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EFFECT OF BIOSURFACTANTS ON THE DEGRADATION OF SELECTED HYDROCARBONS BY *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Thirty two of different bacterial strains including *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas lemoignei, Alcaligenes faecalis, Alcaligenes eutrophus* and *Bacillus subtilis* degraded tetradecane, hexadecene, pristane and 2-methylnaphthalene hydrocarbons with different percent. *Pseudomonas aeruginosa* I strain was the best strains in degradation of hydrocarbons and the only strain produced biosurfactants. Biosurfactants were produced by the isolated *Pseudomonas aeruginosa* mainly at the beginning of stationary phase of growth. After 7 days of incubation in a non-hydrocarbon medium, about 10 ml of crude surfactant could be removed from 1000 ml of culture supernatant. Biosurfactants lowered the surface tension of water to 35 dyn/cm, achieved critical micelle concentration (CMC) at 20 μg/ml and exhibited an emulsifying activity of 315 U/ml. Addition of 100 μg/ ml of concentrated biosurfactant increased the biodegradation. Addition of biosurfactant to culture medium improved biodegradation of tetradecane from 70% to 95%; pristane from 50% to 70%; hexadecene from 40% to 60% and 2-methylnaphthalene from 10 % to 20%. However, the inocula addition caused only 10% increase in percentage of tetradecane, hexadecene and pristane degradation and no increase in percentage of 2- methylnaphthalene biodegradation.

INTRODUCTION

Hydrocarbons such as oil products, petroleum products, and halogenated compounds form an serious class of pollutants on global scale. This occures through continuous input, spillage, improper handling or waste disposal, and natural seepage. These compounds normally enter and penetrate the soil and ground water and the environments [1]. Once released, hydrocarbons tend to be dispersed over diffuse areas.

The presence of hydrocarbons in the environment is of considerable public health and ecological concern due to their persistence, ability to be bioaccumulated and toxicity to a wide variety of biological systems [2,3]. Therefore, there is a great demand to clean up soils and ground water which have been contaminated with hydrocarbons.

Recently, interest in microbial surfactants has been increased considerably especially due to their potential application in enhanced oil recovery. The production of surfactants by microorganisms is well established [4-6]. Their potential for enhanced oil recovery is based on their application as agents for emulsification, micellar flooding and viscosity reduction of heavy crude oils [7].

Hydrocarbons are widely used as the substrate for the production of biosurfactants. It has been postulated that the biological function surface active compound is related to hydrocarbon uptake, and therefore a spontaneous release occurs with these substrates [8,9]. The surface activity was related to a glycolipid moiety called rhamnolipids. These were produced when hydrocarbons, glycerol, glucose or peptone was the substrate [10,11,9,12].

Several *Bacillus* species which exhibited extracellular emulsifying activity have been isolated[13]. The aim of the present work was the optimization of biosurfactant production by *Pseudomonas aeruginosa* and enhancement of biodegradation of selected hydrocarbons.

MATERIALS AND METHODS

Microorganisms :

The following bacterial strains were isolated from soil samples contaminated with hydrocarbons near crude oil extraction company (El-Suez and Red Sea) in Egypt: *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas lemoignei, Alcaligenes faecalis, Alcaligenes eutrophus* and *Bacillus subtilis.* All isolates were maintained on agar slants (Sigma Chemical Company) at 4°C and subcultured every two weeks.

Degradation of selected hydrocarbons :

The selected hydrocarbons were: tetradecane (a linear aliphatic alkane), pristane (a branched alkane), hexadecene (a linear aliphatic alkene) and 2-methylnaphthalene (an aromatic hydrocarbon). These compounds are the components of crude oil [14] obtained from Sigma Chemical Company. Mixed hydrocarbons were used with the following concentrations (w/w): tetradecane 40%, pristane 25%, hexadecene 25% and 2-methylnaphthalene 10%.

Hydrocarbon degrading bacteria were routinely grown in basal mineral salt (BMS) medium containing 0.2% of hydrocarbons as carbon source [15]. The BMS consisted of the following: 137 mg/L of $NaNO_3$; 22 mg/L of $MgSO_4 . 7H_2O$; 55 mg/L of KCL; 55 mg/L of NaCL; 2.75 mg/L of $CaCL_2 . H_2O$; 27.5 μg/L of $FeSO_4 . 7H_2O$; 82.5 ug/L of $ZnSO_4 . 7H_2O$; 82.5 μg/L of $MnSO_4 . H_2O$; 16.5 μg/L of H_3BO_4 ; 8.5 μg/L of $CoCl_3 . 6H_2O$ and 8.5 μg/L of $CuSO_4 . 5H_2O$.

The residual hydrocarbons were extracted with carbon tetrachloride, centrifuged at 4000 x g for 30 min

to remove the biomass and analyzed [16] using a gas chromatograph equipped with flame ionization detector. For determination of the reduction of different component peaks of degraded hydrocarbons, corresponding peaks of untreated hydrocarbons (control) was used as 100 % values.

Cultivation condition for biosurfactants production :

A series of 1000 ml flasks were set up. Each flask contain 250 ml of BMS medium which consisted of the same components used in hydrocarbon degradation but 0.2% glucose (20 g/L) was used as carbon source instead of hydrocarbons. The pH of the medium was adjusted to 7.0 before autoclaving. $CaCL_2$ was autoclaved separately before adding to the medium. To each flask a loopful of bacterial cells was added, incubated at 25 °C with shaking at 200 rpm. Samples (1 ml) were removed at intervals, diluted with distilled water and surface tension measured using a Fisher Tentiomat. Biosurfactant production was also assayed by using the drop- collapsing test (17, 18).

To study the effect of medium composition such as nitrogen concentration on production of biosurfactant, 0.5 mg/L of yeast or 220 mg/L of NH_4SO_4 was added to BMS medium. The concentration of $FeSO_4$ was increased to 1,000 ug/L to study effect of iron concentration on production of biosurfactant.

Surfactant extraction :

After 6 days of growth, cells were removed from the medium by centrifugation at 16,000 x g for 15 min (Sorval, GSA rotor). The pH of the medium was lowered to 2.0 using 1.0 N HCL. The biosurfactant was extracted with diethyl ether [11]. Briefly, to the culture supernatant (about 900 ml) was added an equal volume of diethyl ether and the mixture placed in a separating funnel and shaken vigorously for 5 min.

The mixture was held stationary for 5 min to allow phase separation. The top diethyl ether layer containing the surfactant was removed and the bottom aqueous layer was re-extracted with diethyl ether until solutions from the bottom layer no longer exhibited drop collapsing ability. This usually required 5-8 extractions. The aqueous phase was further concentrated by drying under vacuum using a rotoevaporator at 50 °C to 20% of its original volume. The diethyl ether extraction process was repeated with the smaller volume of aqueous phase until all surfactant activity was removed by measuring surface tension, emulsifying activity and the drop collapsing ability.

One unit of emulsifying activity was defined as the amount of emulsifier giving an absorbance of 1.0 at 540 nm. This material was further placed under vacuum for 1 h followed by heating at 80°C for 15 min to remove other volatile substances in the extract (6,18).

Surface activity of the concentrated extract :

The potency of the concentrated extract was assayed by measuring its CMC value. Various dilutions of the extract were prepared and their apparent surface tensions measured by a tentiomat. The concentration of the extract at which the apparent surface tension dramatically increased was recorded as CMC value.

Effect of biosurfactant or inoculum on degradation of the selected hydrocarbons :

Degradation of mixed hydrocarbons was studied by adding 0.2% of hydrocarbon mixture to growth medium instead of glucose as carbon source. The medium was inoculated with a loopful of *Pseudomonas aeruginosa* and incubated at 20 °C. At monthly intervals, the content were analyzed for residual hydrocarbons according to a previous report (16).

To study the effect of adding inocula on hydrocarbon degradation, *Pseudomonas aeruginosa* was grown in tryptone soy broth at 25 °C with shaking at 200 rpm as described above. Cells were harvested at the mid- log phase (about 14 h) by centrifugation at 4000 x g and washed twice with 0.85 % (w/v) saline solution before adding to an inoculated medium containing 0.2% of mixed hydrocarbons.

To study the effect of adding surfactants on hydrocarbon degradation, the concentrated biosurfactants were extracted as described above, dissolved in water by heating at 50°C for 30 min with continuous stirring. A stock 0.1% solution of biosurfactant was prepared and diluted with 0.85 % (w/v) saline solution.

RESULTS AND DISCUSSION

Hydrocarbon degrading bacteria :

Table (1) listed hydrocarbons degrading bacteria which were isolated from different places contaminated with crude oil in Egypt. 15 strains of *Pseudomonas aeruginosa*, 6 strains of *Pseudomonas fluorescens* 2 strains of *Pseudomonas lemoignei*, *Alcaligenes faecalis*, 4 strains of *Alcaligenes eutrophus* and 5 strains of *Bacillus subtilis* degraded the selected hydrocarbons with different percent.

Atlas et al., (19) reported the isolation of hydrocarbon- utilizing bacteria and fungi from soil and the application of oil or oily wastes to soil resulted in increased numbers of bacteria and fungi.

Pseudomonas aeruginosa I was the best strain showing the highest percentage of hydrocarbons degradation. It was also the only biosurfactant producer strain from all isolated strains. *Pseudomonas aeruginosa* degraded 70% of the tetradecane, 50 % of pristane, [illegible] % of hexadecene and 10 % of 2 methylnaphthalene hydrocarbons.

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Table (1): Hydrocarbons degrading bacteria:

Isolated bacteria strains	% of hydrocarbon degradation			
	Tetradecane	Pristane	Hexadecene	2-methylnaphthalene
Pseudomonas aeruginosa				
Ps. aeruginosa A	50	35	30	0
Ps. aeruginosa B	40	30	20	10
Ps. aeruginosa C	30	20	20	0
Ps. aeruginosa D	40	30	25	0
Ps. aeruginosa E	45	40	30	10
Ps. aeruginosa F	55	45	25	5
Ps. aeruginosa G	35	25	20	3
Ps. aeruginosa H	35	20	15	10
Ps. aeruginosa I *	70	50	40	10
Ps. aeruginosa J	45	45	35	2
Ps. aeruginosa K	50	40	30	4
Ps. aeruginosa L	25	15	15	3
Ps. aeruginosa M	25	25	20	0
Ps. aeruginosa N	25	20	10	0
Ps. aeruginosa O	20	10	0	0
Pseudomonas fluorescens				
Ps. fluorescens P	40	30	30	10
Ps. fluorescens Q	40	25	20	5
Ps. fluorescens U	30	20	10	5
Ps. fluorescens V	20	10	10	0
Ps. fluorescens W	10	10	5	0
Ps. fluorescens X	10	5	0	0
Pseudomonas lemoignei				
Ps. lemoignei Y	15	10	0	0
Alcaligenes faecalis				
A. faecalis Z	35	25	15	10
A. faecalis ZA	30	20	10	10
A. faecalis ZB	25	20	5	0
A. faecalis ZC	10	0	0	0
Alcaligenes eutrophus	25	15	5	5
Bacillus subtilis				
B. subtilis ZD	45	40	30	10
B. subtilis ZE	40	30	20	5
B. subtilis ZF	30	25	0	0
B. subtilis ZG	25	10	0	0
B. subtilis ZH	10	0	0	0

* Biosurfactant producer strain.

Hydrocarbons differ in their susceptibility to microbial attack, they have been ranked in the following order decreasing susceptibility alkanes > branched alkanes > alkenes > aromatics [20]. Biodegradation rates have been shown to be highest for the saturates, followed by the light aromatics, with high molecular weight aromatics and polar compounds exhibiting extremely low rates of degradation [21].

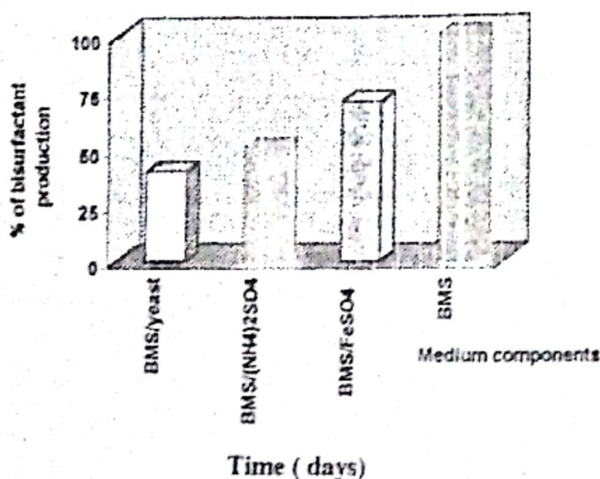
Biosurfactant production by *Ps. aeruginosa*:

Biosurfactants were produced by the isolated *Pseudomonas aeruginosa* mainly at the beginning of stationary phase of growth. After 7 days of incubation in a non-hydrocarbon medium, about 10 ml of crude surfactant could be removed from 1000 ml of culture supernatant. The concentrated material appeared as yellow oily material caused drop collapse on an oily plate.

Biosurfactants lowered the surface tension of water to 35 dyn/cm, achieved CMC at 20 ug/ml and exhibiting an emulsifying activity of 315 U/ml. These results suggested potent surfactant activity. The production of surfactant from *Bacillus subtilis* and the effect of continuous product removal and cation additions were previously studied [22].

Effect of medium composition on biosurfactant production :

The isolated strain produced surface-active compounds when cultivated in basal mineral salt medium as indicated by the lower surface tension values of the culture. Figure 1 indicated that several medium components influenced the biosurfactant production. Addition of yeast extract to BMS medium decreased biosurfactant production from 100% to 40%. Addition of $(NH_4)_2SO_4$ to BMS medium decreased biosurfactant production from 100% to 50%, while the increase of iron concentration decreased biosurfactant production from 100% to 70%. A minimal iron concentration, no yeast extract and low nitrogen concentration optimized the biosurfactants production.



Time (days)

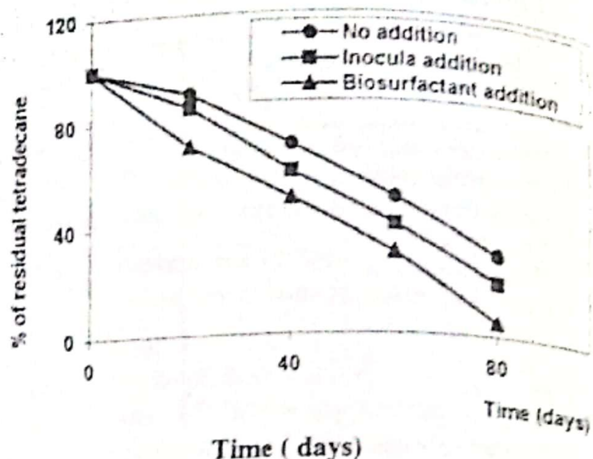
Fig. (1): Effect of medium composition on biosurfactant production.

By applying medium limitations other than carbon (iron and nitrogen), it was possible to direct cellular metabolism to product formation. Since medium limitations were also leading to polysaccharide production [23, 24]. Because of these facts, the use of complex medium additives, such as yeast, has to be avoided.

Effect of Inocula and biosurfactant additions on degradation of hydrocarbons :

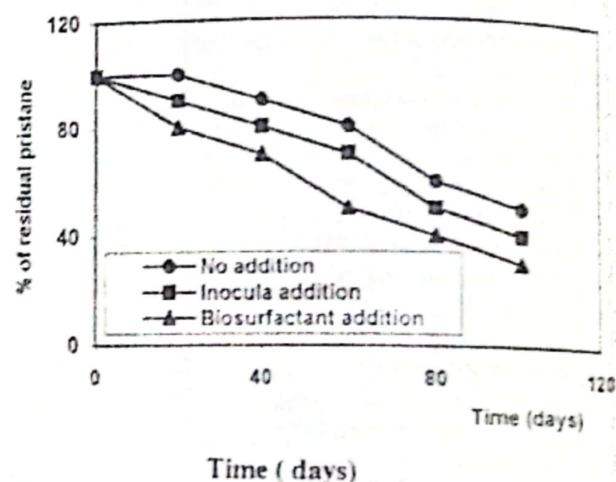
Considerable tetradecane degradation was observed as shown in Fig 2. About 50% was degraded in two months incubation. This value increased to 70% after 80 days incubation. Addition of biosurfactant to culture medium improved biodegradation, about 95% of tetradecane was degraded after 80 days incubation, however, Addition of inocula caused 10% increase in degradation (from 70% to 80%).

Fig 3 showed that about 50% of pristane was degraded after 100 days incubation. Biosurfactant addition improved the degradation from 50% to 70%. However, inocula addition showed about 10% increase to the degradation.



Time (days)

Fig. (2) : Effect of inocula and biosurfactant additions on degradation of teradecane.



Time (days)

Fig. (3) : Effect of inocula and biosurfactant additions on degradation of pristane.

In four months incubation, the increase in percentage of hexadecene degradation was from 40% to 60% after addiion of biosurfactant and from 40 % to 50% after addition of the inocula as shwon in Fig. 4.

Over four months, a low percentage of 2-methylnaphthalene was (less than 10%) degraded. Addition of inocula to culture medium had no effect on the biodegradation, while addition biosurfactant increased percentage of 2-methylnaphthalen degradation from 10% to 20 % (Fig. 5).

Several researchers have studied the effect of exogenous addition of surfactants on hydrocarbons biodegradation. **Atlas and Bartha** [25] found that the use of chemical surfactants had no effect on oil biodegradation in sea water. It was previously reported that the addition of phosphatidylcholine enhanced degradation of alkane mixture up to 30% [26]. **Cooper and Paddock** [27] studied the production of biosurfactants from *Torulopsis bombicola* and reported that these biosurfactants enhanced the biodegradation of crude oil.

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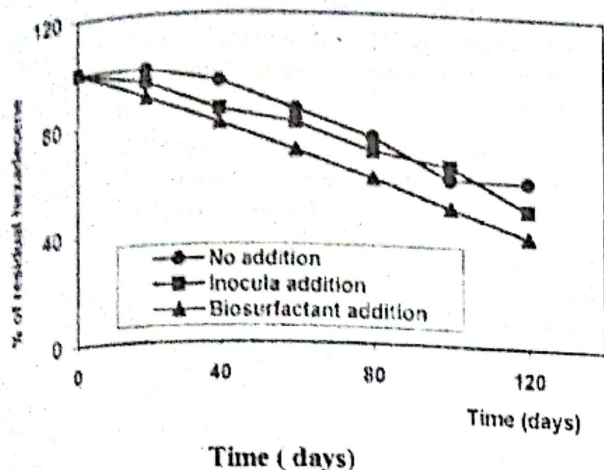
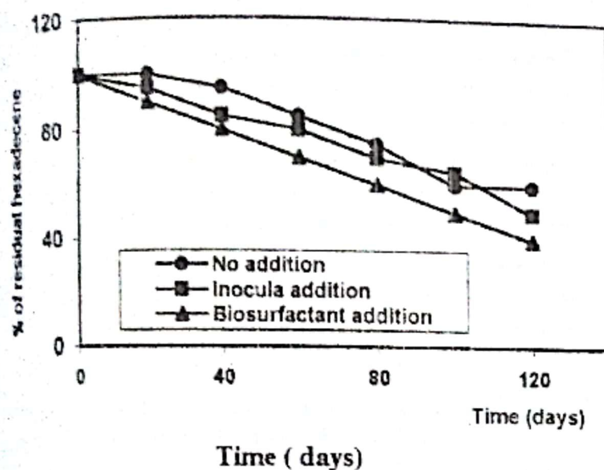


Time (days)

Fig. (4) : Effect of inocula and biosurfactant additions on degradation of hexadecene.



Time (days)

Fig. (5) : Effect of inocula and biosurfactant additions on degradation of 2-methylnaphthalene.

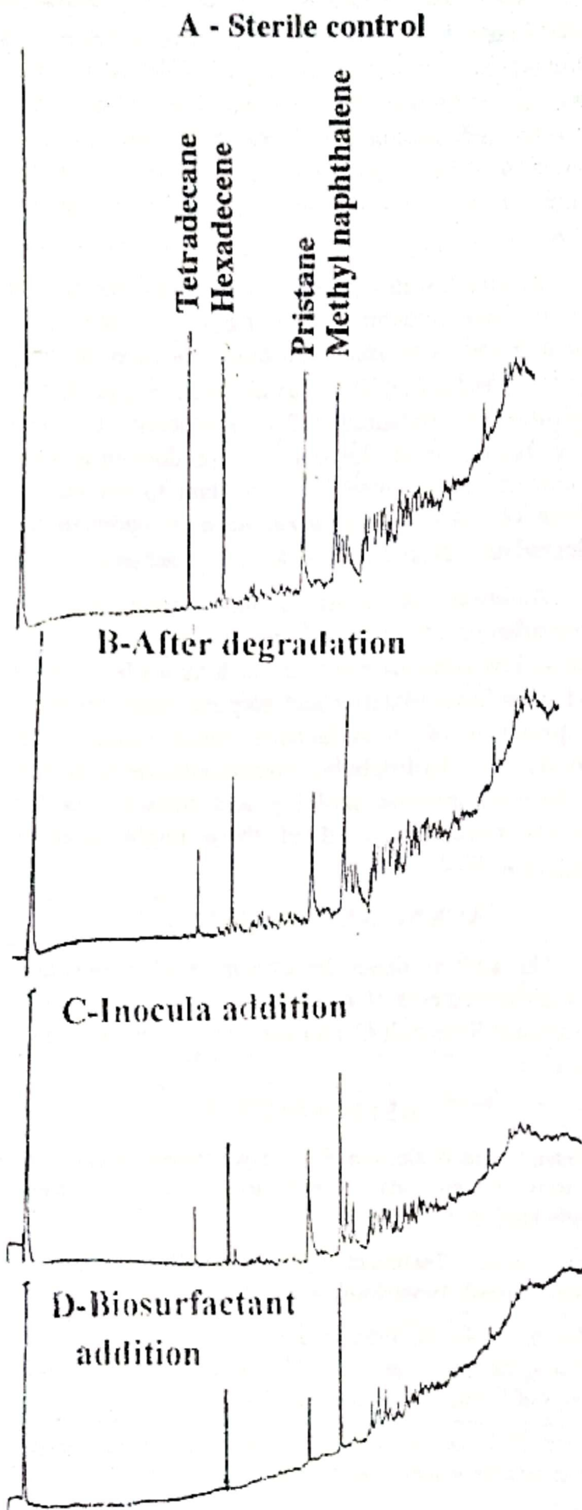


Fig. (6) : Gas chromatographic profiles A) Sterile control, B) Hydrocarbons after degradation by *Ps. aeruginosa*, C) Inocula effect on hydrocarbon degradation , D) Biosurfactant effect on hydrocarbon degradation.

Hydrocarbon polluted soils have been decontaminated by physical or chemical methods such as thermal evaporation, extraction, flooding, adsorption, and immobilization [2]. Enhancement of biological degradation based on seeding with microorganisms. Bioremediation is an attractive and ecologically sound method of decontaminating hydrocarbon polluted soils and ground water and has been claimed to be efficient, economical and versatile [28].

The present study clearly showed the use of isolated *Pseudomonas aeruginosa* for degradation of crude oil hydrocarbons, biosurfactant production and the effect of adding either *Pseudomonas aeruginosa* inocula or biosurfactants on biodegradation of mixed hydrocarbons. The results revealed that the addition of 100 ug/ml biosurfactants enhanced the degradation of the aliphatic tetradecane, pristane, hexadecene and aromatic 2-methylnaphthalene, (the most water-soluble) of the four compounds [29].

The addition of inocula to the culture medium had a little effect on biodegradation of aliphatic hydrocarbons but not on aromatic hydrocarbons. The reasons of lesser effect of inocula addition than biosurfactant addition may be due to the combined toxicity of the four hydrocarbons as well as the inability

of *Pseudomonas aeruginosa* strain to produce its biosurfactants (14) found that the more water-soluble hydrocarbons can be easily degraded while the less water-soluble hydrocarbons are only metabolized when the interfacial tension was lowered by biosurfactants production. These observations suggested that the requirement for surfactant solubility may differ among hydrocarbons.

In conclusion the results suggested the use of BMS medium containing 0.2% of hydrocarbon mixture as carbon source to study the biodegradation of these hydrocarbons by *Pseudomonas aeruginosa* , the use of BMS medium containing 0.2% of glucose as carbon source for maximum biosurfactant production and the addition of concentrated biosurfactant to the culture medium of *Pseudomonas aeruginosa* to optimize the biodegradation of hydrocarbons.

Addition of biosurfactants might enhance biodegradation of hydrocarbons in soil and ground water as low aqueous solubility of hydrocarbons could affect their bioavailability and sorption characteristics. The presence of biosurfactants could increase the solubility of hydrophobic compounds, decrease the face tension, increase mobility and surface area for microbial contact and all of these might promote degradation **(30, 31)**.

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تأثير المواد المقللة للتوتر السطحى على التحلل الميكروبى لبعض المركبات الهيدروكربونية بواسطة السودوموناس اروجينوزا

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تم فى هذه الدراسة عزل وتنقية وتعريف ٣٢ سلالة بكتيرية شملت السودوموناس اروجينوزا ، السودوموناس فلورسنس ، السودوموناس ليموجينى وألكالجين فيكاليز ، ألكالجين إيتروفاز والباسيلس سبتيلس لهم المقدرة على تحليل بعض مركبات الهيدروكربون المكونه للزيت الخام وهى التراديكان والبريستان,الهكساديكين و ٢-ميثيل نفثالين بنسب مختلفة .

وقد وجد أن سلالة السودوموناس اريجينوزا المعزولة هى أفضل السلالات فى تحليل مركبات الهيروكربون المختارة وتنتج مادة حيوية تقلل التوتر السطحى (SAA) للماء إلى ٣٥ وحدة لكل ملى ويمكن فصلها وتركيزها واضافتها للهيدروكربون المختار ليزيد من تحلله الميكروبى.

يتأثر إنتاج هذه المادة بأختلاف الوسط الغذائى واختلاف مكوناته وقد وجد أن الوسط الغذائى BMS هو أفضل الاوساط الغذائية لانتاجها وأن اضافة الخميرة أو زيادة مصدر النيتروجين والحديد يقلل إنتاج المادة الحيوية المقللة للتوتر السطحى.

وقد أثبتت الدراسة أن اضافة ١٠٠ ميكروجرام / مل من المادة الحيوية المقللة للتوتر السطحى يزيد التحلل الميكروبى لمركبات التترادیکان من ٧٠٪ إلى ٩٥٪ ومركب والبريستان من ٥٠٪ إلى ٧٠٪ والهكساديكين من ٤٠٪ إلى ٦٠٪ أما مركب ٢- ميثيل نفثالين فقد زاد نسبة تحلله من ١٠٪ إلى ٢٠٪ فقط. بينما لم تؤثر اضافة العترة المركزة (Inocula) تأثيراً ملحوظاً على تحلل تلك المركبات وخاصة مركب ٢ ميثيل نفثالين .

وقد قمنا بإجراء هذا البحث لأهميته بالنسبة للصحة العامه والبيئة فى التخلص البيولوجى من الملوثات الهيدروكربونيه للمياه والتربة .