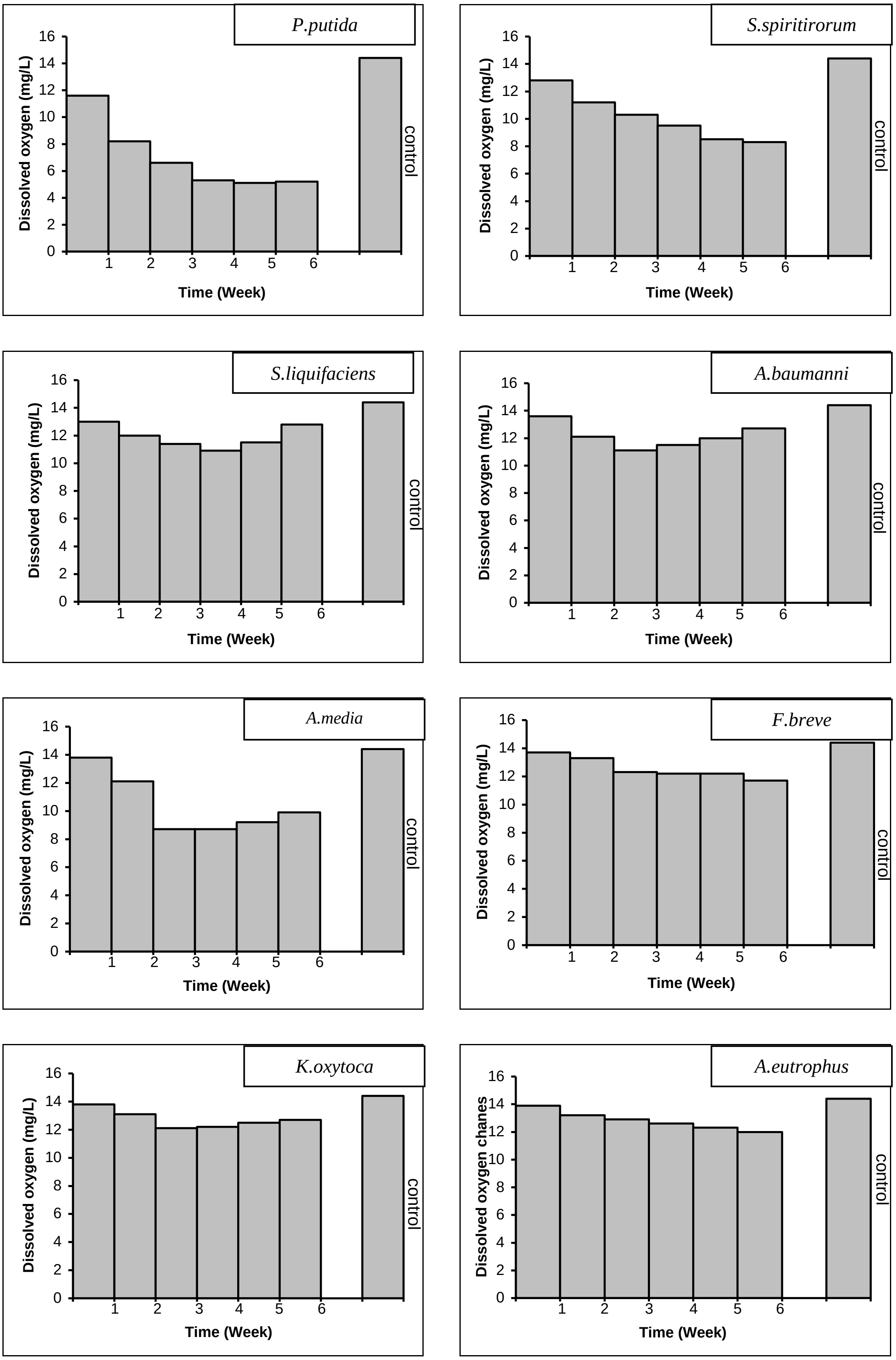


Fig. 10. Dissolved oxygen changes of isolates grown in mineral salts medium containing 2% of HC.

at the final week of incubation, *S.liquifacians* decreased the dissolved oxygen concentration to 10.9mg/L at the first four weeks, then became 12.8mg/L at the final week of incubation, while *A.media was* reduced dissolved oxygen amount at the first two weeks, to 12.1 mg/L. Then this value was decreased to 8.7mg/L at third and fourth weeks , than it returned to rise again to 9.9 mg/L at the fifth and sixth week.

F.breve consumed the dissolved oxygen to 12.2mg/L at the fourth and fifth week. It was subjected to further decreasing at the final week of incubation to 11.7.mg/L.

A.baumanni and *K.oxytoca* exhibited unsignificant differentes in dissolved oxygen concentration of culture medium. So they reduced it to 11.1 mg/L and 12.1 mg/L, respectively , at the end of third week, these values returned to increase again to reach 12.7 mg/L for both of them at the final week.

A.eutrophus consumed only 2.7 mg/L of dissolved oxygen to change its value to 12.0 mg/L at the final week of incubation (Figure.10).

4.3.7. Hydrocarbons loss percentage

All isolates exhibited a significant reduction of hydrocarbon mixtre weight, but the individual isolates showed variations of their hydrocarbon degrading ability. The maximum hydrocarbon loss appeared at the first and second weeks of bacterial growth , while the amount continued to decrease at the following weeks but in slow rates.

The most efficient isolate in hydrocarbon loss was *P.putida* which removed 52.2% of the original hydrocarbon amount, followed by

S.spiritivorum and *S.liquifaciens* which consumed 47.5% and 46.3% of hydrocarbon, respectively. *A.media* was reduced 39.8% of hydrocarbon mixture during the first five weeks and this value was preserved at the next week.

F.breve utilized 38.5% of hydrocarbon amount at the first five weeks, but this value continued at the last week.

K.oxytoca and *A.baumanni* decreased 34.7% and 32.2% of hydrocarbon amount, respectively at the end of incubation period. Finally, *A.eutrophus* consumed 24.6% of hydrocarbon at the first four weeks of incubation, and this percentage stilled without changing during the next two weeks. (Figure 11).

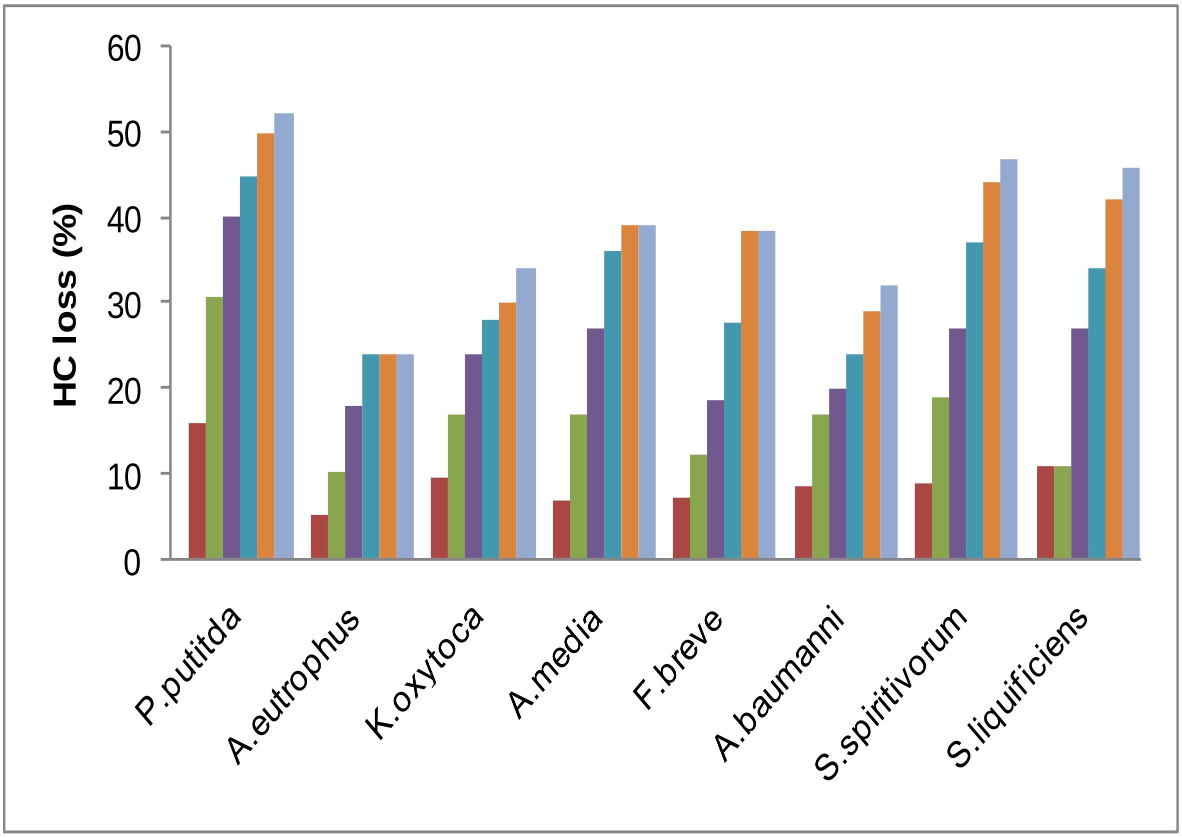


Fig. 11. HC loss of the hydrocarbons mixture which used as a sole source of carbon and energy for the bacterial isolates

4.4. High performance liquid chromatography analysis

The identification and characterization of hydrocarbon mixture before and after treatment by the isolated bacterial species helped to obtaining further confirmed investigation of hydrocarbon degrading isolates efficiency in mineral salts medium containing 2% of hydrocarbon substrate. After 6 weeks of incubation at 37°C in orbital shaker (120 rpm), the samples of hydrocarbon mixture before and after treatment of bacterial isolates, individually, were analyzed using HPLC technique by using C_{18} colomn with UV-detector at 254nm.

Table (4) represents retention times (R.T), peak areas (P.A) and their percentage for hydrocarbon compounds which composed the hydrocarbon mixture before treatment of bacterial isolates (control sample) Figure (12), while tables (5-12) represent the HPLC readings for the hydrocarbon mixture treated with bacterial isolates .

The different isolates exhibited a significant efficiency for degradation of hydrocarbon mixture components, so *P.putida* consumed100% of C_6, C_7 and C_8compounds , while it consumed 92.9% and 90.3% of C_{14} and C_{12} compounds respectively. The least utilizing effecieney appeared with C_{22} aromatic compounds when consumed 36.5 % Table .5 (Figure 13).

S.spiritivorum exhibited a high efficiency to utilize (100%) of C_6, C_7, while it consumed 83.3% of both C_8 and C_{10} aromatic compounds , 83.8%of C_{12} and 81.8% of C_{14} compound, but the least consumption showed for C_{22} when reduced only 14% of it.(Table.6 and Fig. 14) .

S.liquifaciens showed a significant efficiency to utilize C_6 and C_7 when consumed 100% of these compounds , and 83% of C_8 aromatic compound the least utilization is for C_{22} when consumed 25.1% (Table 7and Fig.15).

A.media exhibited a good ability for biodegradation of aromatic compounds, especially for C_6, C_7 and C_{12} when it consumed 100, 100 and 85.0% of these compounds , respectively , but it was less efficient to degrade C_{10} and C_{20} when it consumed 12.3 % (Table 8 and Fig. 16).

F.breve exhibited a considerable ability for biodegradation of aromatic hydrocarbons, especially for C_6, C_8 and C_7 when it consumed 79.3, 79.1 and 73.9% of these compounds , respectively, but it was less efficient to degrade C_{20} and C_{22} when degrade 20.8 and 18.9 % of these compounds, respectively (Table 9 and fig. 17) .

K.oxytoca was more efficient to utilize the low molecular weight aromatic hydrocarbon than the high molecular weight hydrocarbons, so it consumed 93.1% of C_6 compound while it utilized only 3.4% of C_{22} aromatic hydrocarbon (Table 10 and fig. 18) .

A.banmanni was consumed 100% of C_{10} compound and 79.3% of C_6 hydrocarbon compound, but it consumed only 12.7% of C_{22} aromatic hydrocarbon (Table 11 and fig. 19) .

Finally, *A.eutrophus* showed the lowest ability to degrade aromatic hydrocarbons among the eight isolates.The most consumption was 63.7% of C_{12} aromatic hydrocarbon and the lowest consumption was 28.3% of C_{20} compound (Table 12 and fig. 20) .

4.5. Comparison Study between the isolated and the imported consortium

The eight species (*P.putida, S.spiritivoum, S.liquifacions, A.media, F.breve, K.oxytoca, A.baumanni,* and *A.eutrophus)* grown together in order to make an isolated consortium a to compare the efficiency of hydrocarbon degradation with the imported one.

The same parameters which were used to determine the biodegradation activity of the isolated bacterial species consortium were repeated under the same conditions of temperature, aeration and incubation period on the imported consortium to get a comparative view between them to evaluate the final results.

4.5.1. pH measurements

The pH profile of the isolated consortium showed a strong reduction during the experiment duration, especially at the first and second weeks of incubation, while the pH value continued to decrease at the next weeks but in slow rate to reach 4.8 at the final of the growth period. The pH value of the imported consortium culture medium was decreased gradually during the overall incubation period till it reached to 2.2 (Figure 21).

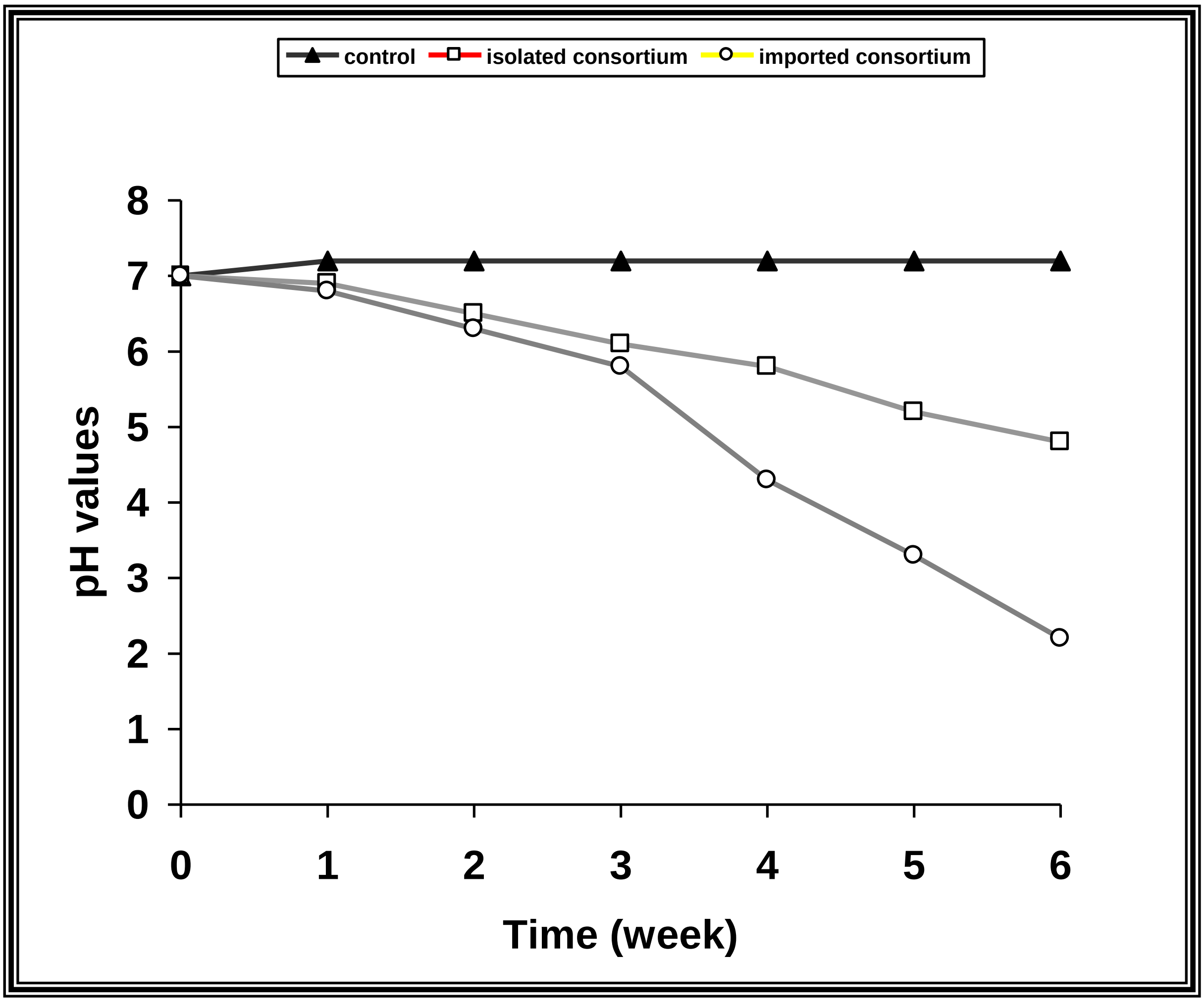


Fig.21. pH values of isolated and imported consortiums grown in mineral salts medium containing 2% of HC mixture

4.5.2. Optical density values

The maximum optical density value was for imported microbial consortium at the last week of incubation period (1.8 nm) while the O.D. of the isolated bacterial consortium was (1.1 nm) at the fifth week of incubation and remaine stable at this value until the last week of incubation (Figure 22).

4.5.3. Surface tension measurements

After six weeks of bacterial inoculation in mineral salts medium contains 2% of crude hydrocarbons, the surface tension of culture medium was lowerd of both isolated and imported consortiums, beging from the first week of cultivation. The results wear demonstrated that the surface tension values decreased during the six weeks of incubation continuously to reach to 41.8 dyns/cm , but the imported consortium reduced the surface tension of culture medium to reach to 22.3 dyns/cm at the end of growth period compared to control sample value which was 71.2 dyns/cm (Figure 23).

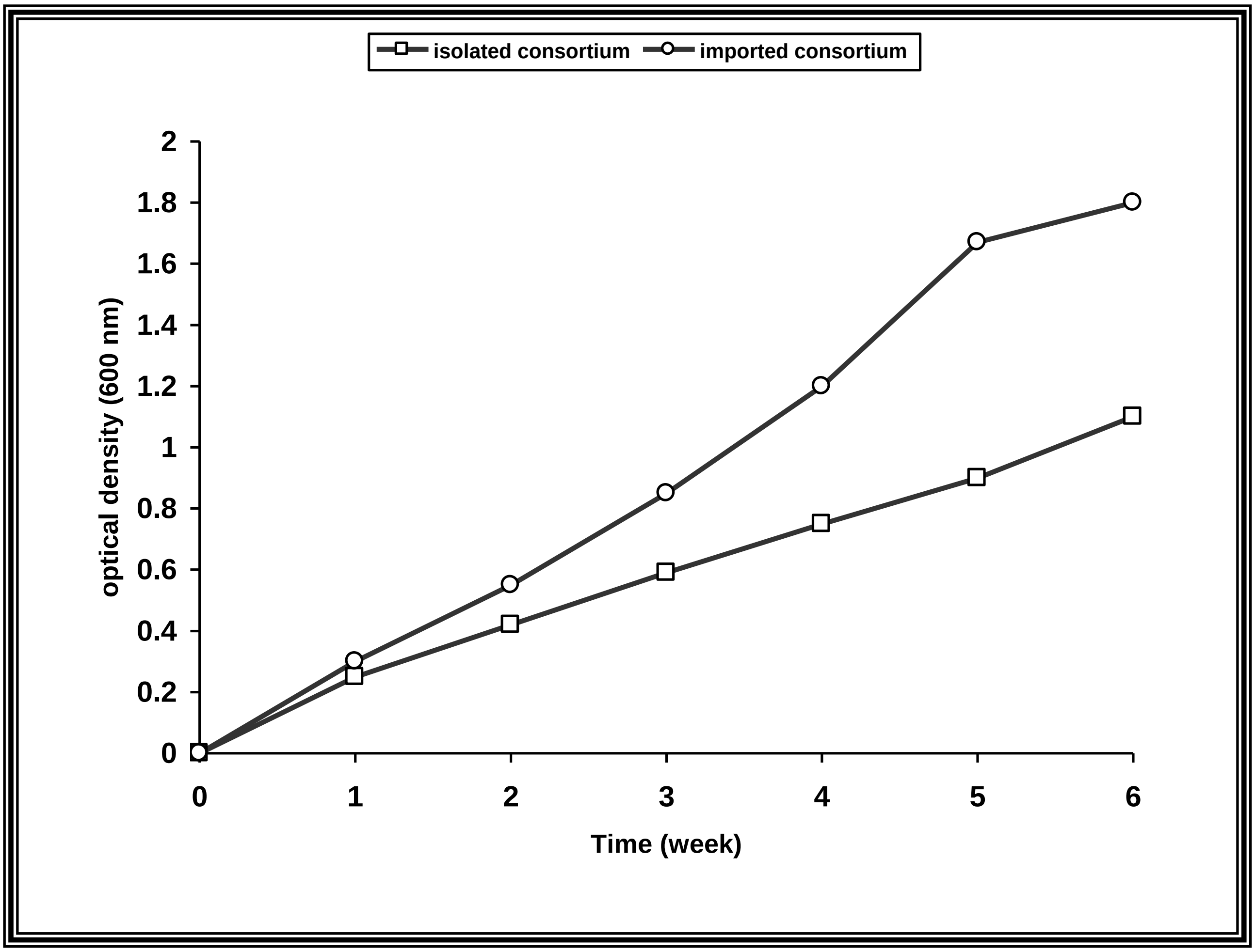


Fig.22. The Optical density of isolated and imported consortiums grown in mineral salts medium containing 2% of HC mixture

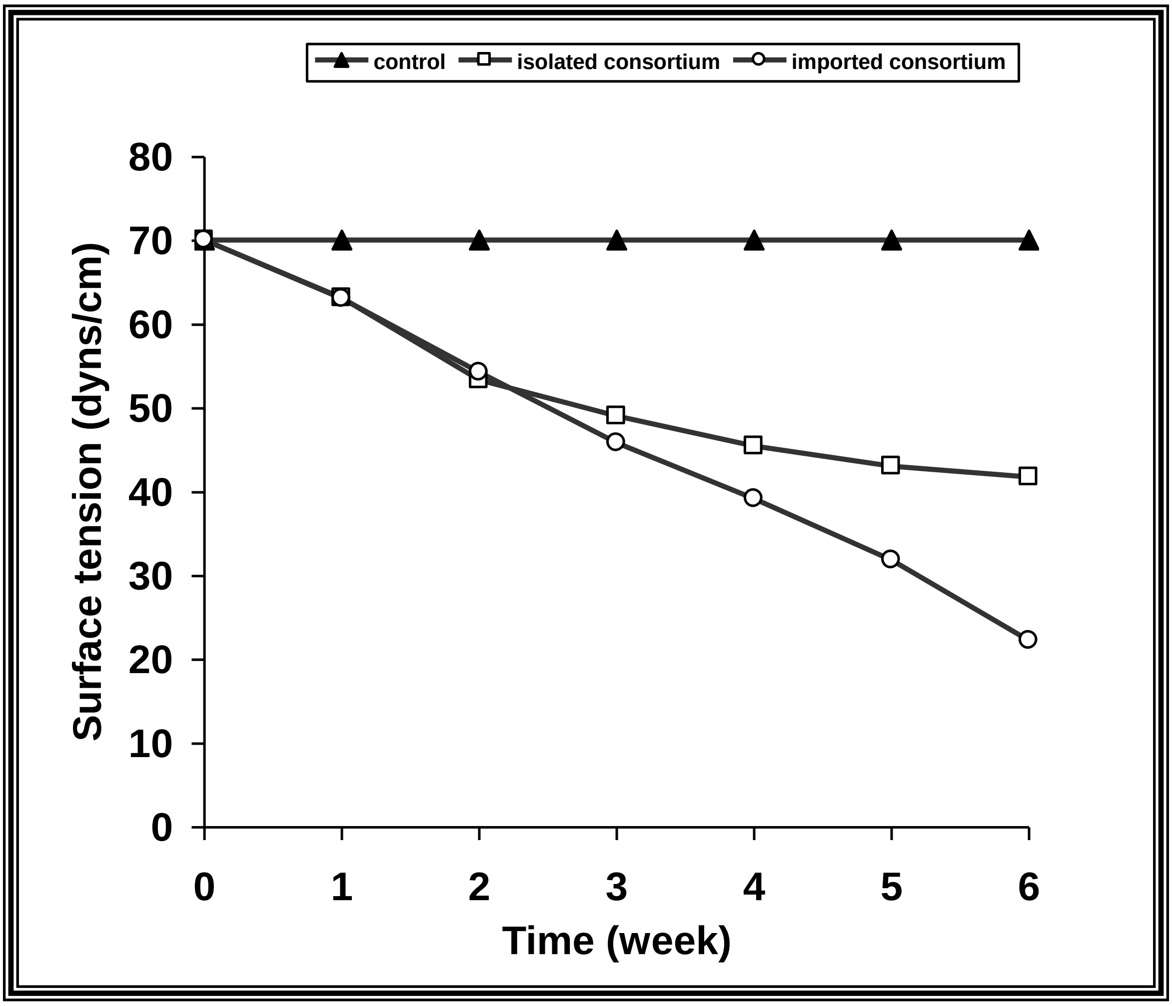


Fig.23. Surface tension values of isolated and imported consortiums grown in mineral salts medium containing 2% in HC mixture

4.5.4. Measurements of biosurfactant production

The amounts of biosurfactant that formed by the isolated and imported consortiums, when grown on hydrocarbons substrate (2%) within the growth period of six weeks, were measured.

The results showed that both consortiums produced biosurfactants but in different values that caused substantial decreasing of culture media surface tension. The isolated consortium continued to release biosurfactants during five weeks to reach to 285 mg/L , then these values remained without changing at the last week, while the production of biosurfactant continued to increase during the growth period of the imported microbial consortium to achieve 475 mg/L at the end of experiment (Figure 24).

4.5.5. Measurements of crease and oil

Weight method was applied for evaluating of crease and oil content of broth cultures in order to calculate the changing of the values during experiment period.

Both isolated and imported consortiums reduced crease and oil content of their culture medium but at different efficiency. The isolated bacterial consortium lowered the crease and oil concentration to 23.4 mg/L at the end of growth period compared to the same parameter of control flask which was 55.5 mg/L , while the imported microbial consortium reduced this value to 12.9 mg/L at the last week of incubation (Figure 25).

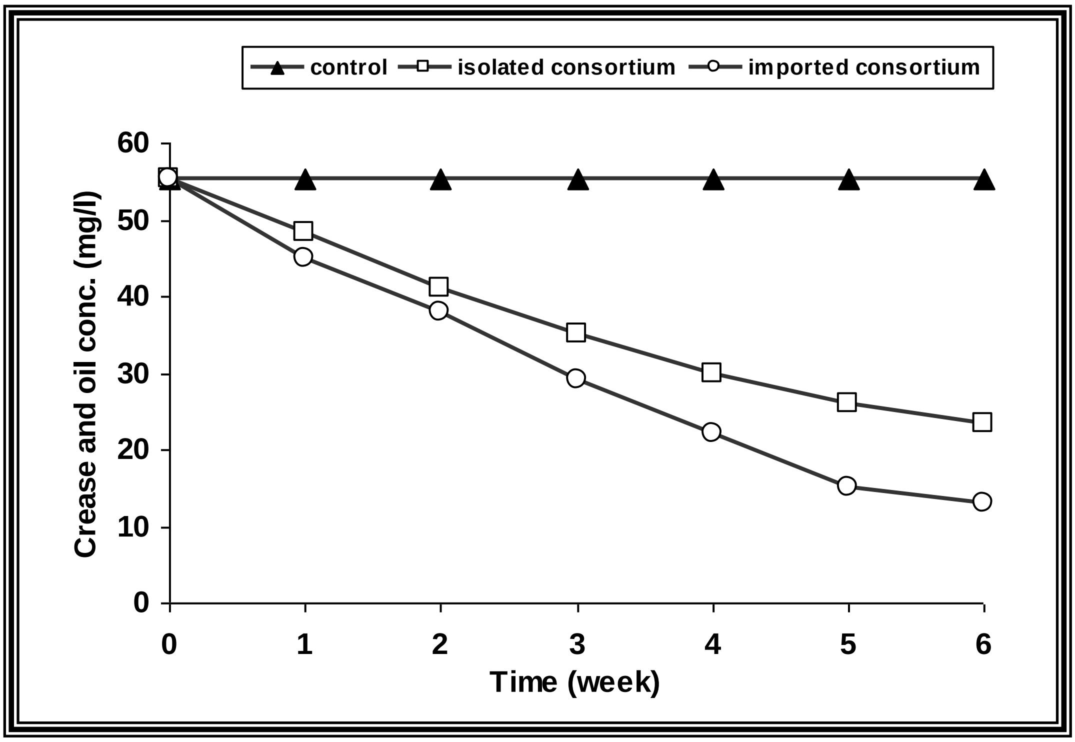


Fig.24. Biosurfactant concentration of isolated and imported consortiums grown in mineral salts medium containing 2% of HC mixture

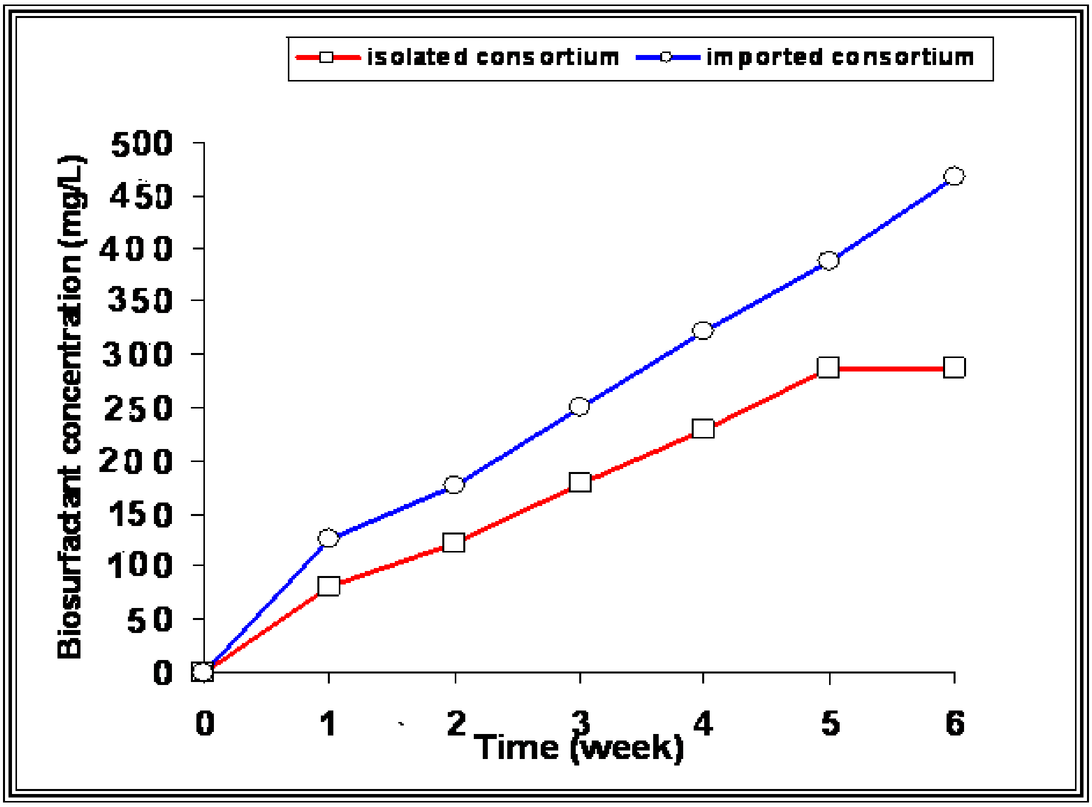


Fig.25. Crease and oil content of isolated and imported consortiums grown in mineral salts medium containing 2% of hydrocarbon mixture

4.5.6. Dissolved oxygen values

Dissolved oxygen amounts of culture medium which inoculated with either isolated or imported consortium, were evaluated during six weeks of cultivation.

The dissolved oxygen amount reduced in culture media due to the action of bacterial consumption for growth and hydrocarbon utilization. The isolated consortium lowered D.O concentration (6.1 mg/L) at the fifth week of incubation. Then this value was increased to 7.2 mg/L at the sixth week, while the imported microbial consortium was reduced it to 1.8mg/L at the final growth period compared to 15.1 mg/L of control sample (Figure 26).

4.5.7. HC loss percentage

The isolated bacterial consortium exhibited a good efficiency to hydrocarbon biodegradation when grown in mineral salts medium contains 2% hydrocarbon substrate and it showed a significant reduction of it's amount when consumed about 61.3% of the hydrocarbon mixture, while the imported microbial consortium was consumed 83.4% at the end incubation period (Figure 27.)

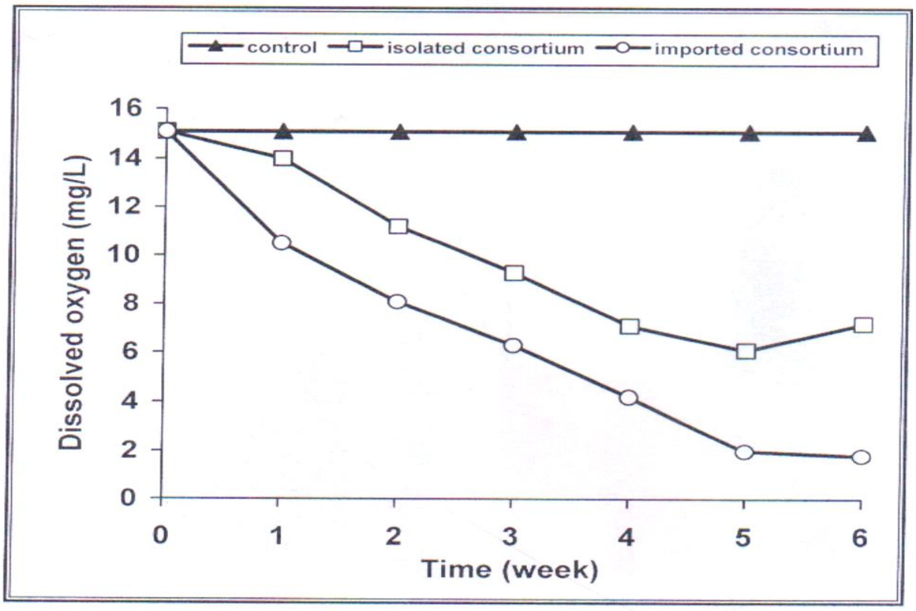


Fig.26. Dissolved oxygen concentration of isolated and imported consortiums grown in mineral salts medium containing 2% of HC substrate

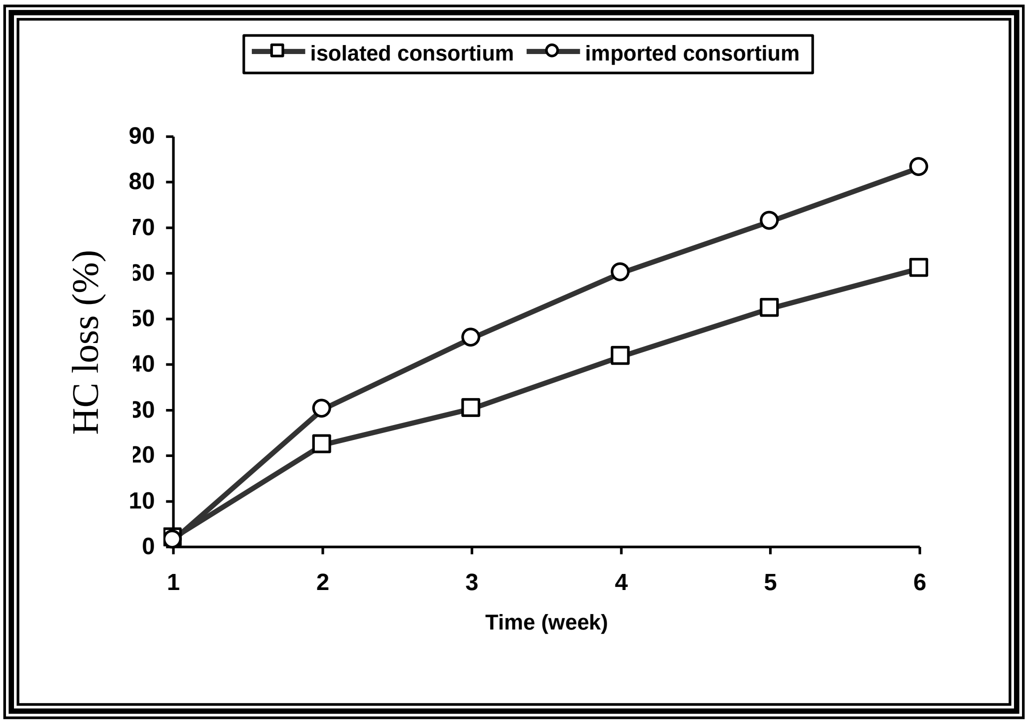


Fig.27 . HC loss percentage of isolated and imported consortiums grown in mineral salts medium containing 2% of hydrocarbon mixture.

4.6.HPLC analysis

The hydrocarbon substrate component were analyzed using HPLC technique after the growth of the misolated consortium composed of ***P.putida ,S.spiritivorum ,S.liqnifaciemn A.meida ,F.breve ,A.baumanni , K. oxytoca and A.eutrophus*** and the imported one for six weeks.

Both of the two consortiums were consumed 100% of C_6, C_7, C_8, and C_{10} of aromatic hydrocarbons , in addition to C_{12} compound in case of the imported one.

There are distinct differences in the ability of degradation of high molecular weight poly aromatic hydrocarbons , so the isolated consortium was utilized 57.8,55.8 and 40.0% of C_{18}, C_{20} and C_{22} (Figure.28 and table .14.) while the imported consortium was consumed 80.2, 80.8 and 75.1% of these compounds, respectively (figure .29and table.15).

5.Discussion

5.1. Wastewater samples :

It is a general knowledge that the activities of oil producing companies affect the environment and health of the people living within the immediate vicinity of the crude oil processing plant . The attendant hazards may trigger processes that may have adverse effects on the ecosystem of such areas. Waters used during extraction of crude oil are contaminated and become wastewater that may contain varying quantities of organic matter , heavy metals , volatile hydrocarbons (such as benzene , xylene , and toluene) and may other potentially toxic compounds . This wastewater contains sludge , which is disposed off in unfriendly manner (Reed and Johnsen , 1995) .

Our samples of wastewater were brought from effluent in Al – Dora Oil Refinery .

Our study was a trail to isolate an efficient local bacterial strains to be used in biological treatment of effluent wastewater from different crude oil refinies in the country .

There are many studies that dealt with biological treatment of hydrocarbon polluted environments . Challiant *et al* . , 2004 explained that extensive petroleum hydrocarbons often result in the pollution of the environment , which could lead to disastrous consequences for the biotic and abiotic components of petroleum contaminated systems could be achieved by biological or physicochemical methods . However , the attendant negative consequences of physicochemical approach are currently directing greater attention to the exploitation of the biological alternatives . Also , the extensive use of petroleum products leads to the contamination of almost all compartment of the environment for this reason Gogi *et al* , (2003) were used biodegradation of hydrocarbons by natural populations of microorganisms to be the main process acting in the removal of hydrocarbons – polluted environment . The mechanism of which has been extensively studied and reviewed (Snape *et al.*, 2001). Crude oil can be accidentally or deliberately released into the environment leading to serious pollution problems often result in huge disturbances of both biotic and abiotic components of ecosystem , more so that hydrocarbon compounds have been known to belong to a family of carcinogenic and neurotoxic organpollutants (Hallier – Soulier *et al* , 1999 ; Mueller *et al* ., 1992) .

On the other hand , hydrocarbons may be formed due to incomplete combustion of organic substances such as a coal , gas , petrochemical industries and oil refining. The residues generated by these activities contain a Rariety of PAHs such as anthracene , phenanthrene , and pyrene . release of PAH residues into the environment can lead to the contamination of natural resources . Their complex molecular structure and low solubility in water limit the application of conventional remediation techniques (Boopathy , 2000) .

Although some microorganisms can metabolize hydrocarbons to carbon dioxide and water , most of microorganisms consumed these compounds partially to give other organic or inorganic intermediates (Hueseman *et al* . , 2002) .

5.2. Isolation and identifiction of bacterial isolates

The positive hydrocarbon degrading isolates were 8 genera (36.3%) all of these were Gram negative , whereas the negative hydrocarbon degrading isolates were 14 (63.6 % ; Table .1.) . These results agreed with Martinez (2006) who reported that 66 % of the isolated strains from HC-contaminated sites in Puerto Rico were Gram negative, while 34% were positive . Also , it agreed with Al- Taher (2002) who reported that 76.5 % of his isolates isolated from oil emulsions were G -ve, while 33.5 % of those were G +ve .

Another study published by Hussein (2006) who isolated only Gram negative HC- degrading bacteria from industrial wastewater .

The Gram negative isolates in the present study were *p.putida* , *S.spritivorum* , *A. media* , *S.liquifaciens* , *A.baumanni* , *K.oxytoca* , *F.breve* ,*A. eutrophus*, and these observations were in accordance with Brito *et al* ., (2006) who explained that the typical groups already known for their capacity to degrade hydrocarbons including *Psendomanas* , *Sphingomonas and Alcaligenes* ,but were in contrary to Bartha (2003) who isolated *Micrococcus and Bacillus* as a hydrocarbon degrading bacteria .This may be attributed to methodological differences useing to enumerate petroleum – degrading microorganism or most probably to the types of hydrocarbons used (Horowitz *et al* ., 1975) .

Oboh *et al* ., (2006) isolated *pseudomonas* , *Bacillus* , *Alcaligenes spp*. *and Citrobacter* from Nigerian Oil Refinery effluent wastewater .

In iraq , Matti (1993) isolated *Pseudomonas, Nisseria* , *Flavobacterium* and *Staphylococcus* from oily polluted region in Shatt-Al-Arab and his study is accepTable with respect to the present study in predominance of *Pseudomonas spp* . as a wide distributed in hydrocarbon polluted sites (Whited *et al*., 1994 ; Deutz and Gibson , 1991) .

The dominance of *Psendomonas spp.* among the studied isolates is consistent with their wide spread distribution in water and soil polluted by hydrocarbonic compounds (Goodwin *et a.*, 2005).

Parales *et al* . , (2000) reported that *Pseudomonas* can adapt to diverse substrates and possess recalcitrant substances . Some studies on *P.putida* identified metabolic pathways dedicated to hydrocarbon compounds like benzenes , toluene , ethylbenzene , and xylene (BTEX) (Bodour *et al* .,2003; Quresh and purohit , 2002) .

There are many investigations showed several types of microorganisms which biodegrade various components of petroleum hydrocarbons such as poly aromatic hydrocarbons , including anthracene and naphthalene , in addition to monoaromatics HC such as toluene , benzene , and xylene (BTX) , or aliphatic hydrocarbons such as n-alkanes which are readily isolated from the environments , particularily from petroleum – contaminated sites (Olsen *et al* . , 1994)

All HC-degrading isolates in our study were Gram negative bacteria (Table.3.) This dominancy was due to their chemical structure of the cell wall and its outer membrane . The outer leaflet of which is primarily composed of lipopolysaccharide (LPS) .The presence of smooth LPS results in a relatively hydrophilic cell surface that is permeable to small hydrophilic molecules (MW < 600 Daltons) but excluding hydrophobic molecules (Nikaido and Narra , 1985 , poxton,1993). Several studies have shown that it is possible to modify the outer membrane of Gram negative bacteria by mutation or by the addition of chemical agents , such as hydrocarbons . This results in overall changes in cell surface properties (Makin and Beveridge , 1996) .

The loss of LPS from the outer leaflet of the outer membrane may cause a temporary exposure of the hydrophobic phospholipid fatty acid tails associated with the inner leaflet of the outer membrane . Alternatively , loss of LPS may decrease the compaction of the outer leaflet of the outer membranes , allowing increased passage of large hydrophobic compounds . Nikaido and vara (1985) further suggest that lose of LPS may be replaced with phospholipids , resulting in a cell suface with increased hydrophobic character.

The ability of *Pseudomonas spp* . to utilizes HCs as a source of carbon and energy is well known. In most cases , however , the rate of utilization is slow compared to readily soluble compounds like sugars .

Bacteria are also known to adapt to changes in environmental conditions , such as growth in the presence of hydrocarbon substrate , by altering the lipid composition of the cytoplasmic membrane in order to maintain or adjust membrane bilayer fluidity (Sikkema *et al* . , 1995 ; Ara –Rajas ,2004) .Alteration to the fatty acid moieties of membrane lipids are thought to be the most effective state membranes , which is essential for optimal membrane function .

These changes act as protective mechanisms against hydrocarbons associated toxicity by rendering the membrane less permeable to hydrocarbons (Herbert ,1990). Conversion of Cis- fatty acids to their transforms regarded as an additional protective mechanism to reduce hydrocarbon toxicity observed in a number of *Pseudomonas spp* . (Wu *et al* ., 2001 ; Yang *et al* ., 1999 .

In 1995 , Sikkema *et al* .,published an extensive review on the mechanisms of membrane toxicity of hydrocarbons for a variety of organisms . They outlined the toxicity of lipophilic compounds ,including hydrocarbons (alkanes , cyclic hydrocarbons). Hydrocarbons tend to reside in the hydroacyl chain of phospholipids , with partitioning being related to the hydrocarbon-water partition coefficient of the lipophilic compound . Hydrocarbon insertion alters membrane structure by changing fluidity and protein conformations and results in disruption of the barrier and energy transduction function while affecting membrane – bound and embedded enzyme activity (Wang and Fingas ,2001).

In terms of general stress responses , bacteria may from biofilms , altering their cell surface hydrophobicity to regulate their partitioning with respect to hydrocarbon – water interface or , in Gram – negative bacteria , gaining protection from hydrophilic lipopolysaccharid component , that offer high transfer resistance to lipophilic compounds . In addition , energy dependent repair mechanisms may be used to compensate losses in membrane integrity resulting from the partitioning of lipophilic compounds . As well as , membrane fluidity can be decreased through increased membrane ordering by affecting Cis / trans phospholipids isomerization by decreasing unsaturated fatty acid content , and by altering phospholipids head groups (Soharabi *et al.* , 1999) . These changes may be associated with an overall increase in phospholipids content and increased phospholipids biosynthesis in solvent – stressed cell (Upatham , 1998 ; Watkinson and Morgan , 1990 .

These alterations serve to produce a physical barrier to the intercalation of hydrocarbons in membrane . Thus offsetting the passive influx of hydrocarbon , into the cell. It is generally believed that hydrocarbon interacts with microorganisms nonspecifically and more passively into the cell (Stokes *et al* . , 1998) . Hydrocarbon – degrading microorganisms must necessarily come in contact with their substrates before any transport , either active or passive , may take place .

5.3. Growth conditions of HC- degrading isolates

The mineral salts medium (MSM) used in the present study was Bushnell and Hass medium (1941) . This medium known to give more stable results because it contains required minerals for bacterial growth , especially phosphorus and nitrogen that are considered as a limiting factors essential for microorganisms activity (Guerin and Boyed ,1992; Head and Swannel,1999) . In addition , this media can determine the exact source of carbon and energy by the certain substrate to be studied . So it is suitable to know the ability of isolate to consume it or not . there are several studies referring to other types of MSM which contains more components that may affect negatively on hydrocarbon nature (Hill , 1988 ; Budiziski *et al* . , 1998).

The culture flasks of all experiments in this study were incubated at 37 C° in orbital shaker incubator . Inspite of hydrocarbon biodegradation can occur over a wide range of temperatures . The rates of biodegradation generally decreased with decreasing temperature , so the incubation temperature for all isolates was limited at 37 C° because of the occurring of the highest degradation rates in the range of 30 – 40 C° in water environment (Bossert and Bartha , 1984 , Cooney , 1984) .

Moreover , Nedwell (1999) reviewed the effect of low temperature on the affinity of microorganisms for substrate .

Generally, it is accep table that uptake affinity of microorganisms for hydrocarbon substrate is markedly reduced when the temperature falls below their optimum for growth . At low temperature , the microorganisms try to preserve the vital fluidity of their cell membranes by altering the membrane structure and composition . This is done by increasing the proportion of unsaturated fatty acids , or by shortening their chain length or by replacing iso fatty acids with the corresponding anteso in membrane fluidity (or increase the efficiency of lipids) .

The other affecting factor on bacterial growth is oxygen , which considered as an important factor in hydrocarbon biodegradation process , because microbial utilization of hydrocarbons requires an exogenous electron sink . In the initial attack , this electron sink has to be molecular oxygen . In the subsequent steps too , oxygen is the most common electron acceptor . In the absence of molecular oxygen , further biodegradation of partially oxygenated intermediate may be supported by nitrite or sulfate reduction. Little or no hydrocarbon metabolism occur in strictly anoxic environment (Dibble and Bartha , 1976 ;Rosseert and Brtha ,1984; Dean- Ross,1989)

5.4. Bacterial ability to degrade hydrocarbon

The ability of microorganisms to degrade hydrocarbons and using them as a sole carbon and energy source has been studied worldwide (Atlas , 1991 ; Pritchard *et al* . , 1992 ; Maki *et al*.,2005 ; Otenio *et al*.,2006). Figure.3. exhibites that none of the hydrocarbon – degrading isolates showed a lag phase in their growth curve except three isolates *A.baumanni* , *K* . *oxytoca* , and *A* . *eutrophus* . This result may be attributed to genetic make up due to the constitutive expression of hydrocarbon catalyzing enzymes or physiological owing to previous exposure to exogenous hydrocarbons present in the effluent tanks (Osidanjo , 1992; Okerentugba and Ezeronye , 2003) .

Cells pregrown in the presence of hydrocarbons had a lag phase lesser than 1 day prior to initiation of hydrocarbon degradation (Churchil *et al* . , 1999) pointed to the presence of lag phase of isolates when grown on hydrocarbon substrate in order to be adapt with such compounds .

Suehara *et al* .,(2005) mentioned that exposure of a microbial community to hydrocarbons have been shown to result in an increase in a number of bacterial plasmid types . Catabolic plasmids are non- essential genetic elements in so far as viability and reproduction of an organism is concerned. But they do provide metabolic versatility normally present in the cell . Such genetic potential allows for the evolution of integrated and regulated pathways for hydrocarbons degradation . Plasmids enhanc the metabolic potential of microorganisms in ecosystems . Conjunctive plasmids are important agents of genetic changes and evolution in bacteria , and could be packed up from or brought together in different organisms as groups of genes , which through mutations and re-combination can specify new metabolic functions (Samanta *et al*., 2002;Zhang *et al*., 2004) .

The present study revealed that *P.putida* was the most efficient hydrocarbon-degrading isolate among the total isolates in account of cell density which reached 52.1x10^7 CFU/ ml at the end of the fifth week of incubation then this value decreased to 35.9x10^7 CFU/ml at the end of incubation period. The same phenomena was observed with *S. spirivorum and S.ligifaciens* which reached with the maximum value of cell number at the fifth week (26.1x 10^7 and 21.9x10^7 CFU/ml) , respectively then they reduced to (13.2 and 9.2 x10^7 CFU/ml) at the sixth week of incubation (Figure . 5). This observation may be attributed to the rapid growth firstly when concentration of substrate was high and the other conditions like pH was suitable for bacterial growth. But in the following period the substrate concentration of hydrocarbons and pH began to decrease to be around 5.0 , 5.3 and 5.7 for *P.putida* , *S.spiritivorum and S.liquifacene*_respectively (Figure.5.) , as a result of accumulating organic acids in culture media that produced during hydrocarbon biodegradation process which probably convert the medium to unsuitable for a good growth . (Whyte *et al* ., 1996; Oboh *et al* , 2006)

*A.media , F.breve and A.baumanni*_cells number reached 17.3 x107, 15.3 x107and 13.9 x107 CFU/ml (Figure .5.) at the fifth week and this value is still constant during the final week .

This result in return to the growth curve of these isolates which showed that the cells are at the stationary phase mean that the newly born cells were equal to the dead cells at this time . Also , due to stability of cells density at the last two weeks , the pH of their culture media was still without change at this time 5.7 , 5.8 , 6.0 (Figure .6.) for these , respectively. This may be due to decreasing amount of produced organic acids that make distinct changes in pH value (Ojumu *et al* . , 2005) . *A.baumanni and A.eutrophus* exhibited another pattern of growth rate , so they continued to increase in cell number , gradually , to reach 12.1x$10^7$and 9.6 x10^7 CFU/ ml (Figure .4.) , respectively , concomitant with decreasing of pH values to 6.0 and 6.1 , (Figure .6.). This observations may be attributed to the slow growth rate during incubation period leading to a little decrease in pH value which in turn affect slightly on growth rate of bacteria (Wilson and Jones , 1993).

Surface tension values of all isolates culture media were decreased during the growth period, but in different values , the lower surface tension happened with *P.putida* at the end of incubation period which was 32.2 dyns / cm (Figure.7.) This reduction may be attributed to the high production of biosurfactant 69.7 (mg/L) by this isolate (Figure .8.). Biosurfactants are microbially produced surface active compounds with both hydrophilic and hydrophobic regions which give them the property of aggregate with fluids such as water and hydrocarbons , and have the ability to lower surface tension of medium (Fietcher , 1992 ; Lin 1996 ;Yousseff *et al.* , 2005) .

This result agreed with Bossio *et al* . , (1998) who pointed to the capability of *P.putida* to produce surface active agents that emulsify the low soluble compounds such as hydrocarbons in order to facilitate their uptake by bacterial cell to support its growth. *S.spritivorum* growth reduced the surface tension of the medium to 48.2 dyns/cm compared with 72.2 dyns / cm of control sample (Figure . 7) , in result of biosurfactant production which increased with bacterial growth to reach 65.6 mg/ L at the end of incubation period . This assessment agrees with Van Hamm *et al.* , (2003) who indicated that *S.spritivorum* release surface active agents in the form of glycolipid type molecules which was able to change the liquid surface tension even in small amounts releasing.

S.liquifaciens produced 60.2 mg/l of biosurfactant (Figure .8.) which reduced the surface tension value of culture medium to 52.2 dyns/cm (Figure.7.). This result was accepted with Cerniglia , (1993) who reported that *Serratia sp* . produces significant amounts of biosufactant which are used for increasing biodegradation rates of hydrocarbons . *A.media* growth caused decreasing in surface tension value to 52.2 dyns/ cm at the fourth week and this value remained without changing during the following period. This result may be due to production of biosurfactant which remained also without change at 48.2 mg/L (Figure.8) during the last three weeks of bacterial growth . This assessment was pointed by Hussien (2006) who explained that *Aeromonas spp.* release a less amounts of biosurfactants , and this result was related to the total viable counts which remained constant in the last two weeks .

F.breve was one of the indigenous bacterial isolates which reduced the surface tension of medium to 61.1 dyns / cm at the final week inspite of the production of biosurfactants which was constant at the last three weeks at 48.2 (mg/L ; Figure .7) , this results may be return to decrease of surface tension value due to another agents , or probably due to the constriction of a new metabolites which was responsible for this reduction (Kazunga and Aitken , 2000; Jouanneau and Meyer, 2006) .

Bodour *et al.* , 2004 reported that *Flavobacterium* produce flavolipid which is considered as a novel class of biosurfactant .

K. oxytoca growth caused reduction in surface tension value to 51.2 dyns/cm at the last two weeks , this result was due to the constant production of biosurfactant which reach 37.8 mg/L at the last two weeks , and these observations were related to the total viable counts which still constant at the same period , this may be explained by the stopping of bacterial growth which affecte physiological activity of bacteria including production of biosurfactants (Bai *et al* 1997;Wasserman and Durian 2006).

On the other hand, the present results accepted with Bastiaens *et al*., (2000) who explained that *Klebseilla* spp. release a small amounts of rhaminolipids (type of biosurfactant) when exposed to hydrocarbon substrate .

On the other hand , Andreoni *et al* . , (2004) pointed to noticeably relationships between the viable numbers of bacterial cells and the amount of biosurfactant produced .

Surface tension value of *A.baumanni* culture medium was reduced from 72.2 dyns / cm to 41.2 dyns / cm at the third and fourth week then this value return to increase again to reach 48.0 dyns / cm (Figure.7) , but the biosurfactant production was constant at 40.1 mg/L at the last three weeks , this result may be attributed to the appearance of a new metabolic products results from the breakdown of an intact hydrocarbons , and this compounds affect surface activity of the liquid and raise it again (Guerin and Boyed , 1992) . These findings were accepted with Rosenberg (1991) who reported that *Acinetobacter sp.* has biological activity with hydrocarbons decomposition . This emulsion further enhances biological activity by increasing water contact , and decreasing surface tension of culture fluids, as well as , the biodegration rate can be related to the oil / water surfaces. In addition, Roony-Varga *et al*., 1999 suggested that potential mechanisms of microbially facilitated desorption in *A.baumanni* culture medium include solubiization of the hydrocarbons via production of biosurfactant , and development of concentration gradients between the solid phase and interfacial hydrocarbons . It has also been suggested that microorganisms capable of attachment exhibit higher rates of degradation of a sorbed substrate (Harms and Zahnder , 1994 ; Wolfardt *et al*., 1998).

The surface tension value of *A.eutrophus* culture medium was decrease gradually to reach 52.3 dyns / cm (Figure .7) as a result of biosurfactant release at 19.6 (mg/L) (Figure.8) which causes emulsification of hydrocarbon substrate , even in a small amounts . The present result was corroborated by previous research being reported by Okpokwasili and James (1995) which indicated that *Alcaligenes sp* . is able to produce biosurfactant but in a small amount leading to limited hydrocarbon biodegradation .But this result was in contrary with Alexander , (2000) ; Atagana , (2004) ; Braddock *et al* . , (1997) who referred to *Alcaligenes sp.* as the most active hydrocarbon-degrading microorganisms .

P.putida was the most crease and oil removal isolate , since it reduced the oil and crease content of culture medium from 54.3 mg/l to 22.5 mg/L at the end of growth period (Figure .9) , but the greater removal was occurred at the first two weeks and this may be due to rapid growth at this period .

Oil and crease contents of petroleum wastewater and sludges are an important consideration in the treatment of the material for ultimate disposal . Oil and crease are singled out for special attention because of their poor solubility in water and their tendency to separate from the aqueous phase . Although its characteristic is advantageous in facilitaiting the separation of oil and crease by use of floatation devices , it does complicate the transportation of the wastes through pipelines , their distraction in the biological treatment unit , and their disposal into receiving waters (Anderoni *et al* , 2000).

Dissolved oxygen uptake was an indicator on the bacterial activity of this isolate in the action of different types of mono-and dioxygenases that involved in hydrocarbon degradation processes (Leahy and Colwell , 1990;Pitter and Chudoba ,1990), besides its requirements for physiological activity of bacterial cell (Riis *et al* .1996.).

The isolates *K.oxytoca* and *A.baumanni* exhibited reduction in dissolved oxygen content at first four week to 12.2 and 11.5 mg/L , respectively , then this value return to increase again to 12.7 mg/L for both isolates (Figure.10) , this result return to weak growth rate of these two isolates during this period . On the other hand , *S.spiritivorum* consumed up to 6.1 mg/L during growth period in spite of continuous aeration of medium by shaking , this observation proved the high activity of this isolate because the initial steps of transformation of hydrocarbons are often catalyzed by extracellular enzymes include a large range of oxidoreductase and hydroxylases which are produced by microorganisms.

These enzymes require oxygen for their function , and it transform the polymeric substrates partially degraded or oxidized oligomers or monomers (Tabatabai and Dick , 2002) .

*F.breve*_was consumed up to 2.2 mg/L of dissolved oxygen at the fourth and fifth weeks of incubation , then the consumption became 2.7 mg/L at the end of incubation . (Figure .10) .

This result can be explained by the decreasing in growth rate which lead to lower the amount of oxygen uptake . this explanation was agreed with (Macnaughton *et al* . , 1999), who reffered to the strong link between microbial activity and oxygen uptake . Also , this result accepted to Ridgway *et al* . , (1990) who reported that *F.breve* consumed 3.6 mg/L of oxygen during it's incubation period.

A.media consumed up to 5.7 mg/L of dissolved oxygen during the third and fourth week , then this value became 5.2 mg/L at the fifth week and 4.5 mg/L at last , this fluctuation in its value return to the cessation of bacterial growth during the last week two weeks.

Bruheim and Eimhjellen (1998) reported that increased oxygen consumption for endogenous respiration at the higher cell concentration could reduce the amount of oxygen available for hydrocarbon degradation , oxygen limitation also could lead to aerotactic movement of bacteria away from the hydrocarbons source , since *A.media* has aerotaxis phenomenon .

S.liquifaciens reduced dissolved oxygen concentration. to 11.5 mg/L at the fifth week , then this content return to increase to 12.8 mg/L . this observation was associated with reduction of cell growth rate at this time and continuous providing of oxygen through aeriation . *A.eutrophus* reduced dissolved oxygen content in gradual rate to 12.0 mg/L at the final week related with gradual increasing of it's cell number during incubation period. This result was agreed with Santas *et al* . ,(1999) who suggested that oxygen limitation can govern biodegradation rates and these can mitigate any positive influence of chemotaxis or other mechanisms of improving hydrocarbon availability .

P.putida exhibited the more hydrocarbon removal from the culture medium when consumed 52.2% of initial hydrocarbon amount followed by *S.spiritivorum* and *S.liquifaciens* which removed 47.5 and 46.3% then *A,media* and *F.breve* which utilized 39.8 and 38.5 %, respectively, followed by *K.oxytoca* , *A.baumanni* and *A.eutropus* which consumed 34.7,32.2 and 24.6 % of hydrocarbon initial amount respectively (Figure 11) .

There are many studies that pointed to *P.putida* as an active hydrocarbon degrader . Simon *et al* (1993) and Kurkela *et al*.,(1988) explained that *Pseudomonas spp*. Posses different degardative genes which are involved in the metabolism of aromatic and aliphatic hydrocarbons .

The alkane hydroxylase of *P.putida* has broad specificity can convert a range alkanes , alkanols , alkenes , and other substrates into interesting product , these enzymes may be participated in *Pseudomonas* efficiency of hydrocarbon biodegradation . Many oxidative enzymes have been the target of directed evolution (Grim and Harwood , 1997) .

Cytocheome P-450_{cam} monooxygenase from *P.putida* has successfully involved to function more efficiently in the hydroxylation of naphthalene and dioxygenases with improved thermostability and substrate specificity have been reported (Haryama *et al* 1999 ; Marx and Aitken , 1999; Holden , *et al* . , 1997) .

Foght *et al*., (1990) postulated that *Pseudomonas* having multidegradative capacity . In addition Bouchez – Naitali *et al* . , (1999) explained that it possess five aerobic hydrocarbon degradation pathways encoded by Tol plasimd which converted aromatic hydrocarbon to benzylachol , benzoate and catechol , which further undergoes metacleavage by an extradiol dioxygenases , or catechol 2, 3 – dioxygenase . In addition , *P.putida* has alkine hydroxylase complexes involved in the degradation of long chain alkanes and a few strains carry genetic information for a third alkans hydroxylase complex , with a monooxygenase which enable these strains to have an extended spectrum of alkane utilization , from C 16 _ C22 alkanes . whilst saturated alkanes with C12- C18 chains or longer are deadly attacked by alrage variety of bacteria .

On the other hand, in an environment rich in particular hydrocarbonic compound , a selective pressure may lead to a quisition and maintenance of plasmid that specifies a corresponding catabolic genes may present in the same cell (Haramaya and Timmis , 1989) .

Recently Story *et al* . , (2003) isolated *P.putida* that is able to thrive in the presence of hydrocarbons . In early studies, it was found that in order to increase membrane rigidity a converting of Cis-9,10-methylene hexadecanoic acid to 9-Cis- hexadecanoic acid and subsequently to the corresponding trans isomer must be done . these changes increase lipid ordering to restore membrane integrity and reduce organic solvent partitioning in the membrane .

In addition , *P.putida* has chemotaxis shown to enhance the degradatioin of hydrocarbon , consistent with theoretical perditions that bacterial chemotaxis towards nutrient sources can increase the rate of nutrient consumption (Venosa *et al*., 1996 ;Tolker *et al*., 2000;) .

April *et al*., (2000) reported that an increase in cell density can lead to an increase in the degradation rate of the attractant near the interface , as well as , motility – enhanced adhesion of bacterial cells can similarly enhance the rate of biodegradation . Others have shown that motility is an important factor in attachment can stimulate bacterial growth and increase rates of biodegration (Harms and Zehander , 1994) .

Chemotaxis clearly is a mechanism by which bacteria can increase the availability of nutrient associated with non aqueous sources such as hydrocarbons (Whyte *et al*., 1997).

Marx and Aitken (2000) explained that motile bacteria such as *Pseudomonas , Sphingomonas , Alcaligenes , Serratia and Aeromonas* are able to control their spatial position with respect to various stimuli such as hydrocarbons by a variety of mechanisms .

The metabolic versatility makes *Pseudomonas* an excellent tool for biodegradation of environmental pollution, due to its hydrocarbon-degrading properties *Pseudomonas sp.* is commonly found in contaminated sites and restoration processes (Chaineau *et al*.,2005; Bent *et al*., 2006).

5.5. Degradation of hydrocarbons by consortium:

It is well known that single species of bacteria are often able to degrade a limit number of hydrocarbonic compounds , but consortium which is composed of many different microbial species is usually involved in hydrocarbon degradation (Dasliva *et al* . , 2004). From this consideration , a consortium of the eight hydrocarbon – degrading isolates was made to study its efficiency for hydrocarbon – degradation , and then comparing their result with that of the imported one .

Jacques *et al*., 2007 considered the consortium capacity to degrade hydrocarbons more active than monoculture because it posses a multiple metabolic capacities and due to cometabolism, i.e : the metabolism by a microorganism of a compounds that cannot use by one species as a nutrient or an energy source. They isolated microbial consortium composed of *Mycobacterium fortuitum , Bacillus cereus, Microbacterium sp., Gordonia polyisoprenivorans*, two *Microbacteriaceae* bacteria , and fungus species *Fusarium oxysporum* , that has the ability to utilize a variety of hydrocarbons including PAHs (pyrene, anthracene, phenanthrene , and naphthalene) , mono aromatic hydrocarbons (benzene, ethylbenzene , toluene , and xylene) in addition to intermediary metabolites of PAHs degradation (catechol , gentisic acid , salicylic acid , and dihydroxy- benzoic acid) for it's growth .

Many previous studies used microbial consortium for hydrocarbon biodegradation , Okoh , (2003) used a bacterial consortium composed of *P.stutezri* , *P.putida* , *P. mullei* , *B.subtilis* and *Alcaligenes sp*. To degrade kerosene , diesel and naphthalene , and reported a significant efficiency of this consortium .

Another investigation accomplished by Nwachukwu and Ugoji (1995) using a bacterial consortium composed of *P.putida* , *P.aeruginosa* and *Sphingomonas* to remove benzene , toluene , methylbenzene and p-xylene from oil – contaminated environments .

Optical density (O.D600) for both isolated and imported consortium was measured instead of total viable count (TVC) because the imported consortium composed of several types of microorganisms which require many types of counting techniques which may affect the overall results .

Optical demsity (D600) of isolated consortium was 1.1nm at the end of incubation period , while imported consortium gave 1.8 nm (Figure .20). Determining cellular growth by measuring a consortium composed of different types of microorganism , with low densities make it difficult sometimes , however , in this case of hydrocarbon – grown cells , reproducible results obtained by covering the cuvette with parafilm and inverting it five times , and then immediately determining the O.D600 (White *et al*., 1999).

Analysis of pH changes with growth of isolated consortium revealed a significant reduction in pH (Figure.21) at the early weeks of incubation. A rapid decreasing in pH occured at the third week compared with 7.2 of control sample , that corresponded with the onset of decrease in bacterial population , probably due to the accumulation of acidic metabolites . After the third week no drastic reduction in pH was observed . On the other hand , the imported consortium deceased the pH value gradually to achieve 2.2 at the last week of growth .

The differences between the two types of consortium may returned to the composition of each one. The first composed of unique type of microorganism which was bacteria , and its known that bacteria usually prefer the neutral or around neutral pH and as there is more decrease in pH there is a bad effect and more stress on the cells (Jacob , 1996) .

While the imported consortium composed of different types of microorganisms including fungi and yeast which prefer the low pH value , when the growth of bacteria lead to a dropping in pH, fungi and yeast can tolerate this lowering of pH and continue to grow leading for more viable mass and more degradation (Oboh , 2006).

A decreasing of medium pH give another indicator for hydrocarbon degradation due to breakdown of PAHs by microbial mono- and dioxyganases. This degradation continues to form dihydroxy-PAH which are subject to ring cleavage through different fission pathways , resulting in the formation of organic acids (e.g. succinic , pyruvic , fumaric or acetic acids), and the further co-metabolism ,finally leads to intermediates of the tricarboxylic acid cycle and these acids which are mainly responsible for reduction of culture medium pH value .(Wilson and Jones , 2002).

Biosurfactant production of both types of consortium was measured by indirect method , and the determination of it's concentration in culture medium was 285 mg\ L and 475 mg\L for isolated and imported consortiums, respectively , (Figure .23). These surface active agents caused decreasing in surface tension of the medium depending on the concentration of these agents , so the values of surface tension of isolated and imported consortium where 41.8 and 22.3 dyns / cm ,respectively , (Figure.22). Although biosurfactant concentration was increased continuously , surface tension values were decreased in less rate , this finding may be due to the biosurfactant concentration that reach critical micelles concentration (CMC) at which the increasing of biosurfactant concentration not affect the surface tension values (Jolivalt *et al* .,1990).

Biosurfactant of the imported consortium is more than that of the isolated one due to the release of surface active compounds from different types of Fungi , Yeast and Actinomycetes which reflect its composition , (Aronstien and Alexander , 1992 ; Krivobok *et al*. , 1998 ; Rajas *et al* , 1999) showed that *Trichoderma* , *Aspergillus* , *Pencillium* , *Rhizopus* and *Rhodotorula* species were able to release surface active agents , in addition *to Candida* and *Stroptomycetes* .

Biosurfactant may interact with LPS in two ways . the first is the direct of removal of LPS by solubilization .Clearly , biosurfactant has detergency properties , as demonstrated by it's ability to enhance hydrocarbon solubility (Zang and Miller , 1992 ; Sabra *et al* . , 2003)

The second is that biosurfactant causes the indirect removal LPS through complexation of Mg^{+2} in the outer membrane . Magnesium is a metal that is crucial for maintaining strong LPS-LPS interactions in the outer membrane .Its removal results in the destabilization of LPS-LPS interaction and loss of LPS from the membrane . This mechanism is supported by fact that biosurfactant such as rhamnolipid (release from several bacterial species such as *Pseudomonas spp.*) has been shown to effectively complex divalent metal cations as magnesium . Even the minimal LPS release can lead to a development of high adherence to hydrocarbons , this resulted in enhanced biodegradation of the hydrophobic substrate (Herman *et al* , 1995 ; Caiazza *et al*. 2005)

On the other hand, microorganisms can use other strategies to increase the hydrocarbon availability in the aqueous solution , such as formation of biofilms at the surface of the hydrocarbon crystals (Mairer , 2003 ; Johensen and Karlson , 2004) . One Mechanism to promote the availability of hydrocarbons through the microbial biofilm is by production of biotechnological bioremediation process , enhance nutrient transport across membranes , act in host – microbe interactions and provide biocidal and fungicidal protection to the producing organisms (Jennings and Tanner, 2000).

Oxygen supply is important for best growth and rapid degradation rates of hydrocarbon especially with aerobic HC- degrading microorganisms such as strict aerobic bacteria and fungi (Sepic *et al.* , 1996).

Both types of consortiums caused decreasing of dissolved oxygen concentration , in spite of continuous aetiation of culture media , isolated consortium was consumed 7.9 mg/L at the fifth week , then this value decreased to 6.8mg/L, this result maybe due to the rapid growth at the first period of incubation , then the oxygen consumption decreased and this reflect the low activity of the cells (Eriksson *et al* . , 2003). Whereas imported consortium consumed 8.7 mg/L during growth period (Figure .24) this value is quite high and reflect the high activity of cells .

The use of aeration was so important to get a good degradation rates , as Rhykerd *et al*., (1999) who suggested that a good aeration lead to increasing in predominant bacterial cells , in the addition of increasing in rates of hydrocarbons loss to reach 82% after 12 weeks in an aeriated culture compared with 33% of unaeriated culture .

There is appreciable amounts of crease and oil were removed by the action of isolated consortium during growth period (Figure .25) . So it removed 31.9 mg/L at the end of incubation , while imported consortium removed 42.4 mg/L which measured by weight method using chloroform as an organic solvent which have the ability to dissolved not only oil and crease but also other organic substances , however no known solvent will dissolve selectively only oil and crease (Fava *et al* ,.1995).

Oil discharged into the environment typically has deleterious effects . Oily wastes discharge may have objectionable odors , cause undesirable appearance , burn on the surface of receiving water creating potential safety hazards and consume dissolved oxygen necessary to forms of life in water , and it cause turbidity and film , and make filteration treatment difficult .Bioassay data indicate that oil is toxic to fish . In greater quantities , it limits oxygen transfer , hindering biological activity (Desai and Bannat ,1997; Delille *et al* ., 2004).

The existence of pure populations of microorganism is more or less a laboratory phenomenon . In nature most bacteria do not exist as pure cultures , and significant proportions of all microorganism coexist together . It is well known that bacteria interact in the environment and each bacterial species has its own role in the ecosystem (Alexander , 1994). The combined action of several species present in bacterial communities enhances or is required for complete mineralization of complex compounds such as hydrocarbons (Rozgaj and Glancer , 1992).

The Biodegradation of PAHs by mixed cultures has been subject of numerous investigations , often based on the natural microflora of contaminated soils . These cultures frequently have significant degradative capabilities because the single isolates can complement one another due to their physiological properties , with some members of the culture providing degradative enzymes whereas others supply surfactants or growth factors (Marx and Aitken, 2000). Studies have shown that to enhance the rate of biodegradation of toxic compounds , (including hydrocarbons) in the environment , different strategies can be considered such as , chemotaxics , although chemotaxis is a phenomenon that has been known for sometimes , it is a complex process in which bacterial cells detect temporal changes in the concentration of the chemical stimuli. Some toxic organic compounds are chemoattractants for different bacterial species , which could lead to improved degradation (Ozaki *et al* ., 1991; jerabkova *et al* ., 1997 ;Witt *et al* , 1999).

Since the main purpose of the present study is to achieve and maintain a high rate of hydrocarbon biodegradation using a controlled mixed bacterial community , so the hydrocarbon loss percentage was measured , and it appeared to be 61.1 % for the isolated consortium and 83.3% for the imported consortium (Figure . 26) . The differences of two consortium efficiency return to the composition of each and as the isolated consortium composed of unique microbial type which is bacterial species only , the amount of HC loss which was expressed as a percentage (61.1%) may be regarded as a good positive result in comparison to that obtained by the imported consortium as it is composed of different microbial spp.(fungi , protozoo bacteria ; Figures 30 -35). Jimenez *et al*. , 1991 used both mixed culture and complex or mixed substrate and reported that most members of mixed culture have a greater potential to degrade a variety of substrates and trigger it's full potential.The mixed culture and mixed substrates will result in a different physiology and biochemistry than in pure culture and pure substrate . In each of these degrading bacteria inducible systems that are under the right conditions will be triggered to degrade the largest substrate . The microbial degradation of PAH dependent on their physical and chemical properties , concentration and rates of diffusion as well as their bioavailability (Smith *et al* . , 1997) . PAH . have low water solubility , a property which in part is causing their persistance in the environment (Cerniglia , 1992 ; Shittleworth and Cerigilia , 1996) . In addition , water solubility of PAHs decrease with an increase in molecular weight , and therefore , the rate of PAH biodegradation is usually inversely proportional to the number of fused benzene rings in the PAH- molecules (Wilson and Jones , 1993 ; Pothuluri and Cerniglia , 1994). There are many investigations referring to the role of different types of microorganisms in hydrocarbon degradation , white rot fungus , use cytochroma P-450 monooxygenas to oxidize PAH to arene oxides . The high molecular weight (HMW PAH) is also substituted by the lignolytic enzyme of fungi (Gain and Rao , 2004).

Another fungus *Cladophialophora* sp.was capable of growth on a model water – soluble fraction of gasoline that contained all six BTEX components (benzene , toluene , ethylbenzene , and the xylene isomers). Benzene was not metabolized , but the alkylated benzenes (toluene , ethylbenzene , and xylenes) were degraded by a combination of assimilation and co- metabolism , in co-metabolism process , the microorganism are not able to use the organic compounds as an energy source for growth (Alexander , 1999) . *Cladophilophora* was used toluene and ethylbenzene as a sources of carbon and energy , whereas the xylenes were co-metabolized to different extents , o-xylene and p-xylen were converted to phthalates as end metabolites : p-xylene was not degraded , but in combination with toluene , appeared to be mineralized . the metabolic profiles and the inhibitory nature of the substrate interactions indicates that toluene , ethylbenzene , and xylene were degraded at the side chain by the same monooxygenase enzyme. This findings suggest that soil fungi could contribute significantly to bioremediation of BTEX pollution (prenafeta-Boldu *et al.* , 2002).

Bongan and lamar (1996) explained the capability of *phanerochaete levis* for degrading a wide range of PAHs such as athracene . On the other hand , Field *et al.* , (1992) studied the ability of *Phanerochaete chrysosporium* to degrade four types of PAHs (naphthalene , fluoranthene , chrysene and benzo [a] pyrene) .

There are some investigations referred to the role of protozoa in degrading hydrocarbons in oil polluted environments.

Hee-Shin and Woong-kim (2001) pointed to the applicability of an earthworm bioassay as a technique for monitoring the HC-contaminated treatment . They found that *Eisenia foetia* have appreciable efficiency to degrades PAH , and it has promising ability for removing of such contaminations .

Shippers *et al* ., (2005) pointed to some yeast especially *Sacharomyces cerevisiae* that use cytochrome P450s in initial oxidation of alkanes and aromatics of petroleum .

5. 6. High performance liquid chromatography analysis :

There are many techniques used for analysing of chemical components of hydrocarbon mixture. But HPLC spectrum was chosen for several reasons . HPLC is a term that describes technique used to spectral components of mixtures . In this technique, a liquid is used to carry a mixture a cross a bed of material and because the liquid moves it is called the mobile phase . The bed of material on the other had , is called the stationary phase because it doesn't move. As the mobile phase carries the mixture across the stationary phase , some of the component of the mixture ((stick)) to the stationary phase , and exist the stationary phase at different times . The component of the mixture have been separated .

HPLC composed of a solvent reservoir to hold the liquid mobile phase , and injector to allow injection of small volume of the sample mixture under high pressure , column , and a mean to record the detector signals (Al- calde *et al.,* 2002) .

Since it's introduction in the late 1960s , HPLC has became one of the most widely used separation techniques . The ability to choose among a wide array of stationary phase allows one to apply HPLC to an enormous variety of complex mixture from blood serum to hydrocarbon mixture .

The identification of using hydrocarbon mixture before and after treatment by previously mentioned eight isolates separately , compared to control sample was chosen to obtain a further confirmed investigation for hydrocarbon-degrading isolates efficiency .

In addition , all of the isolates were collected together to form bacterial consortium which was grown again on mineral salts medium contains 2 % of hydrocarbon mixture . The HPLC spectrum showed a clear profile about HC mixture before treatment (Figure12 and Table4) .

The different bacterial isolates were exhibited different patterns in consumption the preferred hydrocarbonic compounds due to variation in genetic and physiological properties .Actually all of the eight isolates were exhibited an ability to degrade all the mixture components but not in equal percentages , except *A.media* and *A.eutrophus* which showed an increasing in C10 and *K.oxytoca* in C8 compounds .

P.putida consumed all of C6 , C7 , C8 components but it consumed C18-C22 in a less efficiency (Table .5 and Figure 13) . This result was not surprising , since there are many investigations pointed to diverse capabilities of *P.putida* to degrade a large number of hydrocarbons .

Gaylardes *et al* . , 1999 referred to the capability of *P*. *putida* to degrade both aliphatic and aromatic compounds since it possess *alk* (which degrade C5-C12 of nalkane) , *nah* (naphthalene) and *xyl* (toluene) pathways that generally located on a large catabolic plasmids , and they have been extensively investigated and characterized such as OCT ,NAH and TOL plasmids .

Oteno *et al* . , 2005 showed that *P.putida* can readily consume benzene (C6) , Toluene (C7) and xylene (C8) , and this result corroborated our findings that this isolate consumed 100% of these three low molecular weight aromatic hydrocarbons .

Ridgway *et al* ., 1990 was screened 297 bacterial isolates from soil revealed their ability to degrade hydrocarbons constituent of gasoline , and the predominant species of these was *P. putida* .

P.putida in the present study was consumed 76.3% of C16 compound , this result agreed with Bastianes *et al*. , (2000) who reported the transformation of pyrene (C16) by this species during growth , and it leads to the formation of significant amount of a ((dead end)) metabolites such as PYRdHD (cis-4, 5-dihydro-4, 5-dihydroxy pyrene) which has been identified as an intermediate in the degradation of pyrene by a species that can convert PYRHD to PYRQ (pyrene -4,5- dione , but it give other non mineral products which couldn't further degraded by the known pyrene degradation pathways such as phenanthrene – carboxylic acid (*Cerniglia* , 1992) .

*P.putida*_consumed 80.7% of C10 compound in the current study this result agreed with AL-Taher , (2002) , who reported that this species utilized 99.5% of C10 compounds in oil emulsions treated with this bacteria and he explained that the low molecular weight aromatic hydrocarbons has a high solubility properties and can be served as a carbon and energy source .

S.spiritivorum . exhibited a significant ability to degrade 100 % of C6 and C7 compounds , while it consumed 29.8, 27.7 and 14.4% of C18 , C20 and C22 respectively (Table 6 and Figure 14)

Van Hamme *et al* ., (2003) pointed to the genes responsible for catabolic pathway of naphthalene , phenantherene , anthracene , biphenyl , toluene and m-and *p*-xylene by this bacteria , were *nah* , *bph* , and *xyl* genes clustering which may be typical to *Sphingomonas spp.,*capability of degrading aromatic compounds .Also Grund *et al*.,(1992) explained PNL1 plasmid of this species which is responsible for degrading toluene , xylene , salicylate , biphenyl , diabenzothiophene , flourene , and benzoate . In this plasmid, at least 13 gene clusters are predicted to encode all the necessary enzymes . Moreover, oxygenases with component spread over six gene clusters have been studied (Selifnov *et al* , 1996)

Beyond the genes known to participate directly in PAH metabolism , genes that may provide important support functions are being exist in some species of *Sphingomonas* which was able to use fluoranthene , naphthalene and phenanthrene as a sole carbon and energy source (Zylstra *et al* ., 1997).

Many investigations referred to catabolic activity of *Sphingomonas* , *spp*. for transformation of (HMW PAH) including flouranthene , benz [a] anthracene and benz [a] pyrene , although , it can grow in the presence of these compounds , but it cannot use them as a sole carbon and energy source , so it may subjected these compounds to co-metabolism transformation (Zhou *et al* . , 1996 ; Leys *et al* , 2004).

Rooney _ Varga *et al* . , 1999 studied the HC- degrading ability of *Sphingomonas sp.* and they reported that it has the ability to degrade benzene , toluene , ethylbenzene and p-xylene rapidly under aerobic conditions , but when grown on phenanthrene (C14) in the presence of pyrene (C16) it will accumulate a significant amount PYRdHD and PYRQ as a non mineralized metabolite .

HMW PAH (consisting of four or more benzene rings) are usually co-metabolically oxidized by a restricted number of bacterial species such as *Sphingomonas spp*. (ceriglia , 1994 ; Samantha *et al* . , 2002 ; Bodour *et al* . , 2003) . Moreover, *Sphingonmonas sp* . has an ability to degrade benzo[a] pyrene , chrysene and benzo [a] anthracene (Hamamura *et al* . , 2006)

Although, *S . liquifaciens* was consumed 100% of C6 and C7 compounds , but it show a less efficiency to degrade C12 and C16 when consumed 75 and 64.5 % of these , respectively (Table.7. and Figure.15).

There are many studies pointed to the capability of *Serratia spp.* to utilize benzene (C6) and toluene (C7) as a sole carbon and energy source in a high efficiency, but this efficiency decreased proportionally with the increase of ring number of polyaromatic hydrocarbons (Mcclay *et al* , 1995 ; Tolker *et al* . , 2000) .

This isolate consumed phenanthrene (C14) at 71.8% but consumption of phenanthrene is affected by the accumulation of PYRdHD which could competitively inhibit a dihydrodiol dehydrogenase utilized in the degradation of phenanthrene (Samanta *et al* . , 1999)

Alternatively , the accumulation of PYRQ in excess concentrations would inhibit the degradation of phenanthrene , also .A possible explanation is its potential to mediate redox reactions that could after the balance of vital redox cofactor , as has been observed for other PAH-O–quinines (Abed *et al*., 2002) .

The genus *Serratia* was studied by AL-Taher (2002) who confirmed the HC-degradation efficiency of it to consume PAH .

Some bacteria , however , are capable of transforming one or more PAHs despite an ability to grow on or mineralize the PAH in question. I t is important to evaluate the products of such incomplete PAH metabolism because of their potential effects on PAH-degrading microorganism or on potentially exposed human populations. Identification of common products of incomplete metabolism can also be important in assessing the extent to which natural attention may be occurring at contaminated sites (Kanzunga and Michael , 2000; Beller *et al*.2002 ; Dasilva and Alivarez , 2002) .

A.media showed a good capability to utilize C6 and C7 compounds when consumed 100 % of both of these , but it couldn't consume C10 when it show an increasing in its value about 12.3% (Figure . 16 and Table .8) , this may be due to breakdown of high molecular weight PAHs to a less molecular weight compounds that eluted at the same retention time.

It is well known that the microorganism which was able to degrade benzene would be able to degrade toluene to (Kasai *et al* .,2001; Kim *et al*.,2005) .

Knowledge regarding bacterial biodegradation of HMW PAHs has been advanced in the last decades a number of HMW PAH – degrading strains have been isolated and characterized . During the same period , the number of HMW PAH compounds know to be degraded by these isolates and consortia has also increased and pathways for the degradation of these compounds by some strains have been elucidated . (Van Hamme *et al* 2003) .

The metabolism of toluene and m-and *p*-xylenes is known in *A.media* . Tolune and m , *p*-xylenes are , respectively , metabolized with XylMA by benzyl alcohol and m, p-toluates via their corresponding aldehydes before being subjected to meta cleavage . On the other hand , o-xylene has not accepted as a substrate for xylMA (Zylstra *et al* . , 1997).

o-xylenes is oxidatively degraded by way of pathways that fall into the three classes , produce their corresponding catechol , which are then subjected to aromatic ring cleavage . The first pathway is one initiated by a ring- hydroxylating dioxygenase

Complementation studies on *Aeromonas spp.* revealed the deficiency of naphthalene – inducible dioxygenase gene cluster , this result may explained the inability of naphthalene degradation by *A.media* (Wubbolt *et al*. , 1994) .

K.oxytoca and *A.eutrophus* degrade all hydrocarbon mixture components in different values , except C8 and C10 compounds which increased , to give an increasing percentage of 6.6% in *K.oxytoca* , and 30.7% in *A.eutrophu*, (Figures . 18, 20 and Tables 10 , 12 , respectively, the inability of *K.oxytoca* to degrade naphthalene probably attributed to a mutation process occurring in one or two alleles of *nah* gene that responsible for encoding enzymes catalyzing naphthalene biodegradation (Jeon *et al* . , 2003)

K.oxytoca and *A.eutrophus* consumed C14 compound at 72.4 and 46.3 % , respectively . Cerniglia and Heitkamp , (1990) suggested that *Klebseilla* and *Alcaligenes spp*. can degrade C14 aromatic hydrocarbon such as phenathrene by two pathways where the initial enzyme attack is 1,2 - or in the 3,4- positions of the molecule , and the major isomer formed is cis – 3,4 – dihydroxy – 3,4 dihydrophenanthrene, which is subsequently oxidized to 3,4- dihydroxy phenantherene and further cleaved and converted to 1- hydroxyl-2- naphthoic acid . The product of ring cleavage is then oxidatively decarboxylated to 1,2 dihydroxy naphthalene , metabolize further via the naphthalene pathway to catechol and finally to the intermediates of the tricarboxylic acid cycle .

Most of the isolates were poorly degrade HMW PAH components especially *K.oxytoca* which consumed 3.4% of C22 compounds , this finding was expected since the number of bacterial types that able to degrade hydrocarbons decrease with the increasing in compound complexity and *P.putia* and relatively *Sphingomonas spp*. considered as the famous bacterial species in this regards in addition to some others (Lonon , 1999).

F.berve and *A. baumanni* were degraded all the hydrocarbon mixture but in low percentage , excepts C10 compound which was degraded completely by *A.baumanni* (Figure . 19 and Table 11) . many investigations described *Acinetobacter spp.* as a petroleum hydrocarbon – degrading species and it can readily isolated by traditional culturing and isolation techniques like viable plate count from contaminated sites (Wilson and jones , 1993 ; Mcllister *et al* . , 1996 , White *et al* ., 1996).

A.baumanni is an important component of the hydrocarbon contaminated environments , and it is capable to possess a wide rage of catabolic activities and also has a different physiological and genetic properties that lead to differentiation in HC-degradation mechanisms (Badzinski *et al*. , 1998 ;Wang and Fingas , 2001).

F.breve is one of the hydrocarbon – degrading bacteria and it classified as chemo-organotroph microorganism . The presence of multiple alkane hydrixylase genes, in some strains of *Flavobacterium sp.* In addition to two distinct monooxygenases , a Cu-containing monoxygense and and integral – membrane , binuclear – iron monooxygenase similar to that of *P.putia* (Carmichael and pfaender, 1997) .

F. breve in the present study degraded all hydrocarbon mixture components , but the less degraded compounds were C12 (69.3 %) , C20 (20.8 %) and C22 (18.9%) (Table . 9 and Figure 17) , since it has weak ability to utilize PAHs consisting of four or more benzene rings , and it can transformed but not utilize C12 compounds due to low bioavailability of these compounds (Aitken *et al* . 1998).

Since , the goal of this study is to obtained an efficient bacterial isolates capable to degrade a high number of petroleum hydrocarbon components , in order to be a part of biological treatment used for diminish the huge risks of these compounds by either completely degrading to carbon dioxide and water , or modify the hydrocarbons only partially , leaving .it's carbon skeleton more or less intact , that mean , it may result in detoxification of present compounds generate new molecule(s). Therefore , complete mineralization of hydrocarbons is more desirable outcome rather than disappearance of the original compound from the wastewater .

So , in order to get this promising step , a consortium comprise of the eight Gram negative hydrocarbon- degrading bacterial species that isolated in this study , was made , and to obtain an overall view about its hydrocarbon-degrading efficiency , a spectrum of HPLC analyses , was carried out .

HPLC spectral data showed that isolated bacterial consortium was consumed all the C6 , C7 , C8 and C10 compounds present in wastewater, in addition to 92.3% and 86.6% of C14 and C16 compounds , respectively , but it appeared less in consuming of C20 (55.8%) and C22 (40%) (Figure .28 and Table .13)

High efficiency of the isolated consortium to utilize low molecular weight (three benzene ring or lesser) is not surprising since the combined action of several species present in bacterial communities enhances complete oxidation of hydrocarbons compounds (Muller *et al* . 1989 ; Rozgaj and Olijan , 1992).

The new isolated consortium was compared to imported one which was in use now in biological treatment of local Oil refineries .

As it expected , the imported consortium showed more efficiency in hydrocarbon degradation than ours , this finding return to the multiple-type species of microorganism which compose it , since it consist of bacteria , fungi , yeast , protozoa , beside some Actinomycetes and may be algae , as shown in (Figure s 30-35) .

The fungus species *Trametes Verisocolor_* showed significant levels of oxidation with acenaohthene (100%) , anthracene (100%) , benzo [a]pyrene (94%) fluorine (85 %) and benzo[b] anthracene (60%).

On the other hand , *Myceliophora* achieved a high oxidation yield only with anthracene (70 %) .

The metabolism of anthracene in these fungi was accomplished by laccase enzyme which was accompanied by the appearance of its oxidation product in HPLC chromatogram . This product was identified as a 9 ,10 - anthraquion , based on its spectrum and retention time compared to those of the 9 , 10- anthraquinon standard , and this formation represent the amount of anthracene lost (Alcaled *et al* . , 2002 ; Piepl *et al*.,2004;Vinas *et al*.,2005).

Another fungal species exhibited the ability to degrade PAHs was *Phanerochaete chrysosporium* which has a distinct capability to utilize benzene , toluene , pyrene , phenanthrene and benzo [a] pyrene (Field *et al*. ,1992 ;Sannell *et al* .,1996; Venosa *et al*.,1997)

The ability of fungal populations to degrade hydrocarbons is well documented Mulder *et al*.,1998;Wang *et al*.,1999 Baldrin *et al*. , 2000).

Coclusions

1- All the bacterial isolates in this study that showed a high ability to degrade hydrocarbons were gram negative .

2- *P.putida* is the most efficient hydrocarbon degrader that consumed 52.2% of HC among the isolates, while the less efficient is *A. eutrophus* that consumed 24.6%.

3- HPLC spectral data illustrated ability of bacterial isolates to consume aromatic hydrocarbons .

4- Isolated bacterial consortium has a good efficiency for hydrocarbon degradation represented about 73% of the imported consortium efficiency .

Recommendations

1- Screening for other types of microorganisms that have hydrocarbon- degradation ability such as fungi , algae , actinomycetes , cyanobacteria and protozoa

2- Preparation of a microbial consortium mimic to that imported one to be use din the biological treatment of petroleum contaminated sites, in addition to genetic studies to improve increase potency of the isolated strains for hydrocarbon degradation .

3- In addition to carbon & energy sources , other mineral nutrients such as nitrogen, sulfur and phosphorus which may limit microbial growth and activity , may be studied to determine their role in biodegradation .

4- Petroleum – containing wastes generally fall into the hazardous – wastes category as identified and limited by the (EPA) , but the potential health risk is difficult to asses and evaluate so it is a good to concentrate some efforts on health aspects and potential risks .

References

- Abed , R . M . M ., N . M . D . Safi , J . koster , D . Beer , Y . EL – Nahhal , J . Rullkotter F. Garcia . 2002 . Microbial diversity of a heavily polluted microbail degradation of petroleum compounds . Appl. Environment Microbail . 68 : 1674 1683.

- Abed , R . M . M ., N . M . D . Safi , J . koster , D . Beer , Y . EL – Nahhal , J . Rullkotter F. Garcia . 2002 . Microbial diversity of a heavily polluted microbail degradation of petroleum compounds . Appl. Environment Microbail . 68 : 1674-1683.

- Aislabie, J., M. McLeod, and Farser. 1998. Potentential for biodegradation of hydrocarbon in soil from the Ross Dependency. Antarctica. Appl. Microbiol Biotechnol. 49: 210 214.
- Aitken , M.D., W.T. Stringfellow , R.D. Nagel , C. Kazunga , and S.H. Chen .1998 . Characterization of phenanthrene – degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons . Can. J. Microbiol.44 : 743 – 752
- AL Tahhan , R.A.,T.R. Sandrin , A.A. Batour and R.M. Maier. 2000. Rhamnolipid induced removal of lipopolysaccharide from *pseudomonas aeruginosa* : Effect on cell surface properties and intraction with hydrophobic substrate . Appl. Environ. Microbiol. 66 (8) : 3262 3268.
- Al Shaieb, Z., J. Cairns and Puckette. 1994. Hydrocarbon induced diagenetic aureols: Indicators of deeper leaky reservoirs. Association of petroleum Geochemical Exploration. 10 : 24 48.
- AL Taher , W.A.Z.H. 2002 . Biodeterioration of oil emulsions and it's control by some chemical agents . ph.D thesis . Babylon university . Bablon – iraq.

- AL- Tahhan , R.A.,T.R. Sandrin , A.A. Batour and R.M. Maier. 2000. Rhamnolipid-induced removal of lipopolysaccharide from *pseudomonas aeruginosa* : Effect on cell surface properties and intraction with hydrophobic substrate . Appl. Environ. Microbiol. 66 (8) : 3262- 3268.
- Alcalde, M., Bulter, T., Arnold, F. H. 2002. Colorimetric assays for biodegradation polyeyclic aromatic hydrocarbons by Fungal laccases . J. Biomol. Screen. 7: 547 553.
- Alexander , M . 2000 Aging , bioavailability and overestimation of risk from environmental pollutants . Environ . Sci . Technol . 34 : 4259 4265 .
- Alexander , M . 2000 Aging , bioavailability and overestimation of risk from environmental pollutants . Environ . Sci . Technol . 34 : 4259-4265 .
- Alexander , M.1994 . Biodegradation and Bioremediation .Appl. Microbial. Biotechnol . 37 :510 517.
- Alexander M . 1999 . Biodegradation and bioremediation . Microbiol . Rev.59:143 169.
- Alexander, M. 1982. Most probable number method for microbial populations. In: Methods of soil analysis. Part2. 2nd ed. Edited by A. L. Page. American society for Agronomy and soil science society of America, Madison, wis pp : 815 820.
- Allan , G.B. 1998 . Elementary statistics . A step by step approach .Mc. Graw –Hill .3rd . ed . Copyright.
- Alloway .J . P . and R . K . Ayres . 1993 . Bioremediation of petroleum hydrocarbons in soil . Adv . Agron . 72 : 53 105.
- Alloway .J . P . and R . K . Ayres . 1993 . Bioremediation of petroleum hydrocarbons in soil . Adv . Agron . 72 : 53-105.

- AL-Taher , W.A.Z.H. 2002 . Biodeterioration of oil emulsions and it's control by some chemical agents . ph.D thesis . Babylon university . Bablon – iraq.
- American public Health Association (APHA). 1992. Standard Methods for the examination of water and waste water. 18th ed. Washington, D. C. P.: 1268 1270.
- Andereoni , V.,S. Bernasconi , M.Colmbo , J.B.Vanbeilem and L.Cavalca . 2000 . Removal of oil and crease from industrial effluents .Appl.Environ.Microbiol . 63:151 154.
- Andreoni , V ., L . Cavalca , A . Rao and G . Nocerino . 2004 . Bacterial communities and enzyme activities of PAHs polluted sites . Chemosphere 57 : 401 412.
- Andreoni , V ., L . Cavalca , A . Rao and G . Nocerino . 2004 . Bacterial communities and enzyme activities of PAHs polluted sites . Chemosphere 57 : 401-412.
- Anton , J.A,P. Adam & J. cannon . 2000 . Molecular evidence for biodegradation of macromolecule . Geochem. Cosmochem . Acta . 64 (20) 3525 – 3537.

- Anton , J.A,P. Adam & J. cannon . 2000 . Molecular evidence for biodegradation of macromolecule . Geochem. Cosmochem . Acta . 64 (20) 3525 – 3537.

- April, T. M., Foght, J. M., Currah, R. S. 2000. Hydrocarbon degrading Filamentous fungi isolated from flare pit soils in northern and western Canada. Can. J. Microb. 46(1): 38 49.

Ara Rojas , S.L.2004 . BTEX contaminated aquifer supports -
a diverse microbial community. Microbiol. Ecol . 48 : 589 –
600 .

- Aronstein , B.N. , and M . Alexander . 1992 . Surfactants at low concentrations stimulate biodegradation of sorbed hydrocarbons in samples of aquifer sands and soil slurries . Environ . Toilcol.Chem . 11 : 1227 – 1233 .

- Asia, I. O. 2000. Studies on industrial sludge treatment options. Ph. D. thesis . University of Benin. Benin.
- Atagana , H.I.2004. Bioremediation of creosote – contaminated soil in south Africa by landfarming . J .Appl. Microbiol.96:510 520 .
- Atlas , R.M, and R.Bartha . 1998 .Foundmentals and application of hydrocarbon biodegradation . Biormemd . J. 3 (2) : 81.84.
- Atlas , R.M.1991 . Bioremediation of fossil fuel contaminated soils . Applications and investigatiuons for hydrocarbon and contaminated site remedation current opinion in biotechnology 2 : 440 – 444.
- Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbon. Microbiol. Rev. 45: 180 209.
- Atlas, R. M. 1984 Pathways of hydrocarbon degradation. In petroleum Microbiology. Masmillan Publishing company,3ed ed. New York, USA, pp.1 15.
- Atlas, R. M. and Bartha R. 1998. Fundamentals and applications . In: Microbial Ecology 4th edition. Benjamin/ Cummings publishing company, Inc. California, USA, pp.523 530.
- Atlas, R. M. and Barther , R. 1973. Simulated biodegradation of oil slicks using oleophilic tertilisers. Environ. Sci. Tech. 7: 538 541.

Bai , G.Y. , M.L. Brussean and R.M. Miller - .1997.Biosurfactant – enhanced removal of residual hydrocarbons from soil.J. Contam. Hydrol. 25. 157 170

- Baldrin , P. , C. Wiesche , J. Gabriel, F. Nerud , and F. Zadrazil . 2000. Influance of cadmium and mercury on activity of lingninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus* *ostreatw* in soil .Appl. Environ. Microbiol . 66:2471 2478.

- Balkwill , D . L . , G. R. Draker , R . H . Reeves and C. M. Spadoni . 1997 . Taxonomic study of aromatic – degrading bacteria *Sphingomonas* species . Int . Syst . Bacteriol . 47 : 191 201 .

- Balkwill , D . L . , G. R. Draker , R . H . Reeves and C. M. Spadoni . 1997 . Taxonomic study of aromatic – degrading bacteria *Sphingomonas* species . Int . Syst . Bacteriol . 47 : 191-201 .

- Banat, I. M., R. S. Makkar, and S. S. caneotra. 2000. Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol. 53: 495 508.
- Bartha , H . J . 2003 . The influence of cyanobactenia on oil polluted intertidal soils at the saudi Arabian gulf shores . Mar . pollut . Bull . 46 : 1245 – 1252 .

- Bartha , H . J . 2003 . The influence of cyanobactenia on oil polluted intertidal soils at the saudi Arabian gulf shores . Mar . pollut . Bull . 46 : 1245 – 1252 .

- Bartha R. 1986a. Microbial ecology fundamentale and applications. Microbi Ecol. 12: 155 72.
- Bartha, R. 1986b. Biotechnology of petroleum pollutant biodegradtion. Microb. Ecol. 12: 90 98.
- Bastiaens , L., D. Springael , P. Wattiau , H. Harms , H. verachtert , and L. Diels . 2000 . Isolation of Adherent polycyclic aromatic hydrocarbon (PAH) degrading bacteria using PAH sorbing carriers . Appl . Environ . Microbiol. 66 : 1834 1843 .

- Battistel, E., Luisi, P. L., and Rialdi, G. 1988. protein inactivations during Novel bioseparation techniques. J. phys. Chem., 92: 6680 6689.
- Beller , H . R ., S . Kane and T. Legler . 2002. Areal time PCR method for monitoring hydrocarbon degrading bacteria based on catabolic gene . Environ . Sci .Technol . 36 : 3977 – 3984 .

- Beller , H . R ., S . Kane and T. Legler . 2002. Areal time PCR method for monitoring hydrocarbon degrading bacteria based on catabolic gene . Environ . Sci .Technol . 36 : 3977 – 3984 .

- Beller, H. R., and E. A. Edwards. 2000. Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. Appl. Environ. Microbiol. 66: 5503 5505.
- Benson, H. J. 1998. Microbiological application. Laboratory manual in general microbiology. 7th ed. MaGraw Hill Inc. New York.
- Bent , F.M.A.O.Flavio , C.Benedict , C.Okeke and W.T.Frankeberger . (2006) . Comparative bioremediation of of soils contaminate with diesel oil by natural attenuation , biostimulution and bioaugmentation . Microbiol . Res. 160(3) : 249 255.

benzene degradation in a petroleum – contaminated sites -
.Appl. Environ. Microbiol . 65 : 3056 – 3063 .

- Bodour , A.A. , J.M. wang , M.L. Brusseau , and R.M. Maier .2003. Temporal change in culturable phenanthrene degraders in response to long term exposure to phenanthrene in a soil column system. Environ. Microbiol. 5(10) :888 – 895 .
- Bodour , A.A.,G.C.Barajas , B.V.Jiorle , M.Malcomsom , A.K.Paull , A.K.somogyi , A.Trinh, L.N.Bates and R.B.Maier . 2004 .Structure characterization of flavolipids a novel class of

biosurfactants produced by Flavorbacterium sp . Appl.Environ.Micrbiol.70:114 120.

- Bogan , B . W . and R . T . Lamar 1996 . polycyclic aromatic hydrocarbon – degrading capabilities of *Phanerochaete laevis* and its extracellular lighinolytic enzymes . Appl . Environ . Microbiol . 62 (5) : 1597 – 1603.

- Bogan , B . W . and R . T . Lamar 1996 . polycyclic aromatic hydrocarbon – degrading capabilities of *Phanerochaete laevis* and its extracellular lighinolytic enzymes . Appl . Environ . Microbiol . 62 (5) : 1597 – 1603.

- Boopathy , R . 2000 . Factors limting bioremediation technologies . Bioresour . Technol 74 : 63 67 .

- Boopathy , R . 2000 . Factors limting bioremediation technologies . Bioresour . Technol 74 : 63-67 .

- Bossert , I . and R . BARTHA . 1984 . The fate of petroleum in soil ecosystems . Biotechnol . Bioeng . 62 : 526 – 536 .

- Bossert , I . and R . BARTHA . 1984 . The fate of petroleum in soil ecosystems . Biotechnol . Bioeng . 62 : 526 – 536 .

- Bossio , D . A . , K . M . Scow , N . Gunapala and K . J . Graham . 1998 . Determinants of soil microbial communities . Microb . Ecol. 36 : 1 12.

- Bossio , D . A . , K . M . Scow , N . Gunapala and K . J . Graham . 1998 . Determinants of soil microbial communities . Microb . Ecol. 36 : 1-12.

- Bouchez –Naitali , M ., R . Rakatoza and J . March . 1999 . Diversity of bacterial strains degrading hexadecane J . Appl . Microbiol . 86 : 421 – 428 .

- Bouchez –Naitali , M ., R . Rakatoza and J . March . 1999 . Diversity of bacterial strains degrading hexadecane J . Appl . Microbiol . 86 : 421 – 428 .
- Braddock , J.F., M.L.Ruth , P.H. Catterall , J.L.Walworth , and K.A.Mccrathynd . 1997 . Enhancement and inhibition of microbial activity in hydrocarbon – contaminated arctic soils implications for nutrient amended bioremediation. Environ . Sci . Technol . 31 : 2078 2084.
- Bregnard , T.,P.Hohener , A, Haner , and J.Zeyer .1996 . Degradation of weathered diesel fuel by microorganisms from acontaminted aquifer in aerobic and anaerobicmicrocosms . Environ . Toxical . Chem . 15 : 299 – 307 .
- Brignard , T. , P. Hohener , A. Haner and J. Zeyer.1996. Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer by in aerobic and anaerobic microcosms . Environ . Toxicol. Chem. 15 : 299 307 .
- Brito, E. M., Guyoneaud, R., Goni Urriza, M. Ranchou peyruse, and Duran, R. 2006. Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments in Guanabara Bay. Brazil Research in microbiology 14: 219 230.
- Bruheim ,P.&K.Eimhiellen . 1998 . Chemically emulsified crude oil as substrate for bacterial oxidation : differences in species response .Can . J.Microbiol.44(2):195 199.
- Budziniski , H., T . Nadalig , N. Natalie & M. Gilewicz . 2000 Evidence of two metabolic pathways for degradation of 2MP by Sphingomonas sp. Environ . Toxicol .chem.19(11) : 2672 – 2677.

- Budziniski , H., T . Nadalig , N. Natalie & M. Gilewicz . 2000 Evidence of two metabolic pathways for degradation of 2MP by Sphingomonas sp. Environ . Toxicol .chem.19(11) : 2672 – 2677.

- Budzinki , H., N.Raymond & T.Nadaling . 1998 . Aerobic Biodegradation of aromatic hydrocarbons by abacterial community . Gor.Geochem . 28(5):337348.
- Bushnell, L. D., H. E. Hass, 1941. Utilization of certain hydrocarbon by microorganisms. J. Bacteriol., 41: 653 658.
- Cantor, C. R., Schimmel, P. R. 1980. Biophysical chemistry p III: The behavior of biological macromolecules.2ed ed. San Francisco : WH Freeman and Co., pp. 1327 1371.
- Carmichael , L.M , and F.K. Pfaender .1997. polynuclear aromatic hydrocarbon metabolism in soils . Eviron. Toxicol . Chem 16:666 – 675 .
- Carmichael L.M and F.K. Pfaender . 1997 . The effect pf inorganic and
- Cavicchioli , R. , F.Fregatella , M. Ostrowski , M.Eguchi , and J.Gottschal.1999.Sphingomonads from marine environments . J.Ind.Microbiol. Biotechnol.23:268 272.
- Ceniglia C.E.1992 . Biodegradation of polycyclic aromatic hydrocarbons .Biodegradation 3:351 368.
- Cerniglia , C.E. and M . A . Heit camp . 1990 . Microbial degradation of polcyclic aromatic hydrocarbons (PAH) in the enviornment. J. Mol. 101—110.

- Cerniglia , C.E. and M . A . Heit camp . 1990 . Microbial degradation of polcyclic aromatic hydrocarbons (PAH) in the enviornment. J. Mol. 101—110.

- Cerniglia , C.E.1993 . Biodegradation of polycyclic aromatic hydrocarbons . Curr.Opin.Biotechnol.4:331.338.
- Cerniglia, C. E. 1993. Biodegradation of polycyclic aromatic hydrocarbons. Curr. Opin. Biotechnol. 4: 331 338.

- Chaillan, F., Chaineau, C. H., Point, V. Saliot A., Oudot. J. 2006. Factors inhibiting bioremediation of soil contaminated with weathered oils and drill cuttings Environ. Poll. 37: 1490 1497.
- Chaillan, F., L. E. A. Felche, E. Bury, Y. H. Phantavong, P. Grimont, A. Saliot, and J. Oudot. 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon degrading microorganisms. Res. Microbiol. 755: 587 595.
- Chaineau , C.H. , G.Rougeux , C.Yepremian and J.Oudot.(2005) . Effects of nutrient concentration on the biodegradation of crude oil and associated microbial populations in the soil . Soil.Biol.Biochem.37:1490 1497.
- Challiant , F.,L.E.A.Fleche , E.Bury , T.H. Phantavong , P.Grimont , A.saliot , and J.Oudot , 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon degrading microorganisms .Res. Microbial.755: 587 595.
- Churchil , P . and S . Churchill . 1997 .Surfactant – enhanced biodegradation of soild alkanes . J . Environ . Sci . Health 32 : 293 – 306 .

- Churchil , P . and S . Churchill . 1997 .Surfactant – enhanced biodegradation of soild alkanes . J . Environ . Sci . Health 32 : 293 – 306 .

- Churchill , S.A.,J.P. Herper & R.F. churhill.1999. Isolation and characterization of Mycobacterium species capable of degrading three – and four – ring aromatic microbial. 65 (2) : 594 552.

- Churchill , S.A.,J.P. Herper & R.F. churhill.1999. Isolation and characterization of Mycobacterium species capable of degrading three – and four – ring aromatic microbial. 65 (2) : 594-552.

- Ciazza , N.C,R.M.G. Shanks , and G.A.Tolle . 2005 Rhamnolipids Modulate swarming motility palterns of pseudomonas aeuginosa . J.Bacterid . 187:7251 7361.
- Cooney , J.J.(1984).The fate of petroleum pollutants in freshwater ecosysts . Microbiol.142:1333 1340.
- Cooper , D.G. and B.G. Golden berg . 1987. Sufface active agents from two Bacillus species. Microbiol . Rev . 49 :24 32.
- Cooper , D.G. and B.G. Golden berg . 1987. Sufface active agents from two Bacillus species. Microbiol . Rev . 49 :24-32.
- Cooper, D. G. and D. P. Paddock. 1983. *Torulopsis petrophilum* and surface activity. Appl. Environ. Microbial. 46: 1426 1429.
- Cooper, D. G., C. R. Macdonald, S. J. Duff, and N. Kosaric. 1981. Enhanced production of surfaction from *Bacillus subtilis* by continuous product and Metal cation additions. Appl. Environ. Microbiol. 42(6): 408 412.
- Da silva , M . L . and P. J . Alvarez . 2002 . Effects of ethanol on benzene , toluene , ethylbenzene Eng . 128 : 862 – 867 .
- Da silva , M . L . and P. J . Alvarez . 2002 . Effects of ethanol on benzene , toluene , ethylbenzene Eng . 128 : 862 – 867 .
- Dasilva , M.L.B and P.J. Alvare Z . 2004 . Enhanced aerobic biodegradation of benzene – toluene – ethybenzene – xylene – ethanol mixtures in bioaugmented aquifer colomns . Appl.Enciro.Microbiol.70:4720 4726.
- Daunert, S., G., Barrett, J. S. Feliciano, R. S. Shetty, S. Shrestha, and W. Smith Spencer. 2000. Genetically engineered whole cell sensing systems: coupling biological recognition with reporter genes. Chem. Rev. 100: 2705 2738.

- Dean Ross , D.(1989).Bacterial abundance and activity in hazardous waste – contaminated soil . Bull.Environ.cont.Toxicol.43: 511 517 .
- Delille , D.F.Coulom , and E.Pelletier. (2004) . Effects of temperature warming during a bioremediation study of nutrient – amended hydrocarbon – contaminated subantractic soils .Cont. Reg.Sci.Tech.40:61 70.
- Desai, J. D., and I. M. Bannat. 1997. Microbial Mol. Biol. Rev. 61: 47 64.
- Deutz,A.w,C.Jong,P.A.Williams , and J.G.Andel .1994 competition in chemosat culture between pseudomonas strains that use different pathways for the degradation of toluene . Appl.environ.Microb.60:2858 2863.
- Dibble , J.T, and R.Bartha. 1976 . The effect of iron on the biodegradation of petroleum in seawater . Appl.Enciron . Microbiol.31:544 550.
- Dibble, J. T., R. Bartha, 1979. Effect of environmental parameters on the biodegradation of oil sludge. Appl. Environ. Microbiol. 37(4): 729 739.
- Didgeway , H.F.,J.Safarik,D.pgipps , P.Carles and D.Clark . 1990 . Identification and catabolic activity of well – derived gasoline – degradating bacteria from a contaminated aquifer.Appl.Environ . Microbiol.56:35365 3575.
- DuaM.A.Singh , W.Sethunarhan , and A.K.Johri.2002 .Biotechnology and Bioremedation : successes and limitations . Appl . Microbiol . & Biotechnol 59:143 152.
- Dutta, T. K., and S. Harayama. 2001. Biodegradation of n alkylcycloalknes and n alkylbenzenes via new pathways in *Alcanivorax SP.* Appl. Environ. Microbiol. 64: 1884 1889.

- Eriksson , M.E., S . Sodersten , Z.Yu , G.Dalhammar , and W.W Mohn.2003 . Dergadation of polycyclic aromatic\ic hydrocarbons at low temperature under aerbic and nitrate reducing conditions in eurichment cultures from Northern soil Appl.Environ.Microbiol. 69:275 284.
- Fava , F., P.Aremante , and Kafkweitz.1995.Aerobic degradation and dechlorination of 2,3,4 Chlorophenol by *Pseudomonas* . Appl.Microbiol.Biotechnol. 43:171 177.
- Fayad, N. M., R. L. Edora, A. H. El Mubarak, and A. B. Polabkos. 1992. effectiveness of a bioremediation product in degradation the oil spilled in 1991 Arabian gulf war. Bull. Environ. Cantam. Toxicol.
  49: 787 796.
- Fetzner. S. 1998. Bacterial degradation of pyridine, indole, quinoline and their derivatives under different redox conditions. Appl. Microbial. Biotechnol. 49: 237 250.
- Fiechter , A.1992 . Biosurfactants :moving towards industrial application . Trends Biotenol 10:208 217.
- Field , J.A.,E.Jong , G. , Feijoo – costa and J.M. , de Bont . 1992 . Biodegradation of polycyclic aromatic hydrocarbons by new isolates of while rot Fungi . Appl.Environ Microbiol .75:123 136
- Foght , J.M. , P.M. Fedorak , and W.S.Westlake . 1990 . Mineralization of (C14) – Hexadecane and (C14) phenanthrene in crude oil : specificity among bacterial isolates . Can . J , Microbial. 36:169 175.
- Garrett, R. M. , I. J. Pickering, C. E. Haith, and R. C. Prince. 1998. Photooxidation of crude oils. Environ . Sci. Technol. 32: 3719 3723.

- Gaylarde , C.,C.,F.M. Bento & J. Kelly.1999. microbial contamination of stored hydrocarbon fuels and it's control. Rev. Micrebiol . <u>30</u> (1) : 1 10.

- Gaylarde , C.,C.,F.M. Bento & J. Kelly.1999. microbial contamination of stored hydrocarbon fuels and it's control. Rev. Micrebiol . <u>30</u> (1) : 1-10.

- Ghazali, F. M., Rahman, R. N., Salleh, A. B., Basri, 2004. Biodegradation of hydrocarbons in soil by microbial consortium. Intl. Biodeter. Biodegrad. <u>54</u>: 61 67.
- Giam l.and M.A.,Rao.2004 .Potential of extracellular enzymes in remediation of polluted soils . areview . Enz .Microbiol . technol.<u>35</u>:339 354.
- Gogi , B.K.N.N.Dutta, P. Goswami , and T.R.mohan.2003.Acase study of bioremediation of petroleum –hydrocarbon contaminated soil at a crude oil spill site ,Adv.Environ.Res.<u>7</u>:767 782.
- Goodwin , K . D., R . Tokarczyk , F . Carol and E . Saltzman . 2005 . Description of toluene inhibition of hydrocarbon . biodegradation. Appl . Environ . Microbial <u>71</u> (7) : 3495 – 3503 .

- Goodwin , K . D., R . Tokarczyk , F . Carol and E . Saltzman . 2005 . Description of toluene inhibition of hydrocarbon . biodegradation. Appl . Environ . Microbial <u>71</u> (7) : 3495 – 3503 .

- Grady , C.P.L.and H.C.Lim . 1996 . Biological wastewater treatment theory and application . Water Res.30:742 748.
- Grimm , A . C . and C . S. Harwood . 1997 . Chemotaxis of *pseudomonas* SP. to the polyaromatic hydrocarbon naphthalene . Appl . Environ . Microbiol . <u>63</u>: 4111 4115 .

- Grimm , A . C . and C . S. Harwood . 1997 . Chemotaxis of *pseudomonas* SP. to the polyaromatic hydrocarbon naphthalene . Appl . Environ . Microbiol . <u>63</u>: 4111-4115 .

- Grund, E., B. Denecke, and R. Eichenlaub. 1992. Naphthalene degradation via salicylate and genetisate by *Rhodoccus SP*. strain B4. Appl. Environ. Microbiol. 58: 1874 1877.
- Guerin , W.F. and S.A Boyd .1992 . diffrenetial bioavailability of soil sorbed naphthalene to two bacterial species . Appl. Environ.Microbiol.58:1142 – 1152 .
- Guerin, W. F., and S. A. Boyd. 1997. Bioavailability of naphthalene associated with natural and sunthetic sorbents water Res. 51: 1504 1512.
- Guerrero, R., E. Montesinos, C. Pedros Alio, I. Esteve, J. Mas H. Van Gemerden, P. A. G. Hofman, and J. F. Bakker. 1985. Phototrophic sulfur bacteria in two Spanish lakes. Oceanogr. 30: 919 931.
- Hallier – Soulier , S ., V . Duccrocq , N . Mazure , and N . Truffant . 1999 . Detection and quantification of degradative genes in soils contaminated by toluene . FEMS Microb . Ecol . 20 : 121 133 .

- Hallier – Soulier , S ., V . Duccrocq , N . Mazure , and N . Truffant . 1999 . Detection and quantification of degradative genes in soils contaminated by toluene . FEMS Microb . Ecol . 20 : 121-133 .

- Hamamura , N. , S.H. Olson , D.M. , Ward , and W.P.Inskeep.2006.Microbial population dynamics associated with crude

Hamamura , N. , S.H. Olson , D.M. , Ward , and -
W.P.Inskeep.2006.Microbial population dynamics associated with crude

- Harms , H. , and A.J.B.Zehander 1994 . Influence of substrate diffusion o degradation of dibenzofuran and 3 – chlorodipenzofuran by attached ans suspended bacteria.Appl.Environ.Mirob. 60: 2736 2745.

- Harms , H., and A.J. B.zehnder . 1995 . Bioavailability ode orbed 3 chorodibenzofuran . Appl.Environ.Microbiol. 61:27 33.
- Harris, R. F. & L. E. Sommers. 1998. Plate count Frequency technique for assay of microbial ecology. Appl. Microbiol. 16(2): 330 334.
- Harrison , A . B ., M . B . Ripley and R . K . Dart . 2000 . Effect of protein hydrolysate on the degradation of diesel fuel in soil . Enzyme Microb . Technol . 26 : 388 393 .
- Harrison , A . B ., M . B . Ripley and R . K . Dart . 2000 . Effect of protein hydrolysate on the degradation of diesel fuel in soil . Enzyme Microb . Technol . 26 : 388-393 .
- Harwood, C. S. and Gibson. 1997. Shedding light on anaerboc benzene ring degradation: aprocess unique to prokaryotes? J. Bacteriol 179: 301 309.
- Haryama , S.,H.Kishiray.Kassai,and K.Shutsube 1999.petroleum biodegradation contaminated environments . J.Mol.Microbiol.Biotechnol.1:63 70.
- Haryama , S.and K.N.Timmis . 1989 . Catabolism of aromatic hydrocarbons by pseudomonas . Adv.Biochem.Eng.Biotech. 33:53 93 .
- Head , I .M. , and R.P.J.Swannell.1999.Bioremediation of petroleum hydrocarbon contaminants in marine habitates. Curr.Opin . Biotechnol.10:234 239.
- Hee shin , K.and K.Woong Kim.2001 . Ecotoxicity monitroring of hydrocarbon –contaminated soil using erthwormm (Eisenia Foetida) . Environ . Monit. Assess . 70:93 103
- Herbert, R. A. 1986. The ecology and physiology of psychrophilic microorganisms, p.123 in R. A. Herbert, and G. A. codd (ed.).

Microbes in extreme environments. Academic press, New York, N. Y.

- Herbert, R. A. 1990. Methods for enumerating microorganisms and determining biomass in natural environments. Methods Microbiol. 22: 139 141.
- Herman , D.C.J.F. Artiola , and R.M.Miller . 1995 . Removal of candmuim , lead , and Zinc from soil by rhamnolipid biosurfactant . Environ.Sci.Technol.29:2280 2285.
- Hill,E.C.1988.Microboes & Lubricats . Tribology . 8:221 231.
- Holden , P.A., M.G. Bruce , A.K. Miller and W.G. Lindow . 1997 Biodegradation of hexadecane by *pseudomonas aeruginosa* . Appl . Environ . Microbiol . 68 : 250 258 .

- Holden , P.A., M.G. Bruce , A.K. Miller and W.G. Lindow . 1997 Biodegradation of hexadecane by *pseudomonas aeruginosa* . Appl . Environ . Microbiol . 68 : 250-258 .

- Holt, J. G., N.R. Krieg , P,H.A. Smeath , J.T. staley & S.T. Williams . 1994. Bergeys manual of determinative bacteriology. (9th ed.) Williams & Wilkins Inc., Baltimore, Mary land.

- Holt, J. G., N.R. Krieg , P,H.A. Smeath , J.T. staley & S.T. Williams . 1994. Bergeys manual of determinative bacteriology. (9th ed.) Williams & Wilkins Inc., Baltimore, Mary land.

- Hommel R. F., Ratledge, C. 1993. Biosynthetic mechanisms of low molecular weight surfactant and their precursor molecules. In : Biosufactants: Production, Properties, applications (Kosaric N, ed.) New York, pp: 3 63.
- Hommel, R. K. 1990. Formation and physiological role of biosurfactants produced by hydrocarbon utilizing microorganisms. Biodegradation.1: 107 119.

- Horowitz , A., D. Gutnick & C. Rosenberg . 1975. Sequential growth of bacteria on crude oil. Appl. Microbiol . 30 (1) : 10 19.
- Horowitz , A., D. Gutnick & C. Rosenberg . 1975. Sequential growth of bacteria on crude oil. Appl. Microbiol . 30 (1) : 10-19.
- Huesemann , M . H ., T . S . Hansmann and T . H . Fortman . 2002 . Microbiol Factors rather than bioavailability limit the rate and extent of PAH biodegradation in aged crude oil contaminated model soils . Biorem . J . 6: 321 336.
- Huesemann , M . H ., T . S . Hansmann and T . H . Fortman . 2002 . Microbiol Factors rather than bioavailability limit the rate and extent of PAH biodegradation in aged crude oil contaminated model soils . Biorem . J . 6: 321-336.
- Huesemann, M. H. 1995. Predictive model for estimating the extent of petroleum hydrocarbon biodegradation in contaminated soils. Environ. Sci. Technol. 29: 7 18.
- Hussein , E. 2006 . Investigation into the mechanisms which permit the high rate , degradation of phase and related petroleum hydrocarbons in sequencing batch reactors by attached cells in a controlled mixed bacterial community . Appl. Environ. Microbiol.72: 3531 3542 .
- Irvine , R.L. and I.H.J. ketchum . 1988 . Sequencing batch reactors for biological wastewater treatment . Critical reviews in Environmental control . 18:255 294.
- Itoh, S. and T. Suzuki. 1972. Effect of rhamnolipids on growth of *pseudomonas aernginosa* grown on n paraffin utilizing aboility. Agric. Biol. Chem. 36: 2233 2235.

- Jacob , J. 1996 . The significant of poly aromatic hydrocarbons as environmental carcinogens pure and Appl . Chem . 68:301 308.
- Jacques , R.J.,S.,Okeke , C.Benedict , M.B.Fatima , C.R.Maria , A.O.Flavio . 2007 . Characterization of a polycyclic aromatic hydrocarbon – degradation microbial consortium from a petrochemical sludge – landframing site .Biorem.60 :275 280
- Jacques, R., J. S. Okeke, B. C. Bento, F. M. Peralba, M. C. Camargo, and A. O. Flvio. 2007. Characterization of apolycyclic aromatic hydrocarbon degrading microbial consortium from apetrochemical sludge and farming site. Biorem. J. 6: 321 336.
- Janiyani, K. L., H. J. Purohit, R. Shanker, and P. Khanna. 1994. De emulsification of oil in water emulsions by *Bacillus subtilis.* World J. Microbiol. Biotechnol. *10:* 452 456.
- Jennings , E.M. and R.S.Tanner . 2000 . Biosurfactant producing bacteria found in contaminated soils . producing bacteria found in contaminated soils . Proceedings of the 2000 conference on hazardous waste. Research 70: 299 – 306.
- Jeon , C.O., W. Park , C. Derito , J. R. Snape and E.L. Madsen . 2003 . Discovery of a bacterium with distinctive diooxygenase , that is responsible for in situ biodegradation in contaminated sediment . Int . J. Syst . Evol . Microbiol . 63 : 4516 4522 .
- Jeon , C.O., W. Park , C. Derito , J. R. Snape and E.L. Madsen . 2003 . Discovery of a bacterium with distinctive diooxygenase , that is responsible for in situ biodegradation in contaminated sediment . Int . J. Syst . Evol . Microbiol . 63 : 4516-4522 .
- Jerabkova , H., B.K, Kralova , V., Rejeu , J. Sanchez , and M.G. Riog. 1997 . Use of polyurethane foam for the biodegradation of n –alkanes by immobilized cells of pseudomonas . Biotechnol . technol . 11:391 394.

- Jimenez , L.A.Breen , N.Thomas , T.w. Federle , and G.s.Sayler .1991 . Mineralization of linear alkylbenzene sulfonate by a four – member aerobic bacterial consortium . Appl .Environ.Microbiol.57:1566 1569.
- Johensen , A . R ., L . Y . Wick and H . Harms . 2005 . Principles of microbial PAH – degradation in soil . Environ . poll . 133 : 71 84.
- Johensen , A . R ., L . Y . Wick and H . Harms . 2005 . Principles of microbial PAH – degradation in soil . Environ . poll . 133 : 71-84.
- Johnsen , A.R. , and U . Karlson . 2004 . Evaluation of bacterial stratigies to promote the bioavailability of polycyclic aromatic hydrocarbons.Appl.Microbiol.Biotechnol.63 452 459 .
- Jolivant, C. Minier, M., and Renon, H. 1990. Advances in protein chemistry J. Colloid Interface Sci., 135 (1): 85 95.
- Jouanneau, Y., Meyer, C. 2006. Purification and characterization of a arene cis dihydrodiol dehydrogenase with broad substrate specificity toward polycyclic aromatic hydrocarbon dohydrodiols. Appl. Environ. Microbiol. 72: 4726 4734.
- Juhasz, A. L., and R. Naidu. 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzopyrene. Int. Biodet. Biodeg. 45: 57 88.
- Juneson, C. O. P. ward, and A. Singh. 2002. Microbial treatment of a styrene contaminated air stream in abiofilter with high elimination capacities. J. Ind. Microbial . Biotechnol. 26: 196 202.
- Kampfer, P., M. Steiof, P. M. Becker, and W. Dott. 1993. Characterization of chemoheterrophic bacteria associated with the

insitu bioremediation of a water oil contaminated site. Microb. Ecol. 26: 161 168.

- Kappeli , A . C ., S. Thomas and R . Rouiever . 1991. Bacterial conversion of cyclic alkanes and polyaromatic hydrocarbons . Appl . Microbiol . Biotechnol . 58: 704 711 .
- Kappeli , A . C ., S. Thomas and R . Rouiever . 1991. Bacterial conversion of cyclic alkanes and polyaromatic hydrocarbons . Appl . Microbiol . Biotechnol . 58: 704-711 .
- Kasai, Y.,H.Kishra , k.syutsubo, and S.Harayama.2001.Molecular detection of bacterial populations on beaches contaminated by the Nakhdka. Tanker oil – spill accident . Environ.Microbiol.2:246 255.
- Kayser, K. J., B. A. Bielaga Jones, K. Jackowski, O. Odusan, and J. J. Kilbane. 1993. Utilization of organosulfur compounds by axenic and mixed cultures of *Rhodococcus rhodocrous* IGTs8 J. Gen. Microbiol. 139: 3123 3129.
- Kazunga .C., and M.D.Aitken.(2000). Products from the incompelete metabolism of pyrene by polycyclic aromatic hydrocarbons degradting bacteria.Appl.Environ.Microbiol.66:1917 1922.
- Kim, S., Choi, D. H., Sim, D. S., Oh. Y. 2005. Evaluation of bioremediation effectiveness on crude oil contaminated sand. Appl. Environ. Microb. 61: 45 51.
- Kiyohara, H., Nagao, K., and Yana K. 1982. Rapid screen for bacteria degrading water insoluble solid hydrocarbons on agar plates. Appl. Environ. Microbiol. 43: 454 457.
- Kleinsteuber, S., Riis, V., Fetzer, I., Harms, H., Muller., S. 2006. Population dynamics within a microbial consortium during growth

on diesel fuel in saline environments. Appl. Environ. Microbiol. 72: 3531 3542.

- Knaebel , D . B . and J . R . vestal . 1988 . Acomparison of double vial radiorespiratory to measure microbial mineralization in soil . J . Microbiol . Methods . 7 : 309 317 .

- Knaebel , D . B . and J . R . vestal . 1988 . Acomparison of double vial radiorespiratory to measure microbial mineralization in soil . J . Microbiol . Methods . 7 : 309-317 .

- Krivobok , S., E.Miriouckkine , F.Seigle & J.L.Benoit .1998.Biodegradation of anthracene by soil fungi . Chemosphere . 37(3) .523 530.
- Kropinski , A. m. b, L . chan and F.H . Milazzo . 1978 . Susceptibility of lipopolysaccharide defective mutants of *pseudomonas aeruginosa* to dyes , detergents , and antibiotics . Antimicrob . Agents . Chemo ther . 13 : 494 – 499.

- Kropinski , A. m. b, L . chan and F.H . Milazzo . 1978 . Susceptibility of lipopolysaccharide defective mutants of *pseudomonas aeruginosa* to dyes , detergents , and antibiotics . Antimicrob . Agents . Chemo ther . 13 : 494 – 499.

- Kurkela , S., H. Lehvashaiho , E.T. Palva , and T.H. Teeri . 1988. Cloning , Nucleotide sequence , and charactertization of genes encoding naphthalene dioxygenase of psedumonas putida strain NCI,B9816. Gene 73:355 362.
- Kurola , J . 2006 Micrbiol activities , in boral soils : Biodegradation of organic contaminats .Can. J. Microbiol.46:10 2

Kurola , J . 2006 Micrbiol activities , in boral soils : -
Biodegradation of organic contaminats .Can. J.
Microbiol.46:10 2

- Lang, S., and D. Wullbrandt. 1999. Rhamnose lipids biosynthesis: microbial production and application potential. Appl. Microbial. Biotechnol. 51: 22 32.
- Lang, S., Wagner, F. 1987. Structure and Properties of biosurfactants. In: Biosurfactants and Biotechnology (Gray Ncc ed.). New York, pp.21 45.
- Lazar , I.,Avoicu , S.Dobrota . , M.stefanes , L.sadulescu , G.archir , I.G.Lazar , D.Muceninca , A.Balalia.1995 . Microbial communities of soils , slops , and waters contaminated with waste oil and phenol , and their role in bioreme – of such environments . Appl.Environ.Microbiol.49:429 433.
- Leahy, J.G., and R.R Colwell.1990 . Microbial degradation of hydrocarbons in environment micrpbiol.Rev.54: 305 315.
- Leive , L1974 . The barrier function of the Gram negative envelope . Ann .Acad . Sci . 235 : 109 127.

- Leive , L1974 . The barrier function of the Gram negative envelope . Ann .Acad . Sci . 235 : 109-127.

- Leys , N.M. , E.J. Ryngaert , L . verstraete , W. Top and E.M. springael . 2004 . Occurrence and phylogenetic diversity of Sphinomonas strains in soils contaminated with polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 70:1944 1955 .

Leys , N.M. , E.J. Ryngaert , L . verstraete , W. Top and -
E.M. springael . 2004 . Occurrence and phylogenetic
diversity of Sphinomonas strains in soils contaminated
with polycyclic aromatic hydrocarbons. Appl. Environ.
Microbiol. 70:1944 1955 .

- Liang, Y., Grander, D. R. Miller, C. D., Chen, D., Anderson, A. J., Weimer, B. C., Sims, R. C. 2006. Study of biochemical pathways and enzymes involved in pyrene degradation by *Mycobacaterium SP.* Strain KMs. Appl. Environ. Microbiol. 72: 4726 4734.

- Lim. L. H., R. M. Harrison, and S. Harrad. 1999. The contribution of traffic to atmospheric concentrations of polycyclic aromatic hydrocarbons Environ. Sci. Technol. 33: 3538 3542.
- Lin,S.C.1996 . Biosurfactants : recent advauces .J.Chem . Technol.Biotechnol.66 :109 115 .
- Linos , A.,M.M.Berekaa , R.Reichelt , U. Keller , J.Schmit , H.C.Flemming , R.M.Kroppeus and A.Steinbuchel.2000 .Biodegradation of Cis 1,4 polyisoprene rubbers by distinct acactingomycetes : Microviol.66:1639 1645
- Lonon , M.K.,M.A Banto & R.H. Findlay. 1999 . Apilot study for monitoring changes in the microbiological component of MWFS as a function of time & use in the system. Am. Ind. Hyg . Assoc . J. 60 (40) : 480 – 485

- Lonon , M.K.,M.A Banto & R.H. Findlay. 1999 . Apilot study for monitoring changes in the microbiological component of MWFS as a function of time & use in the system. Am. Ind. Hyg . Assoc . J. 60 (40) : 480 – 485

- MacFaddin, J. F. 2000. Biochemical tests for identification of medical bacteria 3rd. M. G. Lawrence (ed.). Lippinocott & Williams , New York.
- Macnaghton , S.J.,R.J.Stephen , A.D.Venosa, G.A.Davis .Y.J.Chang , and D.C.white . 1999. Microbial population changes during bioremediation of an experimental oil spill.Appl.Environ.Microbial.65:3566 3574.
- Magot, M ., B. Ollivier, and B. K. C. Patel. 2000. Microbiology of petroleum reservoirs. Antonie van Leeuwenhook. 77: 103 116.

- Maier , R. (2003) . Biosurfactants : Evolution abd diversity in bacteria . Adv.Appl.Microbiol. 52:101 121 .
- Maki, H,T.Saski, and S.Haramaya.2005 . Photooxidation of biodegradable crude oil and toxicity of the photo oxidized products chemosph . 44.1145 1151.
- Makin , S.A.and T.J.Beveridge . 1996 . The influence of A band and B band lipopolysaccharide on the surface characteristics and adhersion of pseudomonas aeruginosa to surface . Microbiology 142 : 299 307.
- Makkar, R. S., and S. S. Cameotra. 2002. An update on the use of unconventional substrates for biosurfactant production and their new applications. Appl. Microbiol. Biotechnol. 58: 428 434.
- Makula, R. A., P. J. Lockwood, and W. R. Finnerty. 1975. Comparatire analysis of the lipid of *A cinetobacter* species grown on hexadecane J. Bacteriol. 121: 250 258.
- Margesin , R . 1998 . Biodegradtion and bioremediation of hydrocarbons in extreme environments . Appl . Microbiol Biotechnol . 56 : 650 – 663.

- Margesin , R . 1998 . Biodegradtion and bioremediation of hydrocarbons in extreme environments . Appl . Microbiol Biotechnol . 56 : 650 – 663.

- Martinez , E.M.R. 2006. Microbiol divbersity of fluidzedbed bioreactor treating diesel – contaminated groundwater (Vega – Baja , Puerto Rico) Appl. Environ Microbual 69: 1482 – 1497 .
- Marx , R . B . and D . Aitken . 1999 . Quantification of chemotaxis to naphthalene by *pseudomonas putida* . Appl . Environ . Microbiol . 65 : 2847 – 2852 .

- Marx , R . B . and D . Aitken . 1999 . Quantification of chemotaxis to naphthalene by *pseudomonas putida* . Appl . Environ . Microbiol . 65 : 2847 – 2852 .
- Marx , R.B. , and M.D.Aitken .2000.Bacterial chemotaxis enhances naphtalene degradation in a heterogeneous aqueous system . Environ.Sci.Technol.34:3379 3383.
- Massol , D., A.,J. Wallon , R.F.Hickey , and J.M.Tiedje . 19995 . Channel structures in aerobic biofilms of Fixed – film reactors treating contaminated grown water . appl . Enciron . Microbiol . 61:769 777.
- Matti , S. 1993. Distribution of oil degrading bacteria in the North – West Arabian Gulf . Marine poll. Bull. 21:32 40.
- Matti , S. 1993. Distribution of oil-degrading bacteria in the North – West Arabian Gulf . Marine poll. Bull. 21:32-40.
- Mcclay , K. , S.H. Streger , and R.J. Steffan . 1995. Induction of toluene oxidation activity in pseudomonas mendocina KRI and *Psendomonas sp*. Enbiron . Microbiol. 61 : 3479 – 3481 .
- Meallister K.A.,L.Hung , and J.Trevors. 1996 . Microbial degradation of phenanthrene . biodegradation 7 (1) :1 40.
- Menn, F. M., B. M. Applegate and G. S. Sayler. 1993. NAH plasmid mediated catabolism of anthracene and phenanthrene to naphthoic acids. Appl. Environ. Microbiol. 59: 1938 1942.
- Mueller , J . G . S . M . Resnick , M . E . Shelton , and P. H Pitchard . 1992 . Effect of inoculation on the biodegradation of weathered Prudhoe Bay crude oil . J . Indst . Microb . 10 : 95 102 .
- Mueller , J . G . S . M . Resnick , M . E . Shelton , and P. H Pitchard . 1992 . Effect of inoculation on the biodegradation of weathered Prudhoe Bay crude oil . J . Indst . Microb . 10 : 95 -102 .

- Mueller , J.G,P.J.Chpman , and P.H. Pritchard .1989 . Action of fluoranthene – utilizing bacterial community on polycyclic aromatic hydrocarbon components of creosote . Appl.Environ.Microbiol.55:3085 3090.
- Mueller , J.G.,P.J.Champan , and P.H.prithard . 1989 . Creosote – contaminated sites . Environ.Sci.Technol.23:1199 1201.
- Mulder, H., A. M. Breure, D. Van Honschooten, J. T. C. Grotenhuis , J. G. Van Andel, and W. H. Rulkenes. 1998. Effect of biofilm formation by *Pseudomonas* 8909N on the bioavailability of solid naphthalene. Appl. Microbiol. Biotechnol. 50: 277 283.
- Mulligan, C. N., R. N. Yong, and B. F. Gibbs. 2001. surfactant enhanced remediation of contaminated soil: areview . Eng. Geol. 60: 371 380.
- Murakami, Y., and M. Alexander. 1989. Destruction and formation of toxins by one bacterial species affect biodegradation by a second species. Biotechnol. Bioeng. 33: 832 838.
- Mutnuri, S., N. Vasudevan, and M. Kastner. 2005. Degradation of anthracene and pyrene supplied by microcrystals and non aqueous phase liquids. Appl. Microbial. Biotechnol. 67: 569 576.
- Nadaling, T. N., Raymond, M. Gilewiez & H. Budziniski. 2000. Development of a protocol to study aerobic bacterial degradation of PAHs. Polycyclic Aro. Comp. 18: 177 192.
- Nakahara , T., L. E. Erickson and J.R. Gutierreez . 1977 . Charscterization of hydrocarbon uptake in cultures with two liquid phases . Biotechnol . Bioeng 19: 9 25.

- Nakahara , T., L. E. Erickson and J.R. Gutierreez . 1977 . Charscterization of hydrocarbon uptake in cultures with two liquid phases . Biotechnol . Bioeng 19: 9-25.

- Naziruddin , M.,C.P.lGrady , and H.H.Tabak . 1995 . Determination of Biodegradation Kinetics of volatile organic compounds through th use of respiromentry . Water Environ.Res.67.151 158.
- Nedwell , B.D. 1999 Effect of low temperature on microbial growth. Microbiol . Eco . 30 : 101 111.
- Nedwell , B.D. 1999 Effect of low temperature on microbial growth. Microbiol . Eco . 30 : 101-111.
- Nikaida , H., and T.Takae . 1979.The outer membrane of Gram ngative bacteria. Adv.Microb.Physiol.20: 163 250 .
- Nikaido , H. , and M.Varra . 1985. Molecular basis of bacterial outer membrane permeability Microbiol.Rev.49:1 32.
- Nwanchukwu S.U. , and E.O.Uqoji 1995 . Impacts of crude petroleum spills on microbial communities of tropical soils . Intern .J.Ecol.Environ.Sci.21:169 176.
- Nwanckykwu , S.U. 2001 . Bioremediation of sterile agricultural soils polluted with crude petroleum by application of the soil supplementation . Curr . Microbial .42(2) : 231 236.
- Oavies, J. I. 1967. Oxidative metabolism of naphthalene by soil *Pseudomonas* iochem J. 91: 251 261.
- Oboh, B. O., M. O. Hori, J. O. Akinyemi, S. A. Adebusoye.2006. Hydrocarbon degrading potentials of bacteria isolated from a Nigerian Bitumen deposit. 4(3): 51 57.

oil biodegradation in diverse soils. Appl. Environ. ○
Microbiol. 72:6316 6324.

- Ojo, O. A. 2006. Petroleum hydrocarbon utilization by native bacterial population from waste water canal south west Nigeria. Afr. J. Biotechnol. 5(4): 333 337.

- Ojumu, T. V., Bello, O. O., Sonibare, J. A., Solomon, B. O. 2005, Evaluation of microbial systems for bioremediation of petroleum refinery effluent in Nigeria. Afr. Biotechnol. 4(1): 31 35.
- Okerentugba , P.O. and O.U. Ezeronye.2003.Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria .Africa. J. Biotechnol . 2 :288 292
- Oko,A.I.2003.Assesment of the Potentials of some bacterial isolates for application in the bioremediation of petroleum hydrocarbon polluted soil.Appl.Microb.Biotech.47:615 619.
- Okoh , A.I. 2003. Biodegradation of light crude oil in soil microscoms by some bacterial strains isolated from crude oil fellow stations saver pits .in Nigeria.Afr.J.Biotechnol.2(5) : 104 108.
- Okoh, A. I. 2006. Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotechnol. & Mol. Biol. Rev. 1(2), pp.38 50.
- Okpokwasili , G.C. , and W.A.James . 1995 . Microbial contamination of kerosene , gasoline , and crude oil and their spoilage potentials . Afr.J.Biotechnol.29:147 156.
- Olsen, G.J. , H.J. , H.M atsuda , R.Hagstrom , and R.Overbreak.1994 . Fast DNA ml: a tool for construction of polygenetic trees of DNA sequences using maximum likelihood . Comput. Appl.Biosci.10:41 43 .
- Osidanjo , H.1992 . Pollution studies on Nigerian rivers . Environmental . Iner.5:49 53.
- Otenio , M.H. , M.T.L.Daslva , M.L.O. Marques , J.C.Roseiro , and E.D.Biodia . 2006 . benzene , toluene and xylene biodegradation by pseudomonas putida . Braz.J.Microbiol.36(3):3350 3358.

- Otenio , M.H., M.T. Dasilva , M.L. oliveira and E.D. Bidoia . 2005 . Benzen , toluene and xylene biodegradation by *Psendomonas putida* CCMI 852 . Brazil . J . Microbiol . 36 (3) : 433 442 .
- Otenio , M.H., M.T. Dasilva , M.L. oliveira and E.D. Bidoia . 2005 . Benzen , toluene and xylene biodegradation by *Psendomonas putida* CCMI 852 . Brazil . J . Microbiol . 36 (3) : 433-442 .
- Ozaki , H., Z.Liiu , Y.Terashima . 1991 . Utilixation of microorganisms immobilized with magnetic particles for sewage and wastewater treatment . Wat.Sci.Technol.23:1125 1136.
- Palmentire, J. P., A. J. Britten, G. M. Charbonneau, and F. W. Karasek. 1994. Determination of PAHs in lubricating of base stocks using HPLc. J. chromatogr. 469: 241 251.
- Parales , R.E.,S.M.Resnick,C.L.Yu, D.R.Boyd , N.D.Sharma , and D.T. Gibson 2000 . regioselectivity and enatioselectivity of naphthalene dioxygenase during arene Cis dihydroxylation : Control by phenylalanine 352 in the α subunit .J.Bacteriol . 182 :5495 5504.
- Permuzic E. T. , M. S. Lin, M. Bohenek and W. Mzhou. 1999. Bioconversion reactions in asphaltenes and heavy crude oil. Energy Fuels. 13: 297 304.
- Phelps, C. D., and L. Y. Young. 2001. Biodegradation of BETX under anaerobic conditions: areview. Adv. Agron. 70: 329 357.
- Phillips, M. G. J. and J. E. Stewart. 1974. Distribution of hydrocarbonutilizing bacteria in Northwestern Atlantic waters and coastal sediments. Can. J. Microbiol. 20: 955 962.
- piepel , H.D,V.A.Martins , and P.N. Golyshin 2004 . Genomic and mechanistic insights into the biodegradation of organic pollutants curr . Opin . Biotechnol.15:215 224.

- Pitter , P.and J.Chudoba . 1990 . Biodrgradatbility of organic sustrate in the aquatic environment . Appl.Environ.microbiol.68:5537 5548.
- Pothuluri , J.V. and C.E. Cerniglia . 1994. Microbiol Metabolism of polycyclic aromatic hydrocarbon. Appl . Environ . Microbiol 66 (12): 5410 5418.
- Poxton , I . R .1993. Prokaryote envelope diversity .J. Appl. Bacteriol. 74:1 11.
- Poxton , I . R .1993. Prokaryote envelope diversity .J. Appl. Bacteriol. 74:1-11.
- Prenafeta, F. X., J. Vervoort, T. C. Grotenhuis, and J. W. Van Groenestijn. 2002. Substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) Hydrocarbons by the Fungus *Cladophialophora SP.* strain T1. appl. Environ. Microbial. 68: 2660 2665.
- Prenefeta – Bolu , F.X. , J.Vervoort , J.T.Grotenhuis , and J.w.Vangroenestijin . 2002 . Substrate intractions during the biodegradation of benzene , toluene , ethylbenzene , and xylene (BTEX) hydrocarbons by the fungus Cladophialophora sp. Strain T1 .Appl.Microbiol.Rev.65:315 321
- Pritchard , P.H., C.F.Costa , J.G Muelles m J.C.Rogers , F.V.Kremer , and J.A.G;aser.1992 .Oil spill biodegradation experiences , lessons and results from the Exxonvaldez oil spill in alaska . Biodegradation 3:315 335.
- Qureshi , A.A. and H.J. purohit . 2002. Isolation of bacterial consortia for degradation of poly aromatic hydrocarbons from contaminated soil.Ann. Appl. Biol 140 (2) : 159 162 .
- Raina, M. M. 1995. Biosurfactant faciliated remediation of metal contaminated soils. Environ. Health. Prespect. 103(1): 59 62.

- Rajas , N.G. , V.R. Rodringuez & F.Enriquiez.1999.Transformer oil degradation by an indigenous microflora isolated from contaminated soil .J.Microbiol.Methods 63:215 225.
- Ramos , J.L. , V.Lorenzo , S.Molin & C.ramos . 1994 . The behavior of bacteria designed for biodegration. Biotechnol . 12 : 1349 1356.
- Randall, J. D., and B. B., Hemmingsen. 1994. Evaluation of mineral agar plates for the enumeration of hydrocarbon – degrading bacteria J. Microbiol. Methods 20: 103 113.
- Reed .M.,and S.Johesen 1995 . Produced water: Environmental issues and mitigation technologies . second edition , Newyork; Plenum press .pp.14 56.
- Reseberg , E.1991.The hydrocarbon.oxidizing bacteria . Appl.Environ.Microb.61:45 51.
- Rhykerd , R. , B. Crews , K.Mcinnes & R.W.weaver. 1999 . Impact of bulking agents , forced aeration & tillage on remediation of oil contaminated soil . Bioresour Technol . 67(2) : 279 285.
- Riis , V., D.Miethe & M.Moder . 1996 . Analytical characterization of the persistent redus after microbial degradation of mineral oils . J.Analyti.Chem.358(6):378 384.
- Romine, M. F., J. K. Fredrickson, and S. M. W. Li. 1999. Induction of aromatic catabolic activity in *Sphingomones* aromaticirorans strain F199. J. Ind. MicrobiolBiotechnol. 23: 303 313.
- Rooney – Varga , J.N. , R.T. Anderson , J.L. Fraga , D. Ringelberg , and D.R. Lovely . 1999. Microbial communities associated with aerobic benzene degradation in a petroleum – contaminated sites .Appl. Environ. Microbiol . 65 : 3056 – 3063 .

- Rossert , I., and R.Barth. 1984 . The fate of petroleum in soil ecosystems . Biotechnol.Vioeng.62:526 536.
- Rozgaj , R.and M.Glancer – olijab . 1992 . Total degradation of aminoaphthalene – 2 sulphonic acid by mixed culture consisting of different bacterial genera . J.Geu.Microbiol . 83:165 170.
- Sabra , W. , H.Lunsdorf , and A.P.zeng. (2003) . Alterations in the formation of lipopoly saccharide and membrane vesicles on the surface of *pseudomonas aeruginosa* under oxygen stress conditions . Microbiology 149: 2789 20795.
- Samanta , S . K ., A . K . Chakra and R . k . Jain . 1999 . Degradation of phenathnene by different bacteria . Appl . Microbiol . Biotechnol . 53 : 98 107 .
- Samanta , S . K ., A . K . Chakra and R . k . Jain . 1999 . Degradation of phenathnene by different bacteria . Appl . Microbiol . Biotechnol . 53 : 98-107 .
- Samanta , S.K. , O.V.Singh , and R.K.Jain . 2002 . Polycyclic aromatic hydrocarbon : Environmental pollution and bioremediation . Trends. Biotechnol.20:243 248.
- Sannell , R.P.J.,D.Mitchell , G.Lethbridge , D.Jones , and M.Mcdonagh . 1996.Field evaluations of oil spill bioremediation . Microbial.Rev.60:342 365.
- Santas , R., and P. Stantas . 2000 .Effects of wave action on the bioremediation of crude oil saturated hydrocarbons . Mar.Pollut . Bull.40:434 439.
- Santas , R.,A.Korda,A.Tenente , K.Buchholz , and P.H. Santas . 1999 . Mesocosm assay of oil spill Bioremediation .Mar.Pollut.Bull.38:44 48.
- Schippers, A., K. Bosecher, C. Sproer, and P. Schumann . 2005. *Microbacterium* oleivorans SP. and *Microbacterium*

hydrocarbonoxydans SP. , novel crude oil degrading gram positive bacteria. Int. Syst. Evol. Microbiol. 55: 655 660.

- Schmitt R., H .P . Rohs & P.Eckert 1996 . Biodegradation of aromatic hydrocarbon . Org . Geochem . 25 (1) : 41 50.
- Schmitt R., H .P . Rohs & P.Eckert 1996 . Biodegradation of aromatic hydrocarbon . Org . Geochem . 25 (1) : 41-50.
- Selifonov , S.A. ,M. Grifoll , R.W. Eaton , and P.J. Chapman .1996. Oxidation of Naphthalene and methyl Substituted aromatic compounds by naphthalene 1,2 deoxygenizes. Appl.Environ.Microbiol.62:507 514.
- Sepic , E., C.Trier & H . Leskovesk.1996 . Biodegradation studies of selected hydrocarbons from diesel oil . Analyst . 121:1451 1456.
- Shiaris , M.P.1989 . Seasonal biotransformation of naphthalene , ands benzo[a] pyrene in surficial estuarine sediments . Appl.Environ .Microbiol . 55:1391 1399.
- Shreve , G.S. , S. Ingnva , and S.Gunnam. 1995 . Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *psendomoned aeruginosa*. Mol . Mar. Biol . Biotechnol. 4:331 337.
- Shreve , G.S. , S. Ingnva , and S.Gunnam. 1995 . Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *psendomoned aeruginosa*. Mol . Mar. Biol . Biotechnol. 4:331-337.
- Shuttelworth K.L.<and C.E.Cerniglia.1996.practical methods for the isolation of polycyclic aromatic hydrocarbons (PAH) degrading microorganisms and biodegradation intermediates .Mol.Biol.Rev.67:121 135.
- Sikkema , J. , J.A.M.Debont , and B.Poolman . 1995 . Mechanisms of membrane toxicity of hydrocarbons . Microbiol. Rev.59:201 222.

- Simon , M.J. ,T.D. Osslund , R.Saunders , B.D. Ensley , S.Suggs , A.Harcourt , W.C. Snen , D.L.Cruden , d.t.Gibson , and G.J.Zylstra . Dioxygenase in pseudomonas putida strain G7 and NCIB , 816 4 . Gene 127:31 37.
- Smith . M.J.G.Lethbridge . and R.Burns . 1997 . Bioavailability and biodegradation of polycyclic aromatic hydrocarbons in soils . Microbial . Lett . 152 (1) :141 147.
- Snape , I.M.J.Riddle , J.S.stark , C.M.cole, C.K.king,S.Duquesne , and D.B.gore.2001.managment and remediation of contaminated sites at casey station , Antractica . Intern.Biodegradation . 4:19.25
- Soharbi , M.,A. Mogharei and N.G haseemi 1999. Some aspects of bioremediation of soil contamincted with petroleum hydrocarbons Appl. Environ. Microbiol. 56: 307 – 312

- Soharbi , M.,A. Mogharei and N.G haseemi 1999. Some aspects of bioremediation of soil contamincted with petroleum hydrocarbons Appl. Environ. Microbiol. 56: 307 – 312

- Spence . M ., M . Grifoll and S. Kubota . 2005 . Isolation and characterization of hydrocarbon utilizing bacteria . Appl . Microbiol Biotechnol. 52 : 704 712 .

- Spence . M ., M . Grifoll and S. Kubota . 2005 . Isolation and characterization of hydrocarbon utilizing bacteria . Appl . Microbiol Biotechnol. 52 : 704- 712 .

- Stafford, S., Berwick, P., Hughes, D. W. and D. A. Stafford, 1982. Oil degradation in hydrocarbon and oil stressed environments. In: Experimental microbial ecology. Edited by R. G. Burns and J. H. Salter Blackwell scientific publishers, Oxford. P..591 612.
- Stokes , D.J.,B.L. Thiel & A.M. Donald . 1998.Direct observation of water – oil emulsion systems in the liquid state . chemosphere. 38(15): 3463 3473

- Stokes , D.J.,B.L. Thiel & A.M. Donald . 1998.Direct observation of water – oil emulsion systems in the liquid state . chemosphere. 38(15): 3463-3473
- Story , S.P,S.H. Parker , S.S. Hayaska , M.B. Riley and kline .2003.Conversion of divergent points in catabolic pathways involved in utilization of fluoranthene , naphthalene , anthracene and phenanthrene by *Sphingomonas spp*. J. Ind .Microbiol. Biotechnol. 26 : 369 382.
- Suehara , K.I. , Y.Kaswamoto , E. Fujii , J.Kohda , Y.Nakano and .Yano . 2005 . Biological treatment of wastewater discharged from biodiesel fuel production plant with Alkali – Catalyzed Transestrification.J.Biosc.Bioeg. 100(4):437 442 .
- Sveum, P. and S.ramstand . 1995 . Bioremediation of oil on shorelines with organic and inorganic nutrient . Environ . Technol.20:863 873.
- Tabataai , A.M. and W.A. . Dick . 2002 . Enzymes in soil environment activity . J. Bacteriol . 128 (1) : 485 496.
- Tabataai , A.M. and W.A. . Dick . 2002 . Enzymes in soil environment activity . J. Bacteriol . 128 (1) : 485-496.
- Tolker , N.T , U.C. Brinch , P.C. Ragas , J.B. Andersen , C.S. Jacobsen , and S. Molin . 2000 . Development and dynamics of *Psendomonas sp*. biofilms. J. Bacterial. 182: 6482 – 6489 .
- Twiss, M. R., L. Granier, P. Lafrance, and P. G. C. Campbell. 1999. Bioaccumulation of 2, 2, 5, 5 tetrachlorobiphenyl and pyrene by picoplankton: influence variable humic acid concentrations and pH. Environ. Toxicol. Chem. 18: 2063 2069.
- Upatham, E. S. 1998. Biodegradation of crude oil by soil microorgamisms in the tropic environment . Biodegradation. 9 (2) : 83 90.

- Upatham, E. S. 1998. Biodegradation of crude oil by soil microorgamisms in the tropic environment . Biodegradation. 9 (2) : 83- 90.
- Van Hamme, J. D., Singh, A., Ward, O. P. 2003. Recent advances in petroleum microbiology. Microbiol. Mol. Biol. Rev. 67: 503 549.
- Venosa , A.D., M.T.Suidan , D.King and B.A.Wrenn 1997 . use of hopane as aconservative biomarker for monitoring the bioremediation effectiveness of crude oil contaminating sites .J.Ind.Microbidbiot.18:131 139.
- Venosa , A.D.,M.T. Suidan , B.A.Wrenn , K.L.Strohmeier , J.R.Haines , B.L.Eberhart , D.king , and E.Holder . 1996 . Bioremediation of an experimental oil spill on the shoreline of Delaware Bay.Environ . Sci.Technol.30:1764 1775.
- Verdin A., A. L. H. Sahraoui, and R. Durand. 2004. Degradation of benzopyrene by mitosporic fungi and extracellular oxidative enzymes. Int. Biodeterior. Biodegrad. 55: 65 70.
- Verdin, A., A. L. H. Sahraoui, G. Robinson. And R. Durand. 2005. Effect of polycyclic aromatic hydrocarbon, benzopyrene, on the interacellular protein composition of *Fusarium solani* and *Fusarium* oxysporum . Int. Biodeterior. Biodegrad. 55: 171 174.
- Vinas , M., J.savate , M.Espnny , and A.M.Solanas . 2005. Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote – contaminated soil.Appl.Environ.Microbiol.71:7008 7018.
- Wackett , L . P . 1996 . Biologocal processing of coal . Appl . Microbiol . Biotechnol . 52: 16 24 .
- Wackett , L . P . 1996 . Biologocal processing of coal . Appl . Microbiol . Biotechnol . 52: 16-24 .

- Wang , Z. & M. Fingas . 2001 . Degradation of Petrolenm biomarkers . J. Chromatogr 926 : 275 290.
- Wang , Z. & M. Fingas . 2001 . Degradation of Petrolenm biomarkers . J. Chromatogr 926 : 275- 290.
- Wang, K. W., Baltzis B. C., Lewandoski, G. A. 1996. Kinetics of phenol biodegradation in the presence of glucose. Biotechnol Bioeng. 51: 87 94.
- Wang., Z., M. Fingas, Y. Y. Shu. L. Sigouin, M. Landriault, and P. Lambert. 1999. Quantitative characterization of PAHs in burn residue and soot samples and differentiation of pyrogenic APHs from petrogenic PAHs. Environ. Sci. Technol. 33: 3100 3109.
- Wasserman, and F.Durian (2006) .Characherizeation of hydrocarbon degrading bacterial communities from mangrove sediments in Guanabara Bay, Brazil.Con.J.Microbil.44:195 199.
- Wat Kinson, R., and P. Morgan. 1990. Physiology of aliphatic hydrocarbon degrading microorganisms. Biodegradation 1: 79 92.
- Whited , G.and D.T.Gibson.1991 . Toluene 4 monooxygenese , a three component enzyme system that catalyzes the oxidation of toluene of P cresol in Pseudomonas mendocina KRI.J.Bacteriol.173:3010 3016.
- whyte , C.,J.Saunders , and J.Rodriguez. 1996 . Assessment of the biodegradation potential of Microorganisms . Can . J.Microibiol.42:99 106.
- whyte , L. G. , L. Bourbonniere , and C.W. Greer . 1997 . Biodegradation of petroleum hydrocarbons by psychrotrophic pseudomonas strain possessing both alkane (alk) and naphthalene (nah) catabdic pathways . Appl . environ Microbiol. 63 : 201 211.
- Whyte , L.G. , S.T. Slagman. F. Pietrantonin , L. Bourbonnere , S.F. Koval , J.R. Lawrence , W.E. Inniss , and C.W. Greer . 1999 .

Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus sp*. Strain Q15. Appl. Environ. Microbiol. 65(7) : 2961 2968 .

- Whyte, L. G., J. Hawari, E. Zhou, L. Bourbonniere, W. E. Inniss, and C. W. Greer. 1998. Biodegradation of variable –chain – length alkanes at low temperature by a psy chrotrophic *Rhodococcus SP*. Appl. Environ Microbiol. 64: 2578 2584.
- Wilson , S.C. and K.C. Jones . 1993. Bioremediation of soli contaminated with polynucclear aromatic hydrocarbons . Rev . Environ . poll . 83 : 229 249.
- Wilson , S.C. and K.C. Jones . 1993. Bioremediation of soli contaminated with polynucclear aromatic hydrocarbons . Rev . Environ . poll . 83 : 229-249.
- Wilson S.C.and K.C.jones . 1993 . bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs). Areview . Environ.Poll . 83:229 – 259.
- Witt , M.E., M.J.Dubas , R.M.Worden , C.S.Criddle . 1999 .Motility – enhanced Bioremediation of carbon tetrachloride – contaminated aquifer sediments . Environ.Sci.Technol.33:2958 2964.
- Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, and D. E. Caldwell. 1998. In situ characterization of biofilm exopolymers involved in the accumulation of chlorinated organics Microb. Ecol. 35: 213 223.
- Wu.L.,D.R.Thompson ,G.Li,R.A.Hurt,J.M.Tiede,and J.Zhou.2001 .decelopment and evaluation of functional genes arrays for detection of selected genes in the environment.Appl.Environ.Microviol.67:5780 5790.

- Wubbolts , M. G. P. Reuvekamp , and B. witholt . 1994. TOL plasmid specified Xylene Oxygenase is a wide substrate range monooxygenase capable of olefin epoxidation .Enzyme Microbiol. Technol 16 : 608 615.
- Yang, Y., R. F. Chen, and M. P. Shiaris. 1999. Metabolism of naphthalene , fluorene , and phenanthrene, J. Bacteriol. 176: 2158 2164.
- Youseff , N.H., K.E.Duncan , and M.T.Mclnerney .2005.Importance of hydroxy fatty and composition of lippetides for biosurfactant activity.Appl.Environ.Microbial.71:7690 7695.
- Zajic, J. E., Panchel, C. J. Bio emulsifiers 1976. Rev. Microbiol. 5: 39 66.
- Zhang , y.& R.MILLER 1992. Enhanced octadecame oils dispersion and biodegradation by psendomonas rhmnolipid surfactant (biosurfactant) . Appl. Environ. Microbiol. 58 :3276 – 3282.
- Zhang , y.& R.MILLER -1992. Enhanced octadecame oils dispersion and biodegradation by psendomonas rhmnolipid surfactant (biosurfactant) . Appl. Environ. Microbiol. 58 :3276 – 3282.
- Zhang H.J.,A.,J.Kallimanis , A.I.J.Koukkou , and C.J.Drainas .2004.Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils.Appl.Microbiol.Biotechnol.65:124 131.

Zhou , J . , M.A. Bruns and J.M. Tiedje . 1996. DNA -
recovery from soils of diverse composition . Appl. Environ.
Microbiol. 62: 316 322 .

- ZoBell C. E. 1938. Studies on the occurrence and importance of bacteria in the marine sediments. Bacteriol 36: 662 670.

- ZoBell, C. E. 1972. Microbial degradation of oil. In: The Microbial degradation of oil pollutants. Ahearn & Me center for wetland Resources Louisiana State University Baton pp: 1603 1012.
- ZoBell, C. E. 1973. The microbial degradation of oil pollutants: present status and perspectives. Proceedings center for wetland resource , Lonisiana state University Baton Rouge . pp: 3 16.
- Zylstra , G . J . , E . Kim and A . K . Goyal . 1997 . comparative molecular analysis of genes for polycyclic aromatic hydrocarbon degradation . Genet . Eng . 19 : 257 269 .
- Zylstra , G . J . , E . Kim and A . K . Goyal . 1997 . comparative molecular analysis of genes for polycyclic aromatic hydrocarbon degradation . Genet . Eng . 19 : 257-269 .
- Zylstra ,G.J. , and E. Kim . 1997. Aromatic hydrocarbon degradation by *Sphingomonas Spp*.J. Ind. Microbiol. Biotechnol 19:408 414.

  Zylstra ,G.J.F . Boss and D.T. Gibson.1989.Toluene - degradation by *Pseudomonas Putida* F1 . J . Biol. Chem .264: 14940 14946 .
- Zylstra,G.J., W.R. Mccombie , D.T.Gibson , and B.A.Finette . 1988 . Toluene degradation by pswudomonas Putida FI : genetic organization of the tod operon . Appl.Environ.Microbiol.54:1498 1503.

- Abed , R . M . M ., N . M . D . Safi , J . koster , D . Beer , Y . EL – Nahhal , J . Rullkotter F. Garcia . 2002 . Microbial diversity of a heavily polluted microbail degradation of petroleum compounds . Appl. Environment Microbail . 68 : 1674-1683.
- AL- Tahhan , R.A.,T.R. Sandrin , A.A. Batour and R.M. Maier. 2000. Rhamnolipid-induced removal of lipopolysaccharide from *pseudomonas aeruginosa* : Effect on cell surface properties and intraction with hydrophobic substrate . Appl. Environ. Microbiol. 66 (8) : 3262- 3268.
- Alexander , M . 2000 Aging , bioavailability and overestimation of risk from environmental pollutants . Environ . Sci . Technol . 34 : 4259-4265 .
- Alloway .J . P . and R . K . Ayres . 1993 . Bioremediation of petroleum hydrocarbons in soil . Adv . Agron . 72 : 53-105.
- AL-Taher , W.A.Z.H. 2002 . Biodeterioration of oil emulsions and it's control by some chemical agents . ph.D thesis . Babylon university . Bablon – iraq.
- Andreoni , V ., L . Cavalca , A . Rao and G . Nocerino . 2004 . Bacterial communities and enzyme activities of PAHs polluted sites . Chemosphere 57 : 401-412.
- Anton , J.A,P. Adam & J. cannon . 2000 . Molecular evidence for biodegradation of macromolecule . Geochem. Cosmochem . Acta . 64 (20) 3525 – 3537.
- Balkwill , D . L . , G. R. Draker , R . H . Reeves and C. M. Spadoni . 1997 . Taxonomic study of aromatic – degrading bacteria *Sphingomonas* species . Int . Syst . Bacteriol . 47 : 191-201 .

- Bartha , H . J . 2003 . The influence of cyanobactenia on oil polluted intertidal soils at the saudi Arabian gulf shores . Mar . pollut . Bull . 46 : 1245 – 1252 .
- Beller , H . R ., S . Kane and T. Legler . 2002. Areal time PCR method for monitoring hydrocarbon degrading bacteria based on catabolic gene . Environ . Sci .Technol . 36 : 3977 – 3984 .
- Bogan , B . W . and R . T . Lamar 1996 . polycyclic aromatic hydrocarbon – degrading capabilities of *Phanerochaete laevis* and its extracellular lighinolytic enzymes . Appl . Environ . Microbiol . 62 (5) : 1597 – 1603.
- Boopathy , R . 2000 . Factors limting bioremediation technologies . Bioresour . Technol 74 : 63-67 .
- Bossert , I . and R . BARTHA . 1984 . The fate of petroleum in soil ecosystems . Biotechnol . Bioeng . 62 : 526 – 536 .
- Bossio , D . A . , K . M . Scow , N . Gunapala and K . J . Graham . 1998 . Determinants of soil microbial communities . Microb . Ecol. 36 : 1-12.
- Bouchez –Naitali , M ., R . Rakatoza and J . March . 1999 . Diversity of bacterial strains degrading hexadecane J . Appl . Microbiol . 86 : 421 – 428 .
- Budziniski , H., T . Nadalig , N. Natalie & M. Gilewicz . 2000 Evidence of two metabolic pathways for degradation of 2MP by Sphingomonas sp. Environ . Toxicol .chem.19(11) : 2672 – 2677.
- Cerniglia , C.E. and M . A . Heit camp . 1990 . Microbial degradation of polcyclic aromatic hydrocarbons (PAH) in the enviornment. J. Mol. 101—110.
- Churchil , P . and S . Churchill . 1997 .Surfactant – enhanced biodegradation of soild alkanes . J . Environ . Sci . Health 32 : 293 – 306 .

- Churchill , S.A.,J.P. Herper & R.F. churhill.1999. Isolation and characterization of Mycobacterium species capable of degrading three – and four – ring aromatic microbial. 65 (2) : 594-552.
- Cooper , D.G. and B.G. Golden berg . 1987. Sufface active agents from two Bacillus species. Microbiol . Rev . 49 :24-32.
- Da silva , M . L . and P. J . Alvarez . 2002 . Effects of ethanol on benzene , toluene , ethylbenzene Eng . 128 : 862 – 867 .
- Gaylarde , C.,C.,F.M. Bento & J. Kelly.1999. microbial contamination of stored hydrocarbon fuels and it's control. Rev. Micrebiol . 30 (1) : 1-10.
- Goodwin , K . D., R . Tokarczyk , F . Carol and E . Saltzman . 2005 . Description of toluene inhibition of hydrocarbon . biodegradation. Appl . Environ . Microbial 71 (7) : 3495 – 3503 .
- Grimm , A . C . and C . S. Harwood . 1997 . Chemotaxis of *pseudomonas* SP. to the polyaromatic hydrocarbon naphthalene . Appl . Environ . Microbiol . 63: 4111-4115 .
- Hallier – Soulier , S ., V . Duccrocq , N . Mazure , and N . Truffant . 1999 . Detection and quantification of degradative genes in soils contaminated by toluene . FEMS Microb . Ecol . 20 : 121-133 .
- Harrison , A . B ., M . B . Ripley and R . K . Dart . 2000 . Effect of protein hydrolysate on the degradation of diesel fuel in soil . Enzyme Microb . Technol . 26 : 388-393 .
- Holden , P.A., M.G. Bruce , A.K. Miller and W.G. Lindow . 1997 Biodegradation of hexadecane by *pseudomonas aeruginosa* . Appl . Environ . Microbiol . 68 : 250-258 .
- Holt, J. G., N.R. Krieg , P,H.A. Smeath , J.T. staley & S.T. Williams . 1994. Bergeys manual of determinative bacteriology. (9th ed.) Williams & Wilkins Inc., Baltimore, Mary land.

- Horowitz , A., D. Gutnick & C. Rosenberg . 1975. Sequential growth of bacteria on crude oil. Appl. Microbiol . 30 (1) : 10-19.
- Huesemann , M . H ., T . S . Hansmann and T . H . Fortman . 2002 . Microbiol Factors rather than bioavailability limit the rate and extent of PAH biodegradation in aged crude oil contaminated model soils . Biorem . J . 6: 321-336.
- Jeon , C.O., W. Park , C. Derito , J. R. Snape and E.L. Madsen . 2003 . Discovery of a bacterium with distinctive diooxygenase , that is responsible for in situ biodegradation in contaminated sediment . Int . J. Syst . Evol . Microbiol . 63 : 4516-4522 .
- Johensen , A . R ., L . Y . Wick and H . Harms . 2005 . Principles of microbial PAH – degradation in soil . Environ . poll . 133 : 71-84.
- Kappeli , A . C ., S. Thomas and R . Rouiever . 1991. Bacterial conversion of cyclic alkanes and polyaromatic hydrocarbons . Appl . Microbiol . Biotechnol . 58: 704-711 .
- Knaebel , D . B . and J . R . vestal . 1988 . Acomparison of double vial radiorespiratory to measure microbial mineralization in soil . J . Microbiol . Methods . 7 : 309-317 .
- Kropinski , A. m. b, L . chan and F.H . Milazzo . 1978 . Susceptibility of lipopolysaccharide defective mutants of *pseudomonas aeruginosa* to dyes , detergents , and antibiotics . Antimicrob . Agents . Chemo ther . 13 : 494 – 499.
- Leive , L1974 . The barrier function of the Gram negative envelope . Ann .Acad . Sci . 235 : 109-127.
- Lonon , M.K.,M.A Banto & R.H. Findlay. 1999 . Apilot study for monitoring changes in the microbiological component of MWFS as

a function of time & use in the system. Am. Ind. Hyg . Assoc . J. 60 (40) : 480 – 485

- Margesin , R . 1998 . Biodegradtion and bioremediation of hydrocarbons in extreme environments . Appl . Microbiol Biotechnol . 56 : 650 – 663.
- Marx , R . B . and D . Aitken . 1999 . Quantification of chemotaxis to naphthalene by *pseudomonas putida* . Appl . Environ . Microbiol . 65 : 2847 – 2852 .
- Matti , S. 1993. Distribution of oil-degrading bacteria in the North – West Arabian Gulf . Marine poll. Bull. 21:32-40.
- Mueller , J . G . S . M . Resnick , M . E . Shelton , and P. H Pitchard . 1992 . Effect of inoculation on the biodegradation of weathered Prudhoe Bay crude oil . J . Indst . Microb . 10 : 95 -102 .
- Nakahara , T., L. E. Erickson and J.R. Gutierreez . 1977 . Charscterization of hydrocarbon uptake in cultures with two liquid phases . Biotechnol . Bioeng 19: 9-25.
- Nedwell , B.D. 1999 Effect of low temperature on microbial growth. Microbiol . Eco . 30 : 101-111.
- Otenio , M.H., M.T. Dasilva , M.L. oliveira and E.D. Bidoia . 2005 . Benzen , toluene and xylene biodegradation by *Psendomonas putida* CCMI 852 . Brazil . J . Microbiol . 36 (3) : 433-442 .
- Poxton , I . R .1993. Prokaryote envelope diversity .J. Appl. Bacteriol. 74:1-11.
- Samanta , S . K ., A . K . Chakra and R . k . Jain . 1999 . Degradation of phenathnene by different bacteria . Appl . Microbiol . Biotechnol . 53 : 98-107 .
- Schmitt R., H .P . Rohs & P.Eckert 1996 . Biodegradation of aromatic hydrocarbon . Org . Geochem . 25 (1) : 41-50.

- Shreve , G.S. , S. Ingnva , and S.Gunnam. 1995 . Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *psendomoned aeruginosa*. Mol . Mar. Biol . Biotechnol. 4:331-337.
- Soharbi , M.,A. Mogharei and N.G haseemi 1999. Some aspects of bioremediation of soil contamincted with petroleum hydrocarbons Appl. Environ. Microbiol. 56: 307 – 312
- Spence . M ., M . Grifoll and S. Kubota . 2005 . Isolation and characterization of hydrocarbon utilizing bacteria . Appl . Microbiol Biotechnol. 52 : 704- 712 .
- Stokes , D.J.,B.L. Thiel & A.M. Donald . 1998.Direct observation of water – oil emulsion systems in the liquid state . chemosphere. 38(15): 3463-3473
- Tabataai , A.M. and W.A. . Dick . 2002 . Enzymes in soil environment activity . J. Bacteriol . 128 (1) : 485-496.
- Upatham, E. S. 1998. Biodegradation of crude oil by soil microorgamisms in the tropic environment . Biodegradation. 9 (2) : 83- 90.
- Wackett , L . P . 1996 . Biologocal processing of coal . Appl . Microbiol . Biotechnol . 52: 16-24 .
- Wang , Z. & M. Fingas . 2001 . Degradation of Petrolenm biomarkers . J. Chromatogr 926 : 275- 290.
- Wilson , S.C. and K.C. Jones . 1993. Bioremediation of soli contaminated with polynucclear aromatic hydrocarbons . Rev . Environ . poll . 83 : 229-249.
- Zhang , y.& R.MILLER -1992. Enhanced octadecame oils dispersion and biodegradation by psendomonas rhmnolipid surfactant (biosurfactant) . Appl. Environ. Microbiol. 58 :3276 – 3282.

- Zylstra , G . J . , E . Kim and A . K . Goyal . 1997 . comparative molecular analysis of genes for polycyclic aromatic hydrocarbon degradation . Genet . Eng . 19 : 257-269 .

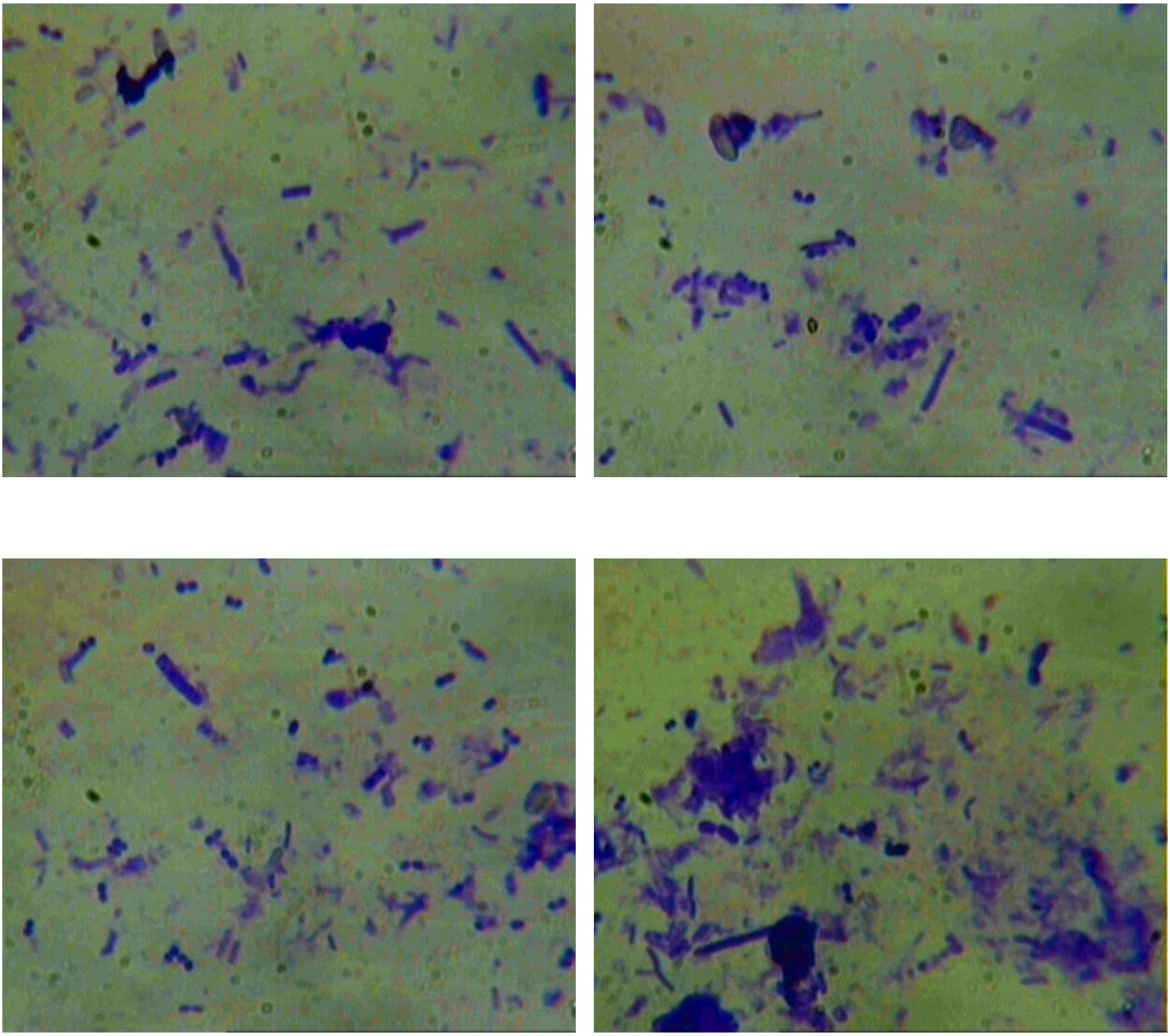


Fig. 30. Simple stain of different types of bacteria found in the imported consortium (1000X, Olympus Microscope).

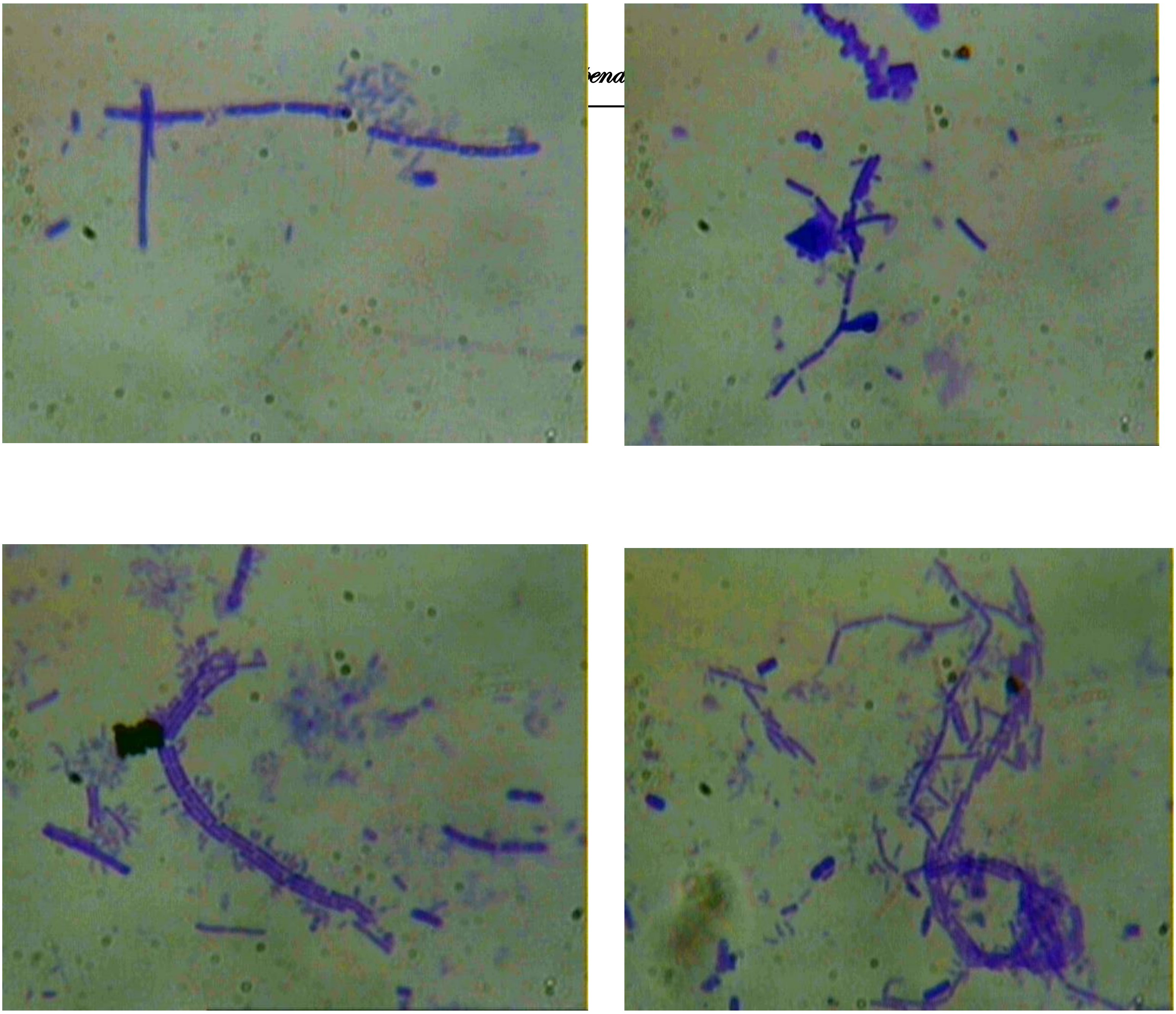
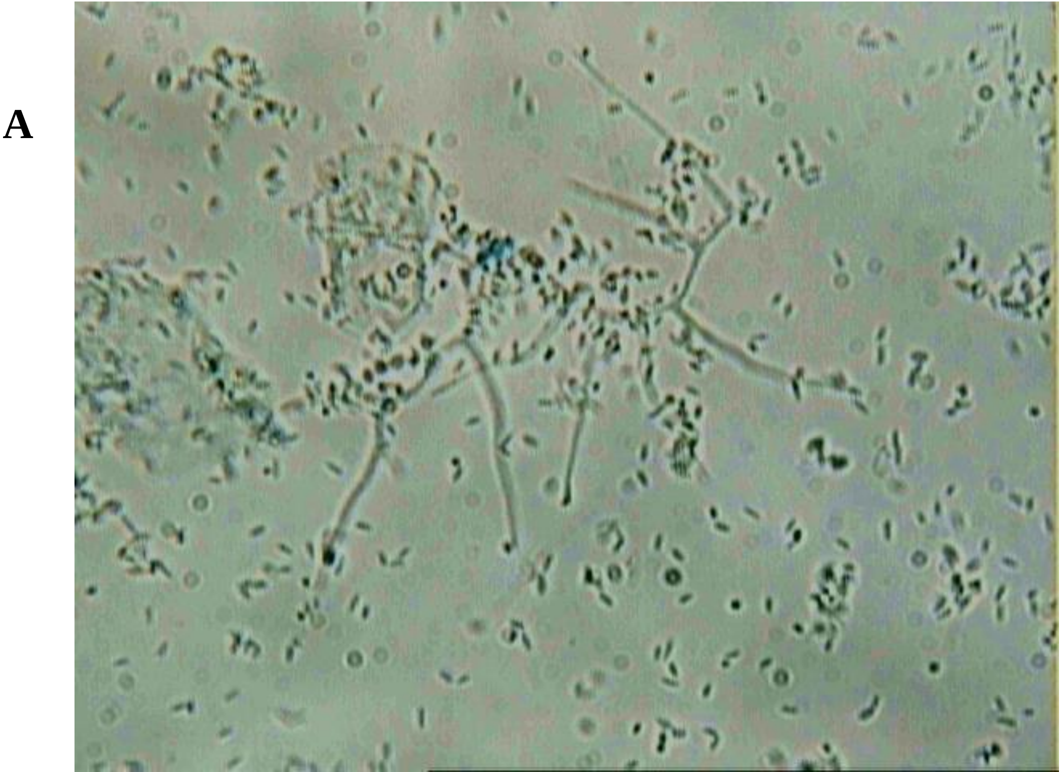


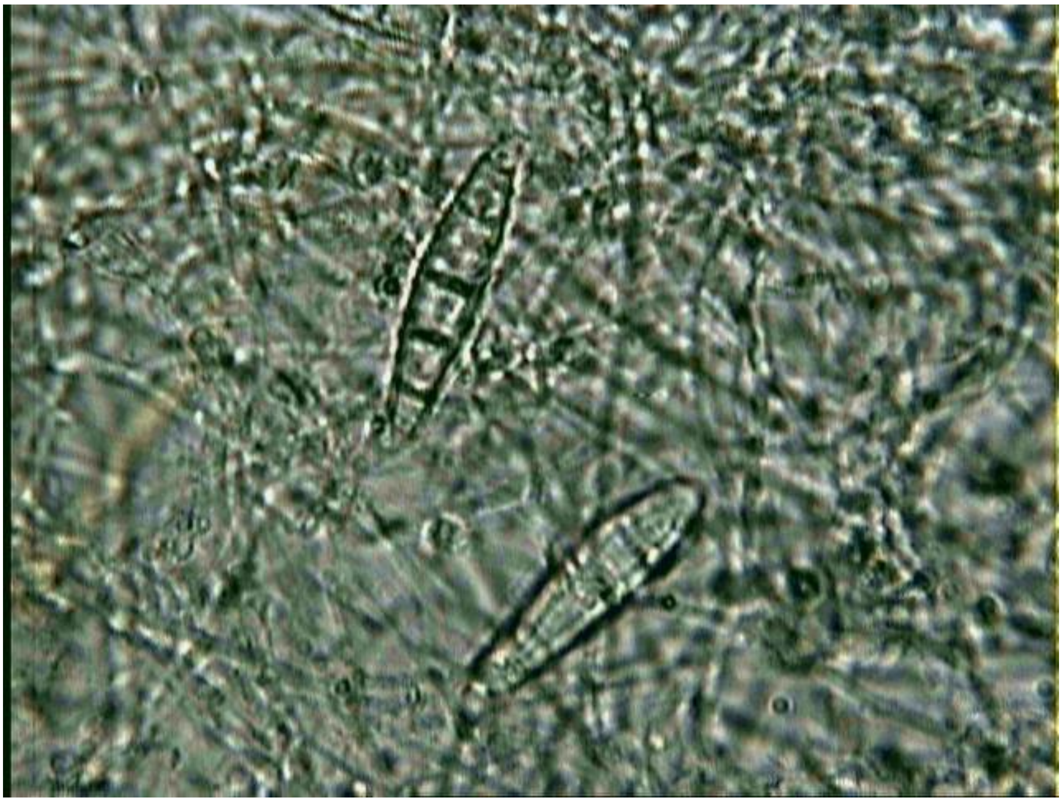
Fig .31. Simple stain of different types of bacterial cells in the imported concortium (1000X, Olympus Microscope)



B

Fig.32. A:Actinomycetes , showing chains of aerial conidia
B: Single conidia on short lateral branches of fungi found in the imported consortium (4000X, Olympus Microscope)

A



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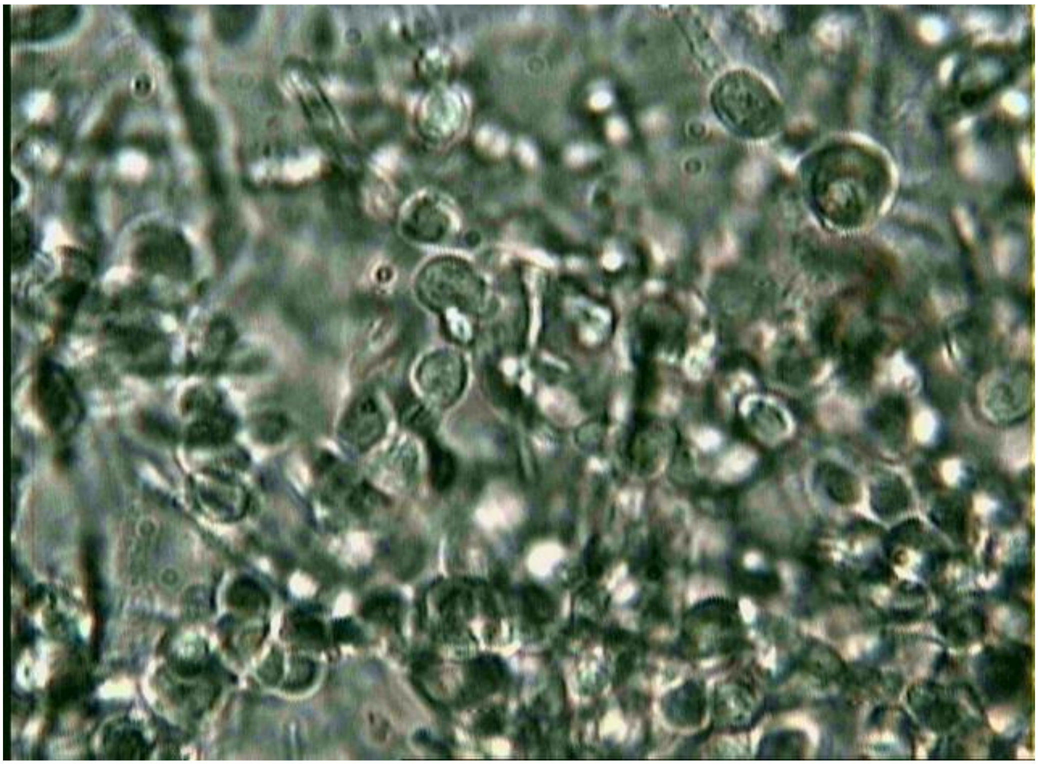


Fig.33: A and B showed different types of macroconidia composed of fungi found in the imported consortium (4000X, Olympus Microscope , Lactophenol blue)

B

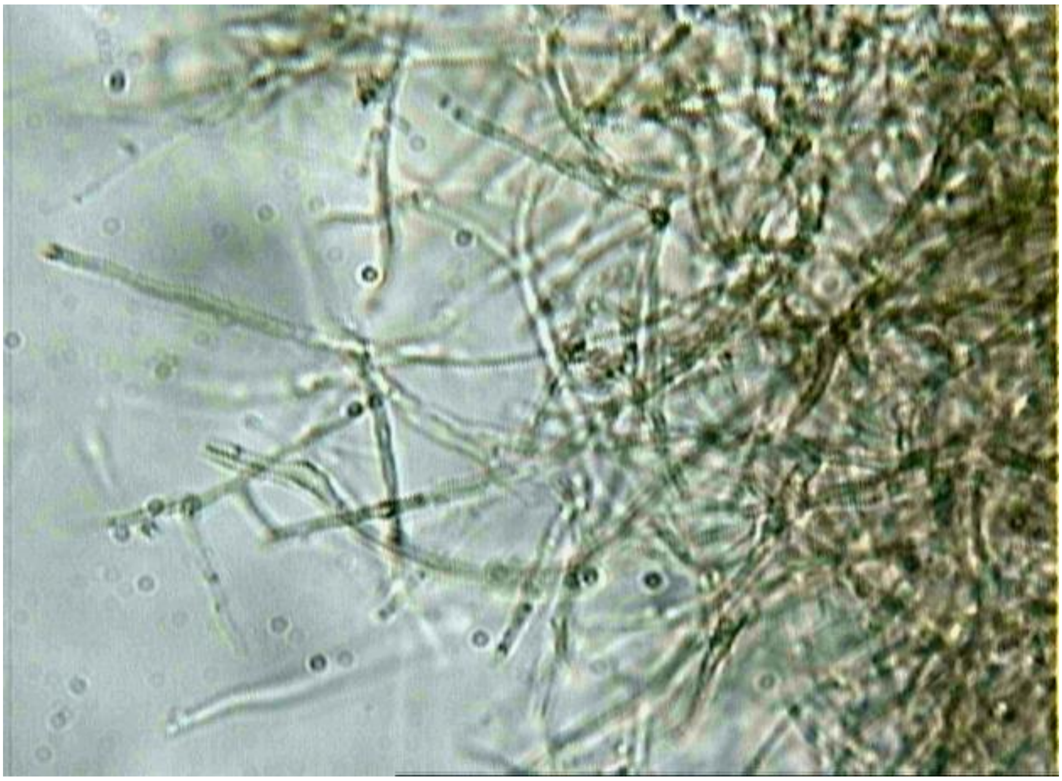
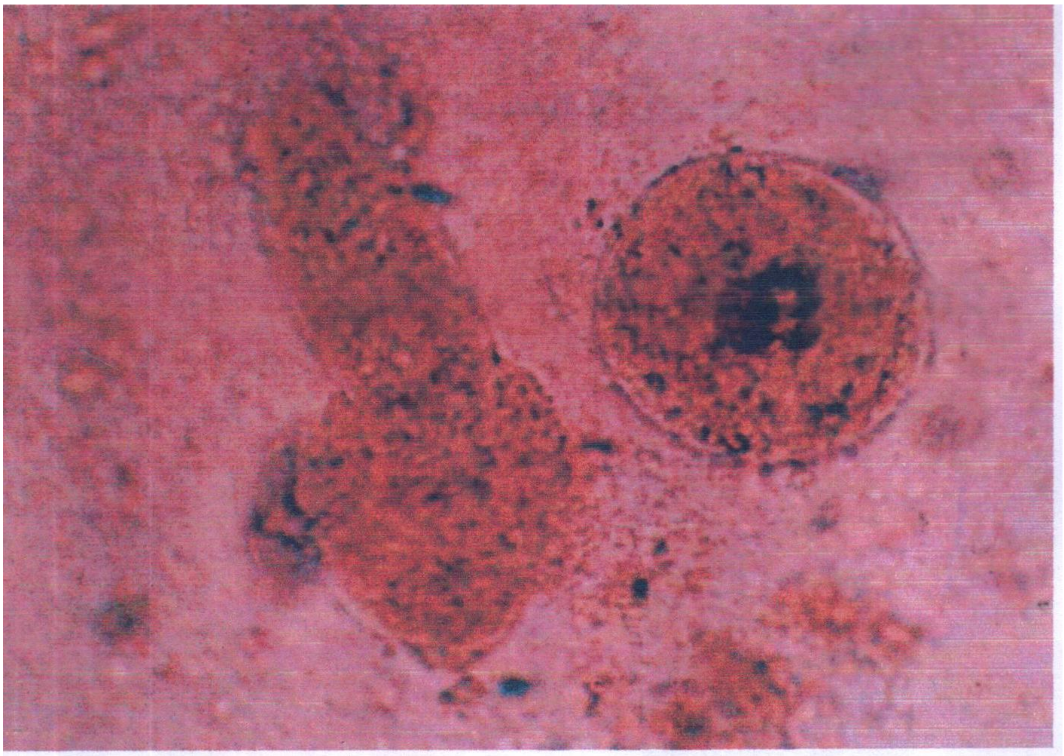


Fig.34. A and B showed different types of microbonidia of fungi found in the imported consortium (4000X, Olympus Microscope, Lactophenol blue)

A



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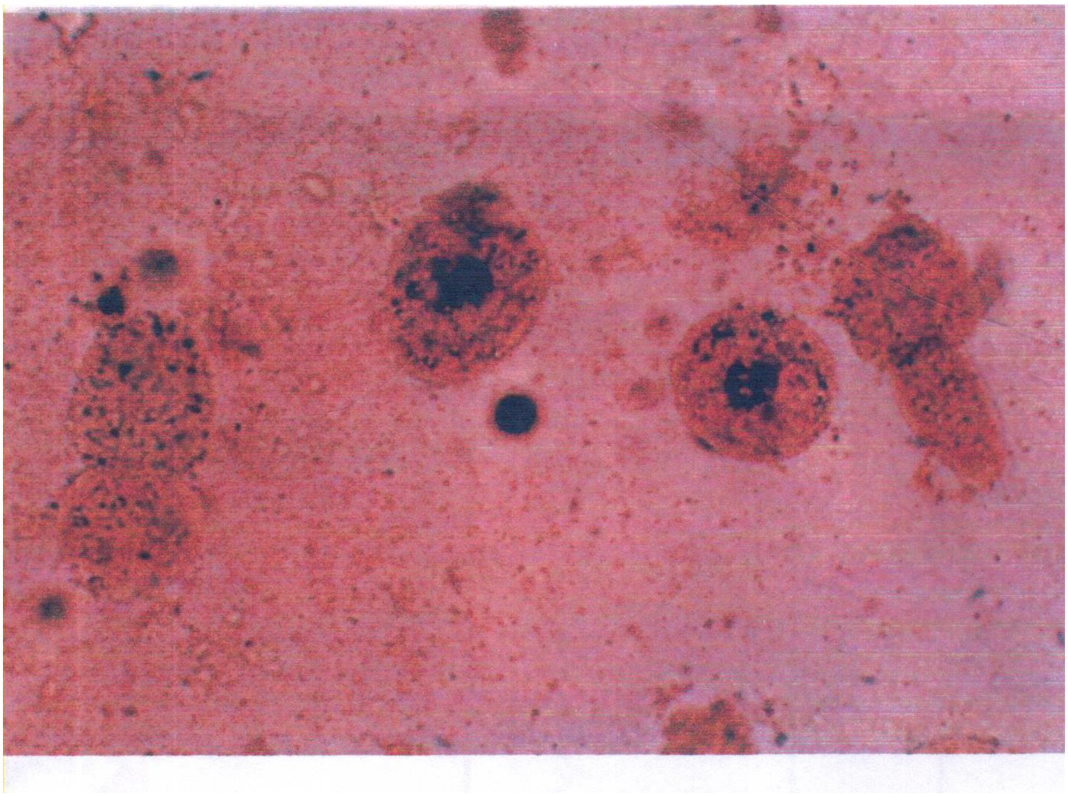

Fig.35. A and B showed different types of protozoa found in the imported consortium(4000X, Olympus Microscope, eosin Y)