Molecular detection of biodegradation and biosurfactant-producing bacteria isolated from hydrocarbon contaminated soils in the Diwaniya city/ Al-Qadisiya governorate

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Abstract:

As the usage of hydrocarbons increase soil contamination with diesel, engine and lubricating oils is becoming one of the major environmental problems this investigation was carried out to determine the bacterial flora of soils contaminated with used oils in the Diwaniya city / Al-Qadisiya governorate . Bacteria were screened for biosurfactants production by using oil spreading technique and hemolytic activity . Isolated bacteria were screened for the presence of one of the hydrocarbon degrading enzyme catechol 2,3 dioxygenase(C23O) and rhamnosyl transferase I (rhIB) enzyme which is involve in the production of biosurfactant by polymerase chain reaction amplification of genes using specific primers.

Enrichment method was employed for the isolation of the bacteria . Soil samples from 17 different repairing car stations and electrical generators in the Diwaniya city were inoculated minimal salt medium (MSM) with crude oil as uniqe carbon source . The results showed that the bacterial species isolated were *Pseudomonas* spp. 14 isolates (66%) , *Bacillus* spp. 3 isolates (14%) , *Micrococcus* spp. 2 isolates (10%) and one isolate (5%) for each *Staphylococcus* spp. and *Streptococcus* spp.

In case of the ability of bacterial isolates to produce biosurfactants , the results showed that using oil spreading technique (among three different oils : crude oil , diesel and kerosene) , kerosene was the best source for the production of biosurfactants in both *Pseudomonas* spp. and *Bacillus* spp. and *Pseudomonas* spp. showed higher activity than *Bacillus* spp. Also the results showed that oil spreading technique was better predicted biosurfactants production than the hemolytic activity . The difference in mean biosurfactant production by using two way ANOVA was found to be statistically significant at the p-values of $p < 0.05$ (at 0.05 level of significance) between different methods and different bacteria .

Molecular detection of *C23O* and *rhIB* genes, 12 isolates (58%) of degrading bacteria isolates from all twenty one bacterial isolates from contaminated soils expressed the *C23O* gene with highest percentage (43%) in *Pseudomonas* spp. This study showed that all *Pseudomonas aeruginosa* ability to produce *rhIB* gene and this DNA came from *P. aeruginosa*.

Introduction :

Hydrocarbons such as diesel fuel, crude oil, lubricating oils and petroleum distillates are some of the world's most widely used primary energy and fuel recourses (Ganesh and Lin, 2009). The presence of different types of automobiles, electrical generators and machinery has resulted in an increase in the use of Hydrocarbon materials (Abioye *et al.*, 2012).Hydrocarbons contain benzene cyclopentadiene, dicyclopentadiene , styrene,toluene and xylene as major components and many other hydrocarbons as minor components (Santhini *et al.*, 2009) .These complex mixtures of molecules are usually highly toxic to many organisms including human beings (Makut and Ishaya , 2010).

Environmental pollution with hydrocarbons has been recognized as one of the most serious current problems especially when associated with accidental spills on a large scale (Nikhil *et al.* , 2013). Which have become a global problem particularly in industrialized and developing countries . If this occurs, hydrocarbons may reach the water table before becoming immobilized in the soil (Borah and Yadav , 2012).

Spills of hydrocarbons may occur from several leakage from tanks and dumping of waste petroleum products (Borah and Yadar , 2012). Among the different technologies used during oil spills responses, a widely preferred and promising technology is bioremediations. Bioremediations has become an alternative way to remediate oil polluted sites where the additions specific microorganism can improve biodegradation efficiency (Opasola *et al.*, 2011) .

A wide variety of bacteria are capable of degrading hydrocarbon fractions and can applied to rehabilitate hydrocarbon contaminated soil (Chaudhry *et al.*, 2005). A large number of *Pseudomonas* strains are capable of degrading hydrocarbons isolated from soil (Luo *et al.*, 2012). Other hydrocarbon degraders includes *Acinetobacter* spp., *Bacillus* spp., *Micrococcus* spp., *Flavobacterium* spp. and *Streptococcus* spp. (Raza *et al.*, 2010 ;Bayoumi *et al.*, 2011; Vijaya *et al.*, 2013).

One of the most characteristics of hydrocarbon degrading bacteria is the ability of emulsifying hydrocarbons in solutions by producing surface-active agents such as biosurfactants(Ganesh and Lin , 2009). Biosurfactants are unique amphiphilic biological compounds produced extracellular or as part of the cell membrane by a variety of bacteria , yeast and filamentous fungi (Liu *et al.*, 2011). These complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, antibiotics, lipopeptides, glycolipids, etc (Singh , 2012).Biosurfactants have several advantages including two toxicity, high biodegradability, low irritant, environmental compatibility, high selectivity an specific activityal extreme temperature, pH and salinity (Plaza *et al.*, 2008). Biosurfactants are directly involved in the process of hydrocarbon removed from the environment through the increased surface area of hydrophobic water-insoluble substances , this lead to produce long chain hydrocarbons to microbes and renders them more access to a microbial enzyme system for the utilization(Vijaya *et al.*, 2013). Rhmnolipids as a potent natural biosurfactant has a wide range of potential applications , including enhanced oil recovery (EOR) , biodegradations and bioremediations (Singh , 2012) . Rhamnolipids (Glycolipids) are a thermo-tolerant biosurfactant produced by *P. aeruginosa* the term is indicative of the fact that these lipids contain one or two rhmanose units , linked glucosidically to one or two molecules of β-hydroxydecanoic acid,thus, the monorhamnolipid from *P. aeruginosa* grown on hydrocarbon is2-o- α-L-rhamnopyranosyl-αL-3-hydroxydecanoyl-3-hydroxydecanoic acid(Arutchelvi *et al.*,2008).The aim of this study was screen and isolate biodegradation and biosurfactant producing bacteria from the hydrocarbon contaminated soil and screen the biosurfactant production by oil spreading technique and hemolytic activity . This research also focused on the screen for presence of catechol 2,3 dioxgyenase (*C23O*)gene (216 bp) that involved in the hydrocarbon degradation and rhamnotransferase I (*rhIB*) gene (723bp) that involved in the biosurfactant production from *P. aeruginosa* .

Materials and Methods

-Sampling and isolation of hydrocarbon degrading bacteria by enrichment method:

Seventeen different oil polluted soil samples (included petrol , diesel , kerosene and lubricating oils) were collected from repairing car stations and electrical generators in the Diwaniya city/Al-Qadisiya governorate. Soil samples were collected at depth within 5 cm from the surface of the soil . They were collected in sterile polyethylene bags and tightly packed . They were then carefully transferred in an ice tank to complete the crude oil utilizing heterotrophic microbial isolation and stored at 4 ^{o}C immediately (Santhini *et al.*, 2009).

Each soil sample 1g was inoculated into 50 ml minimal salt medium (MSM) in a 250 ml conical flask. The medium contained 1 g KH_2PO_4, 0.5 g $MgSO_4$. 0.01 g $FeSO_4$, 1.5 g $NaNO_3$ and 0.002 g $CaCl_2$ per liter. Amount of 1.5g from $(NH_4)_2SO_4$ was supplemented with 5%(V/V) crude oil was obtained from the Shinafiyah refinery (which is located in Al-Qadisiya governorate) as the sole carbon source. Inoculation was performed with shaking 180 rpm at 37^{o}C for 7 days (Liu *et al.*, 2011).

-Identification of the Bacterial isolates:

A volume of 5 ml of enriched media was transferred into freshly prepared media on each week supplemented with 5% crude oil and then incubated at 30^{o}C . The single colonies were streaked onto nutrient agar plates. The plates were incubated at 30^{o}C overnight . Pure cultures of bacterial isolates were identified based on morphological (colonial and cellular observation) and biochemical characterizations were determined according to the manual of determinative bacteriology (Kaplan and Kitts ,2004; Nikhil *et al.* , 2013). For day to day experiments strains were maintained on nutrient agar slants at 4 C^{o} in refrigerator and subcultures at an interval of 30 days.

Screening for biosurfactant producers

The isolated colonies were tested for their biosurfactant production by two methods:

1- Hemolytic activity

Isolates were screened on blood agar plates containing 5% sheep blood and incubated at 37^{o}C for 48h . Hemolytic activity was detected as the presence of clear zone around bacterial isolates (Suganya , 2013). This clear zone indicates the presence of biosurfactant producing bacteria.

2- Oil spreading technique

The selected bacteria were compared by measuring of the diameter of the clear zones occurred when a drop of a biosurfactant containing solution is placed on an oil-water surface . Fifty ml of distilled water was added to a large Petri dish (15 cm diameter) followed by the addition 20 µl of (crude oil, diesel and kerosene) to the surface , 10µ of supernatant of the culture broth (Techaoei *et al.* , 2007).

-Molecular characterization:

-Isolation of total genomic DNA from bacteria:

DNA from all the degrading bacteria isolates were extracted from 1.5 ml of bacterial growth using Genomic DNA mini kit (Geneaid , Korea) according to the manufacturer's instructions . The DNA quality was assessed by 1.5% (W/V) agarose gel electrophoresis .

-Detection of the catechol 2,3 dioxygenase (*C23O*) gene:

The amplification of the degradative catechol 2,3 dioxygenase gene using the primer pair *C23O* Forward 5'-CGACCTGATCATCGCATGACCGA-3' and C23O Reverse 5'-TCTAGGTCAGTACACGGTCA-3' according to Jyothi *et al.*(2012).The amplification was performed in a final volume of 20 µl containing 250 mM of each of the four dNTP, 1.5 µl of 10 pmol/ µl of each primer, 5 µl of extracted DNA and 1 unit of Taq DNA polymerase with 12 µl of PCR water .

Amplification was performed on a Gene AMP thermocycler system . The reaction conditions as follows : an initial denaturation step of 94C^{o} for 5min. was followed by 35 cycles of amplification (94^{o}C for 45 sec., 55^{o}C for 1min. and 72^{o}C for 1 min.) and a final extension step at 72^{o}C for 10 min. .Amplified PCR products was confirmed by electrophoresis on1.5% agarose gel .

-Detection of rhamnosyl transferase I (*rhIB*) gene :

The primers sequences for *rhIB* gene obtained by Mathiyazhagan (2011). The forward primer is 5'-GCCCACGACCAGTTCGAC-3' and the reverse primer sequence is 5'-CATCCCCCTCCCTATGAC-3'.

The amplification reaction mixture for *rhIB* gene contained : 5 µl of DNA template. 1.5 µl of 10 pmol/ µl of each primers, 250µM of dNTP, 1.5 mM of $MgCl_2$, 30 mM of KCl and 1 unit of Taq DNA polymerase with PCR water added to obtain 20 µl final volume in the PCR tube. The reactions were exposed to 94^{o}C for 2min. then to 30 cycles of 94^{o}C for 15 sec., 45^{o}C 15sec. and 72^{o}C for 15 sec. The final extension is done 72^{o}C for 2min. The amplification was done using a Gene Amp PCR system thermal cycler. The PCR product was visualized by electrophoresis through a 2% agarose gel.

Statistical analysis

Statistical analysis was performed by using SPSS 11.5 Windows software . The difference in mean biosurfactant production between different methods and between different bacteria were analyzed by applying two way ANOVA . Mean values were expressed at 0.05 level of significance .

-Results and Discussion:

-Isolation of bacteria from Hydrocarbon contaminated soil:

Bacteria were isolated from Hydrocarbon contaminated soil samples by using minimal salt medium supplemented with 5% crude oil as the source of carbon and energy (Figure 1) .

Figure (1) : MSM + crude oil medium

Then the isolated bacteria were identified morphological and biochemical characteristics (Table 1a . 1b) .

Table (1a): Morphological and biochemical tests of bacteria species able to grow in medium with crude oil as unique carbon source

Bacteria / Features	*Pseudomonas* spp.	*Micrococcus* spp.	*Staphylococcus* spp.	*Streptococcus* spp.	*Bacillus* spp.
Gram stain	-ve	+ve	+ve	+ve	+ve
Cell shape	Rod	Cocci	Cocci	Cocci	Rod
Spores	-ve	-ve	-ve	-ve	+ve
Motility	Motile	Non motile	Non motile	Non motile	Motile
Oxidase activity	+ve	+ve	-ve	-ve	-ve
Gatalase	+ve	+ve	+ve	+ve	+ve
Gelatinase	+ve	-ve	-ve	-ve	-ve
Starch hydrolysis	+ve	-ve	-ve	-ve	+ve
Urease	-ve	+ve	+ve	-ve	+ve

+ve: positive , -ve: negative

Table (1b): Biochemical tests of the *Pseudomonas* spp. isolated from hydrocarbon contaminated soils

Biochemical test	*P. aeruginosa*	*P. inflorescence*
Indole production	+ve	-ve
Methyl red	-ve	+ve
Voges proskauer	+ve	+ve
Citrate utilization	+ve	-ve
H_2S production	-ve	+ve

Total five species of bacteria were isolated , they are *Pseudomonas* spp. 14 isolates (66%) , *Micrococcus* spp. 2 isolates (10%) *Bacillus* spp. 3 isolates (14%) and 1 isolate (5%) for both *Staphylococcus* spp. and *Streptococcus* spp. Initially 21 isolates were isolated from 17 hydrocarbon contaminated soil samples (figure 2) .

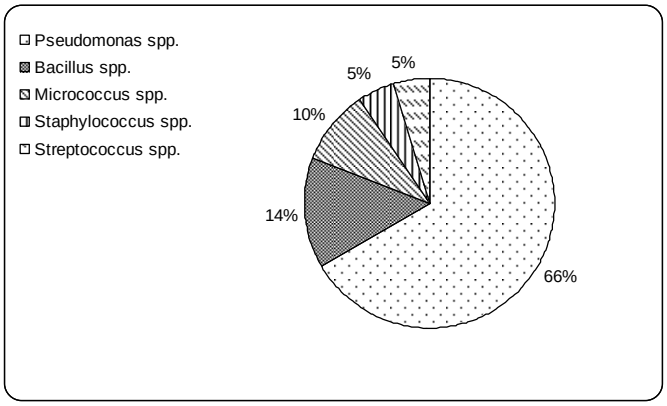


Figure (2) : the percentage of each bacterial species isolated from hydrocarbon contaminated soils

It was observed that *Pseudomonas* spp. and *Bacillus* spp. had highest percentage among bacterial hydrocarbon degraders. These species majorities of which have already been reported by other researchers and have shown biodegradation capabilities (Mittal and Singh, 2009; Makut and Ishaya, 2010; Hamzah *et al.*, 2011; Luo *et al.*,2012) .This may be due to efficient hydrocarbon degrading enzyme system that these organisms possess(Abioye *et al.*, 2012). According to many researches showed that the *Pseudomonas* spp. have various metabolisms , which associated to the presence of degradative plasmids such as ALK (alkanes), OCT(octane), XYL (xylene), CAM(alcanphour), NAH(naphthalene) , TOL (toluene) and SAL(salicylic acid) (Perez – Silva *et al.* , 2006) , that may increase its ability to degradarive hydrocarbons .

The results of the present study was support other studies done by Borah and Yadav(2012) and Vijaya *et al.*(2013) in India where they're isolated *Pseudomonas* spp., *Bacillus* spp., *Streptococcus* spp., *Micrococcus* spp. and *staphylococcus* spp. from various parts of automobile engines and petrochemical contaminated sites in and around Bangalore city.

-Screening for biosurfactant activity:

1- Hemolytic activity :

The blood agar method is often used for a preliminary screening of microorganisms for ability to produce biosurfactants on hydrophilic media (Suganya , 2013). All of the isolated bacteria 21 isolates were tested for hemolytic activity . Among the isolates 14 (66%) showed hemolytic activity (table 2). Selected isolates were used for further screening . Tabatabaee *et al.* (2005) and Karthik *et al.* (2010) were using this method to screen the biosurfactant producing microorganisms.

Table (2) : Hemolytic activity and oil spreading technique for bacteria isolated from hydrocarbon contaminated soils

Bacteria	Isolates No.	Oil spreading technique Zone formation (mm)			Hemolytic activity
		Crude oil	Diesel	Kerosene	
P. aeruginosa	1	18	20	30	+
	2	15	20	20	+
	3	Non zone	Non zone	5	+
	4	16	16	13	+
	5	*	*	*	-
	6	20	22	25	+
	7	7	20	38	+
	8	*	*	*	-
	9	*	*	*	-
	10	20	35	52	+
	11	18	18	22	+
	12	8	10	10	+
	13	8	8	12	+
P. inflorescence	14	*	*	*	-
Bacillus spp.	1	*	*	*	-
	2	15	16	20	+
	3	9	15	18	+
Micrococcus spp.	1	*	*	*	-
	2	5	5	5	+
Staphylococcus spp.	1	Non zone	Non zone	Non zone	+
Streptococcus spp.	1	*	*	*	-

*:test was not done. +:positive , -:negative

2- Oil spreading technique (OST) :

Fourteen bacterial isolates were further screened to conform to their biosurfactant production by oil spreading technique . These isolates were centrifuged and added to different oil (crude oil , diesel and Kerosene) containing plates . The biosurfactant producing organism could only be to displace the oil and the oil dispersed zone was measured in mm (Figure 3).

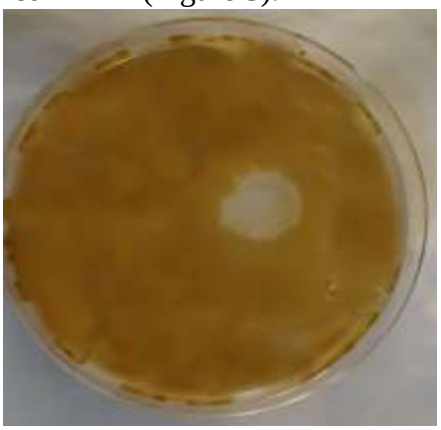


Figure (3) : Zone formation of biosurfactant producing bacteria in oil spreading technique

The results from table (2) and figure(4) showed that *P. aeruginosa* , 10 isolates (77%) , *Bacillus* spp. 2 isolates (15%) and one isolate (8%) of *Micrococcus* spp. as producers for biosurfactant in crude oil , diesel and kerosene . The other organism *Staphylococcus* spp. one isolate (8%) did not show ability as biosurfactant producer.

The results also showed the highest biosurfactant activity was in kerosene which again indicates that produced biosurfactant has better activity against crude oil . the result agreed with study by Samanta *et al.* (2012) who found that kerosene was a better activity against crude oil .

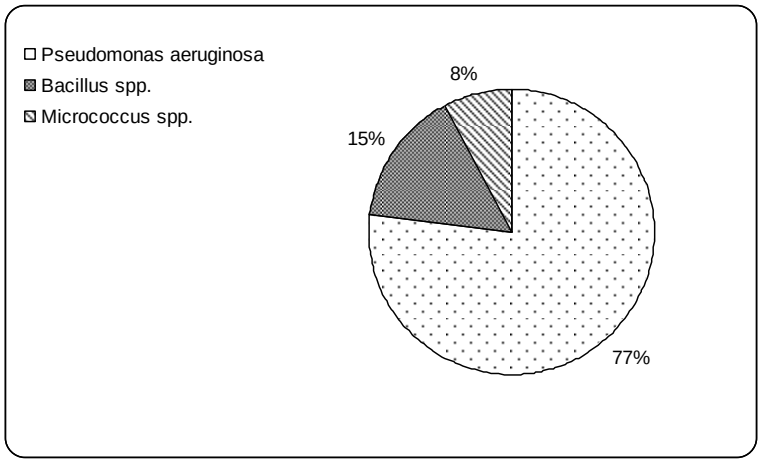


Figure (4) : The percentage of biosurfactant producing bacteria in oil spreading technique

When compared this method(OST) with hemolytic activity (table 2) , the searcher found that some bacterial isolates like P. aeruginosa (3) and *Staphylococcus* spp. (1) Showed haemolysis activity but in oil spreading technique , these isolates showed less or no activity . In another study Youssef *et al.* (2004) found 16% of

false positive results in hemolytic activity for biosurfactant screening due to lyses blood agar by microorganism virulence factors and biosurfactant that are poorly diffusible way not lyses blood cells. Therefore, Kiran *et al.* (2010) suggested that the single screening method is unsuitable for identifying all types of biosurfactants and recommended that more than one screening method should included during primary screening of biosurfactant producers, In the present study used hemolytic activity assay and oil spreading technique. The difference in mean biosurfactant production by using two way ANOVA was found to be statistically significant at the p- values of $p < 0.05$ (at 0.05 level of significance) between different methods and different bacteria .The results of the present study suggested that oil spreading technique was better predicted biosurfactant production than the hemolytic activity because it is very sensitive for detection biosurfactant and it had several advantages in requiring a small volume of samples and rapid and easy to be carried out and do not require specialized equipment.

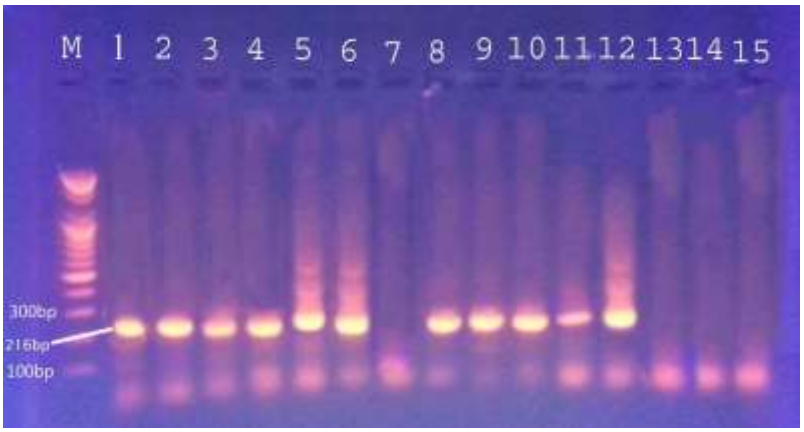
-Detection of catechol 2,3 dioxygenase (*C23O*) gene :

Catechol 2,3 dioxygenase is the one of the exordial enzymes that involved in hydrocarbon degradation by cleaving the aromatic ring between on hydroxylated carbon and other adjacent non-hydroxylated carbonated. In bacteria , this enzyme plays a key role in the metabolism of aromatic compounds . It is responsible for cleavage of aromatic rings during the aerobic catabolism of hydrocarbon compounds (Malkawi *et al.*, 2009).

The presence of the catechol 2,3 dioxygenase enzyme in these identified hydrocarbon degrading bacteria by using C23O specific primers. These primers used to give the (216bp) PCR product. Results of gene amplification by PCR are shown in (table 3 , figure 5).

Table (3): frequency of *C23O* and *rhIB* genes detected from Bacterial isolates

Gene		*Pseudomonas* spp.	*Bacillus* spp.	*Micrococcus* spp.	*Staphylococcus* spp.	*Streptococcus* spp.	Total
C23O	No.	9	2	1	0	0	12
	%	(43)	(10)	(5)	(0)	(0)	(58)
rhIB	No.	13	____	____	____	____	13
	%	(93)					(93)



Figure(5):The amplified *C23O* gene product(216 bp), M:Marker (100 -1200 bp)
- Lane (1-6 , 8-12) positive *C23O* gene
- Lane (7 , 13-15) Negative *C23O* gene

A total of 12 (58%) of degrading bacteria isolates expressed the *C23O* gene with the highest percentage (43% , 10%) in *Pseudomonas* spp. and *Bacillus* spp. respectively. This finding was similar to other studies done by Jyothi *et al.* (2012) and Benedek *et al.* (2011) in which they found that *C23O* gene was involved in hydrocarbon degradation for *Pseudomonas* spp. and *Bacillus* spp. isolated from the waste water and contaminated soils.

The rest of the bacterial isolates 9(42%) were no amplification that indicating the absence of catechol 2,3 dioxygenase enzyme activities for the hydrocarbon degradation.

-Detection of rhamnosyl transferase 1(rhIB) gene:

One major class of biosurfactants is the glycolipids which includes rhamnolipids , trehalose lipids and sophorose lipids. Rhamnolipids are produced only by *P. aeruginosa* (Mazaheri Assadi and Tabatabaee , 2010).

Fourteen *Pseudomonas* spp. isolates were obtained during the hydrocarbon contaminated soil screening. Thirteen isolates were identified as *P. aeruginosa* and on isolates was *P. influorescens* (table 1b). All 14 isolates were subjected to PCR analysis for detecting *rhIB* gene . The results showed (table 3 , figure 6) that all *P. aeruginosa* isolates the amplified of *rhIB* gene. The observed bands were compared with molecular marker (1.2Kbp) and the presence of amplified product of 723bp level confirmed the presence of *rhIB* gene. This finding was similar to other studies done by Elouzil *et al.* (2009) from Libya and Mathiyazhagan (2011) from India in which they found all *P. aeruginosa* isolates had *rhIB* gene.

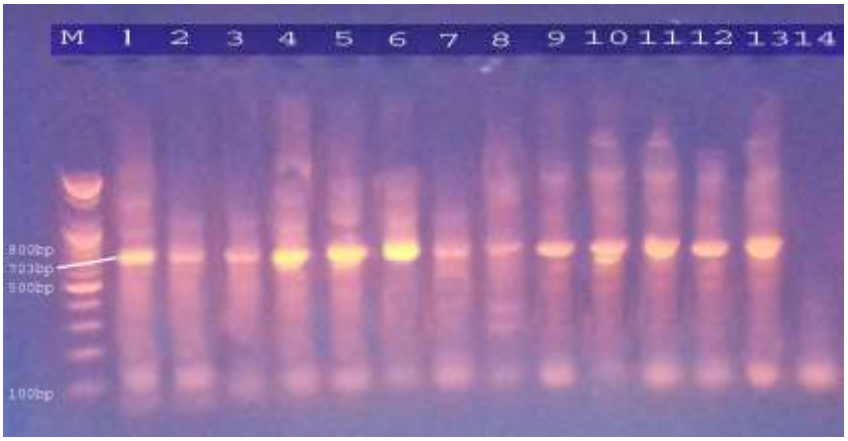


Figure (6):The amplified *rhIB* gene product (723 bp) , M:Marker(100-1200 bp)
- Lane (1-13) positive *rhIB* gene
- Lane (14) Negative *rhIB* gene

Conclusions

The results showed that the soil samples from repairing car stations and electrical generators are good sources for screening of hydrocarbon degrading and biosurfactant producing bacteria and demonstrated that *Pseudomonas* spp. and *Bacillus* spp. were able to degrade crude oil at highest percentage compare to other isolates identification in this study . This study revealed that catechol 2,3 dioxygenase gene was present in almost bacterial isolates and all *Pseudomonas aeruginosa* ability to produce *rhIB* gene.

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التحري الجزيئي عن بكتريا التحلل الحيوي والمنتجة للسطوح الحيوية المعزولة من الترب الملوثة بالمواد الهيدروكاربونية في مدينة الديوانية / محافظة القادسية

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الخلاصة :

نتيجة الاستخدام المتزايد للمركبات الهيدروكاربونية اصبح تلوث الترب بالديزل ووقود المحركات والكيروسين احد المشكلات البيئية الرئيسية .اجري هذا البحث لتحديد بكتريا النبيت الطبيعي في الترب الملوثة بالمشتقات النفطية في مدينة الديوانية / محافظة القادسية , وفحص قدرة هذه البكتريا على انتاج السطوح الحيوية باستخدام تقنية الانتشار بالزيت والنشاط الانحلالي للدم , و التحري عن قدرة هذه البكتريا على انتاج انزيمات التحلل الحيوي للمركبات الهيدروكاربونية وهو انزيم كاتيكول 2,3 ثنائي اوكسجنيز وانزيم الرهامنولبد الذي يشترك في انتاج السطوح الحيوية لبكتريا الزوائف الزنجارية وذلك باستخدام تقنية تفاعل البلمرة المتسلسل لتضخيم الجينات المسؤولة عن تشفير هذه الانزيمات وباستخدام بادئات محددة . استخدمت الطريقة الاغنائية لعزل البكتريا .

تم زرع 17 عينة من الترب الملوثة بالمواد الهيدروكاربونية جمعت من محطات تصليح السيارات ومولدات الكهرباء الاهلية باستخدام وسط الحد الادنى من الملح والحاوي على النفط الخام كمصدر وحيد للكاربون . اظهرت النتائج ان الاجناس البكترية المعزولة هي .*Pseudomonas* spp 14 عزلة (66%) و .*Bacillus* spp 3 عزلات (14%) و.*Micrococcus* spp عزلتين (10%) و عزلة واحدة (5%) لكل من بكتريا .*Staphylococcus* spp و.*Streptococcus* spp .

فيما يخص البكتريا التي لها القدرة على انتاج السطوح الحيوية اظهرت النتائج بان تقنية الانتشار بالزيت (استخدم فيها ثلاث انواع مختلفة من المواد الهيدروكاربونية وهي النفط الخام والديزل والكيروسين) بان الكيروسين كان اكثر كفاءة لتحديد انتاج السطوح الحيوية من قبل .*Pseudomonas* spp و .*Bacillus* spp وان .*Pseudomonas* spp اظهرت كفاءة اعلى في انتاج السطوح الحيوية من .*Bacillus* spp . كما اظهرت النتائج ان تقنية الانتشار في الزيت الافضل في التنبؤ لانتاج السطوح الحيوية من قبل البكتريا المعزولة مقارنة بالفعالية التحليلية للدم .

اظهر التحري الجزيئي عن الجينين *C23O* و *rhIB* امتلاك 12عزلة (58%) من بين 21 عزلة بكتيرية معزولة من الترب الملوثة بالمواد الهيدروكاربونية مع نسبة عالية (43%) لهذا الجين بين عزلات .*Pseudomonas* spp , كما أظهرت جميع عزلات *Pseudomonas aeruginosa* امتلاكها للجين *rhIB* مما يدل على عائدية ال DNA لبكتريا *P. aeruginosa* .