J. King Saud Univ., Vol. 11, *Eng. Sci.* (2), pp. 251-261 (A.H 1419/1999)

PETROLEUM ENGINEERING

Bacterial Isolate from Arabian Gulf Coast Soils in Saudi Arabia Able to Degrade Arab Crude Oil

Abdel-Alim H. El-Sayed and M. S. Al-Blehed

Petroleum Engineering Department, College of Engineering, King Saud University, P. O. Box 800, Riyadh 11421, Saudi Arabia

(Received 15 November 1997; accepted for publication 11 May 1998)

Abstract. By means of the enrichment culture technique, two types of microorganisms were isolated from oil contaminated soil samples gathered from coastal area of Saudi Arabia on the Arabian Gulf. One bacterial isolate has proved its ability to degrade Arab crude oil added to aqueous phases (salts solution, sterile and nonsterile Gulf water) while the other failed to adapt itself in salts solution. The active species was identified as Pseudomonas sp. About 80% of Arab crude oil added to salts solution and nonsterile Gulf water had disappeared within 10 days of incubation by this active isolate. Nonsterile Gulf water yielded higher degradation per cent than sterile Gulf water because of the activation of the indigenous flora and the sufficiency of organic and inorganic nutrients. The bacteria proved optimum degradation per cent at 25° C and 2.5 mg/ml Arab crude oil concentration. Adding nutrients activated the degradation process and phosphorus has proved the best rate among other nutrients. Increasing the inoculum size of bacteria in the presence of sufficient nutrients directly affected the biodegradation per cent of Arab oil added to an aqueous phase.

Introduction

Studies on the ability of microorganisms to degrade hydrocarbon of varied structure existing in petroleum has been started after the wreck of Torry Canyon in the mid 1960's [1;2], where certain number of crude oils of varied composition has been examined. Later researches showed that the ability to degrade petroleum hydrocarbon is not restricted to specific genus, different species of bacteria and fungi were indicated having the ability to degrade hydrocarbon. Bartha and Atlas [3] listed 20 genera of bacteria, one algal genus, and 14 genera of fungi which utilize hydrocarbon. These microorganisms are widely distributed in marine, fresh water and soil habitat. In polluted water ecosystems, bacteria, yeast, and filamentous fungi all appear to be important as hydrocarbon degrader [4].

The biodegradation of crude oil was found to be highly dependent on their composition and environmental conditions. Atlas and Bartha [5] found that the biodegradation rate of heavier crude oil was significantly lower than for the lighter ones. The biodegradation rate was also affected by temperature and toxic components of the crude. Atlas described in his publication [6] that the degradation started after 2 to 4 days lag period and reached its maximum within two weeks. At that time up to 60% of the crude oil and 75% of model hydrocarbon mixture were degraded. Bartha *et al.* [3] listed that nature of the spilled oil, temperature, pressure, oxygen, and mineral nutrients are the environmental constrains that affect hydrocarbon degradation. Bartha reported also that the microbial degradation of oil pollutants is a complex process and the environmental factors have a great influence on the fate of spilled oil, but with an understanding and studying this process in the environment, it is possible to develop strategies for utilizing microbial hydrocarbon degradation activities for the removal of oil spills from contaminated areas. Also, it was reported that the bioremediation was approved to clean weathered and tar like oil which had become impossible to blast from rocky beaches [7]. In Arabian Gulf area, the threat of major oil spill has been recognized by industry and governments of the region in early 1970's, where the Torry Cayon catastrophe first drawn an international attention on the scope of the response required [8]. However, oil spill was reported in the Arabian Gulf thereafter, like that was reported on August 25, 1985, on the coast of Bahrain. A most major one was that which was reported during the Gulf war in 1991. The spill have greatly affected marine life and created a big problem of oil contamination in general and Saudi coastal areas in special.

From this point of view the biodegradation has been suggested as a method for cleaning crude oil contamination. Our laboratory work has been conducted to find out a microbial strain in the Saudi soils that is able to degrade crude oil under natural conditions. The work was directed to isolate oil degrading bacteria from water and soil samples contaminated with crude oil from Arabian Gulf and its coastal areas.

This paper presents the bacteria isolated from Arabian Gulf coast soils in Saudi Arabia, and investigates the growth rate and the biodegradation per cent of Arab crude oil in sterile and nonsterile Gulf water using bacterial isolate. It specifies also the factors affecting this biodegradation such as temperature, crude oil concentration, nutrients and inoculum size.

Materials and Methods

Oil used in this study represents Arab heavy crude oil that was provided by Saudi Aramco. The oil posses 28° API and 17.0 cp viscosity. The oil was weathered and sterilized by heating to 115° C and kept in store for 30 minutes at this temperature. Water used as aqueous phase for the experimental program was specially collected from

Arabian Gulf. The samples were kept in sterilized plastic containers till they were used. For microorganism(s) isolation, oil contaminated soil samples were gathered from different places on the coastal area of Saudi Arabia. The soil was taken from the ground surface by removing 2 inches depth of the surface and collecting about 100 grams from the third inch depth. The samples were kept at 4°C until they were used.

Isolation of microorganisms were obtained by enrichment culture technique from soil samples contaminated with oil over a period of time. Enrichment were carried out in a media composed of (g/l of distilled water) 0.1 KH_2PO_4, 0.2 K_2HPO_4, 0.1 $(NH_4)_2PO4$, 0.1 $MgSO_4$, 0.1 $CaCl_2$ and $2H_2O$. Oxoid and Difco agars were used in identification of the microorganisms [9]. Soil samples (10 g) was incubated with 100 ml salts solution amended with crude oil in 250 ml Erlenmyer flask. The flask was kept incubated at 30°C with shaking for one week. Subcultures were done and microorganisms were isolated. Bacterial isolate was tested for oil utilization by transferring purified colonies into salts solution amended with oil as a sole source of carbon. Growth was remarked by turbidity of the salts solution and monitored by plate count technique.

Experiments were conducted using Erlenmeyer flasks of 250 ml capacity. Flasks were sterilized by autoclaving at 121°C for 15 minutes. Aqueous phase which was salts solution, sterile and nonsterile Gulf water was added in a volume of 50 ml to the flasks. Arab crude oil was added to flasks to give a final concentration of 0.5%. Bacterial isolate was added to flasks to a final cell density of 10^5 cfu/ml. Experiments were carried out for the three aqueous phases and devoted to study the time, temperature, oil concentration, nutrients, and cell density as factors affecting biodegradation.

The flasks were removed from incubation at predetermined intervals of time and the water phase was acidified to pH of 4.0 with concentrated HCl. Two twenty- five ml of methylene chloride were added to each flask. The methylene chloride phase was washed with acidified distilled water of 4.0 pH value. After phase separation, the organic extracts were combined, dried through anhydrous Na_2SO_4, and methylene chloride evaporated to dryness through aspiration at 40°C. After extraction, the residual oil was transferred in a small volume of carbon disulfide (0.001 ml residual oil was added to 0.6 ml carbon disulfide) to capillary column gas chromatography.

The gas chromatography model is GC 3400xc equipped with flame ionization detector and 0.53 mm inside diameter, 30 meter long capillary column. The operating parameters were: injection port 250° , detector temperature 300°C, carrier gas (helium) velocity 20.3 cm/sec, hydrogen 30 cc/min, and injection volume 0.001 ml. The oven temperature was kept at 37°C for 12 minutes following injection and was then programmed at the rate of 5°C/min. to 250°C and held constant for 60 minutes. Individual hydrocarbon were quantified by integration of the corresponding peak area.

Results and Discussions

Microorganisms

Two bacterial isolates were recovered from oil contaminated slug by enrichment culture technique. By testing the degradative capacity of these isolates in salts solution amended with crude oil as a sole source of carbon and energy, one proved its ability to degrade crude oil efficiently, while the other was unable to adapt itself when it was transferred to the new media. The active isolate has been classified as Pseudomonas sp. This isolate suffered a little decay in the new environment then started to grow logarithmically to a level of 10^7 cfu/ml, (Fig. 1).

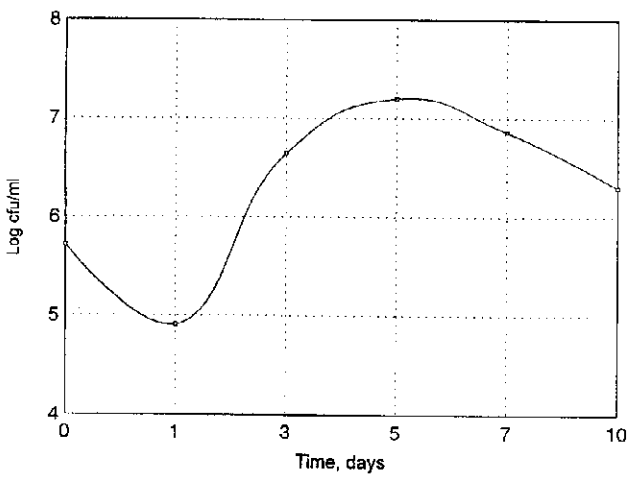

Fig. 1. Growth and survival of bacteria in sterile Gulf water in the presence of spilled oil.

Biodegradation of Arab crude oil in aqueous phase

The biodegradation of crude oil spilled in aqueous phase is shown in Fig. 2. Experiments were conducted for the salts solution, sterile and nonsterile Gulf water. The results showed that the biodegradation was detected quickly in the three aqueous

phases but at different values where about 40% of oil added to salts solution was degraded. Sterile Gulf water resulted in a biodegradation per cent lower than salts solution at the early days and offered a higher extent at the end of the experiment. Biodegradation in nonsterile Gulf water showed the highest degrading capacity after 7 days with an amount equals to that which happened with salts solution after 15 days. Such capacity in nonsterile Gulf water can be attributed to the activation of indigenous flora and the lack of competition for the nutrients at the level of bacterial isolate. The high extent of biodegradation in nonsterile Gulf water is due to the presence of organic nutrients in the Gulf water which activate the bacterial growth and allow high degradation per cent and the activation of indigenous flora. The figure also shows that the extent of biodegradation remains constant after 10 days of incubation. This is due to the survival period of the bacterial isolate where a high decay rate begins after 10 days incubation time and due to the lack of the carbon source in the medium since 80% of the carbon source is already used up. The results obtained encouraged studying the factors affecting biodegradation using sterile Gulf water to avoid the effect of indigenous flora.

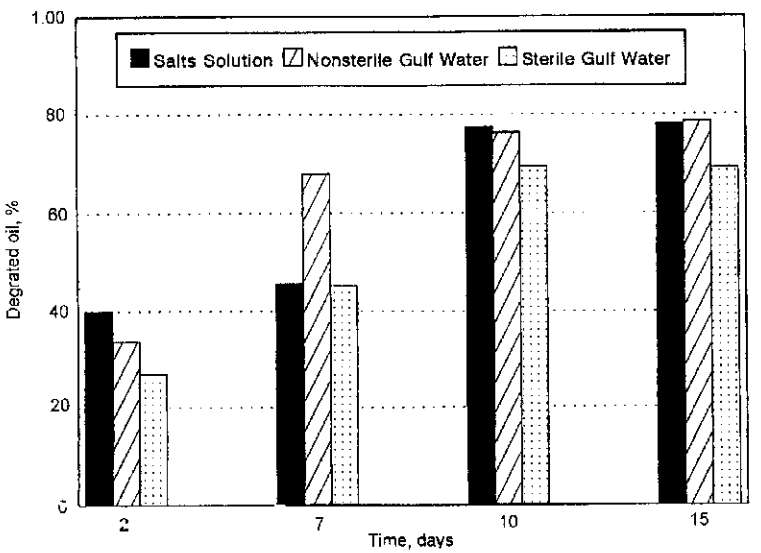

Fig. 2. Biodegradation of Arab crude oil added to aqueous phase.

Effect of added oil concentration on biodegradation

This part of experiments used uninoculated samples as a reference for comparison with inoculated samples at different concentration. Figure 3 shows this comparison at concentrations ranging from 2.5 mg/ml to 15 mg/ml. The results shows that increasing oil concentration adversely affects the biodegradation per cent. This is due to the increase of several components in oil such as toluene and ethylbenzene which have been proved to show inhibition at high concentration. This inhibition may be a reason of low degradation [10]. Another reason may be due to the amount of bacteria which was kept constant at about 10^5 cfu/ml and the amount of nutrient found in the aqueous phase. The uninoculated samples suffered a certain amount of biodegradation which can be attributed to the oxidation of crude oil itself which is dependent on the amount of oxygen available in the sample. Therefore, increasing Arab crude oil concentration reduces the extent of biodegradation as well as oxidation in sample.

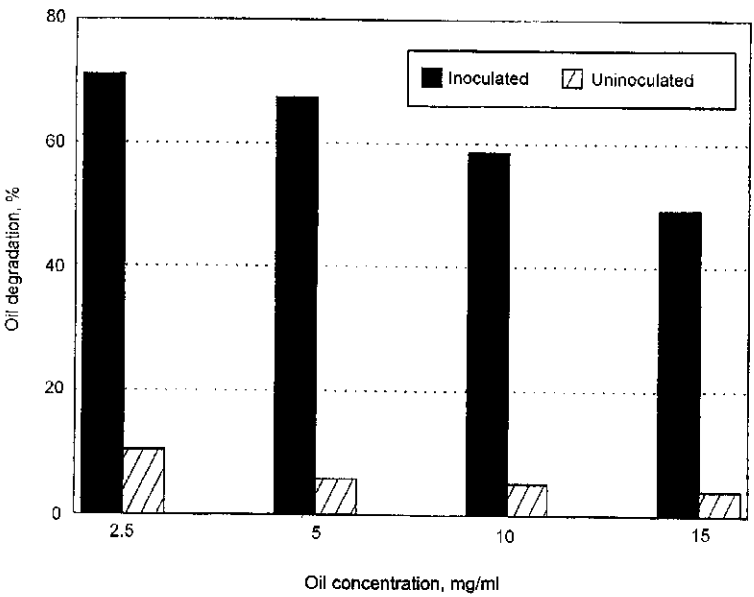

Fig. 3. Effect of Arab crude oil concentration on biodegradation by Pseudomonas sp. in Gulf water.

Effect of temperature on biodegradation

Survey of temperature variation in the eastern part of Saudi Arabia showed that the seasonal temperature changes between 25°C and 45°C [11]. Experiments were conducted at 25°C, 35°C and 45°C. The results are plotted in Fig. 4 for inoculated and uninoculated samples. It shows that the highest degradation was achieved at 25°C, where about 46% of Arab crude oil was disappeared after 5 days. This might be the result of optimum temperature of bacterial growth and decreasing the metabolic activity of bacteria at high temperature. This decrease is due to the increase in toxicity of oil at high temperature [10]. The degradation obtained from uninoculated sample could be attributed to oil evaporation at higher temperatures as well as oil oxidation. In actual case, sea water has enough nutrients and increasing the inoculum size will increase the degradation per cent. This results have been confirmed by Ludzack[12]. He reported that oil degradation was found to be highly dependent on the composition and on incubation temperature. He also mentioned that Pseudomonas sp is able to degrade oil efficiently over a wide range of temperature (20 to 44°C).

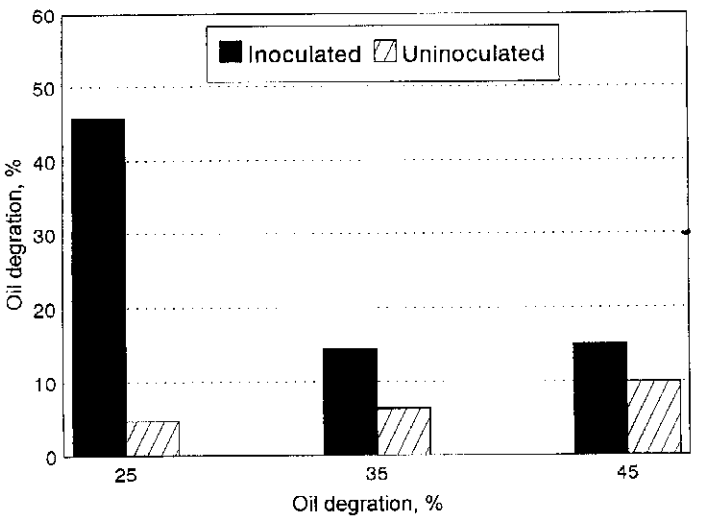

Fig. 4. Effect of temperature on biodegration of 5 mg/ml Arab crude oil by Pseudomonas sp. in Gulf water.

Effect of nutrients on biodegradation

Reishold *et al.* [13] found that adding a mixture of phosphorus and nitrogen at a certain concentration enhances the biodegradation activity. Therefore, three types of nutrients were chosen: one organic (casamino acid), and two minerals (phosphorus and nitrogen). The organic nutrient was added in concentration of 0.05 mg/ml, while the mineral nutrients were added in 100 mg/ml concentration. The result is plotted in Fig. 5 in comparison to unsupplemented sample. It shows that nutrients added fertilize the aqueous phase and activate the biodegradation. The best result was obtained by adding phosphorus to the aqueous phase.

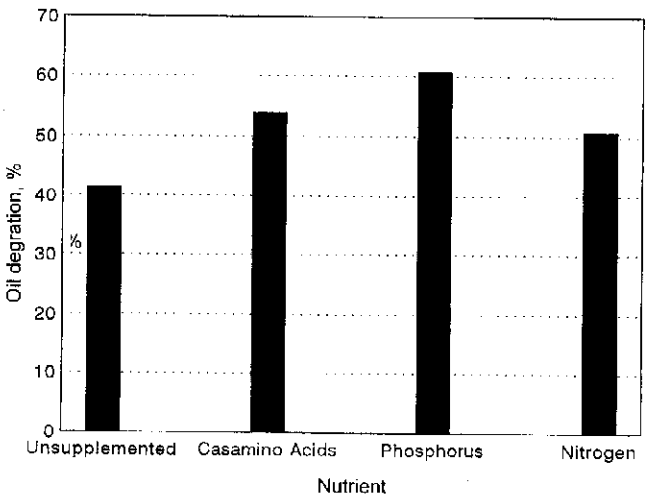

Fig. 5. Effect of added nutrients on biodegradation of 5 mg/ml Arab crude oil by Pseudomonas sp. in Gulf water.

Effect of inoculum size of degrading bacteria

The effect of inoculum size was tested at four levels, i.e. unsupplemented, 10^3 cfu/ml, 10^5 cfu/ml and 10^6 cfu/ml. The results are plotted in (Fig. 6). It is clear that increasing the inoculum size directly affects the degradation of added oil. However, this result is valid when there is sufficient nutrients in the aqueous phase which is necessary for bacterial growth. In case of nutrient lacking the bacterial isolate will not

completely survive the new environment and will suffer low growth rate resulting in low degradation per cent.

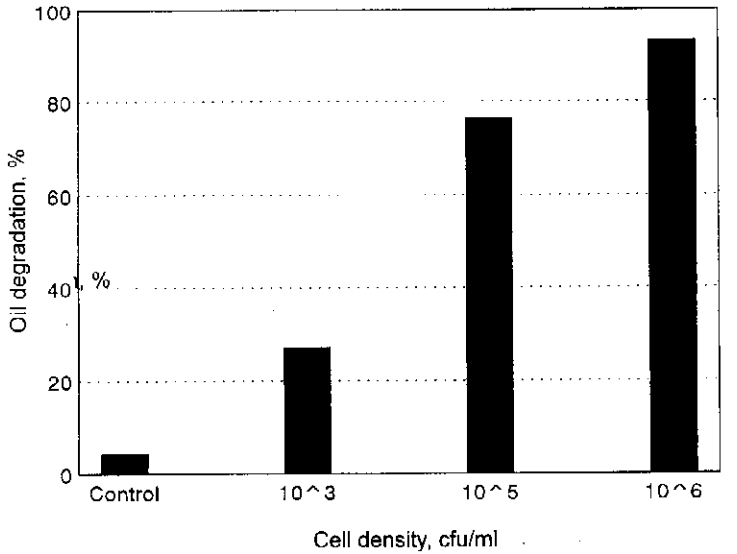

Fig. 6. Effect of inoculum size of pseudomonas sp. on biodegradation of 5 mg of Arab crude oil per ml of Gulf water.

Conclusions

Based on the experimental results and the previous analysis and discussions, the following conclusions are obtained:

1. By means of enrichment culture technique, one bacterial species was isolated from Arabian Gulf coast soil in Saudi Arabia which proved its ability to use Arab crude oil as a sole source of energy.

2. The bacterial isolate was identified as Pseudomonas sp. It adapted the new environment quickly and started to grow logarithmically after 2 days incubation time. It proved its ability to degrade Arab crude oil added to salts solution, sterile Gulf water, and nonsterile Gulf water.

3. About 80% of Arab crude oil added to both salts solution and sterile Gulf water has disappeared within 10 days incubation time.

4. Nonsterile Gulf water yielded higher biodegradation per cent than that of sterile Arabian Gulf water because of activation of indigenous flora and the organic and inorganic nutrients found in the water.

5. Increasing the Arab crude oil concentration reduces the extent of biodegradation.

6. Temperature variations affects the biodegradation per cent. Biodegradation at 25°C was higher than at 35°C and 45°C, which is considered as the optimum temperature of bacterial growth and metabolic activity.

7. Adding nutrients activated the biodegradation. Phosphorus has proved the best one among the nutrients used.

8. Increasing the inoculum size of degrading bacteria when there is sufficient nutrients in the aqueous phase directly affects the degradation of added Arab crude oil.

References

[1] Kallio, RT.E. "Microbial Transformation of Alkanes." *International Ed., Perlman Edition, Fermentation Advances*. New York: Academic Press Inc., 1969, 635-648.

[2] Van der Linden, A.C. and Thijsse, G.J.E. "The Mechanism of Microbial Oxidation of Petroleum Hydrocarbon." *Advanced Enzymol.*, 27 (1977), 469-548.

[3] Bartha, R. and Atlas, R.M. "The Microbiology of Aquatic Oil Spills." *Adv. Appl. Micro.*, 22, No. 3 (1977), 225-226.

[4] Jones, J.G. and Ann Edington, M. "An Ecological Survey of Hydrocarbon-oxidizing Microorganisms." *J. Gen. Microbiology*, 52, No. 5 (1968), 381-390.

[5] Atlas, R.M. and Bartha, R. "Biodegradation of Petroleum in Sea Water at Low Temperature." *Can. J. Microbiology.*, 18, No. 11 (1972), 1851-1855.

[6] Atlas, R.M. and Bartha, R. "Degradation and Mineralization of Petroleum by Two Bacteria Isolated from Coastal Waters." *Biotechnology and Bioengineering*, 14 (1972), 297-308.

[7] Day, J. "Exxon Valdz Spill Cleanup: New Lessons Learned." *Ocean Industry*, (Dec. 1989), 21-25.

[8] Brown, D.J.S. and James, J.D. "Major Oil Spill Response Coordination in Combat of Spills in Bahrain Waters." *JPT*, (Jan. 1985), 131-136.

[9] Kater, H., Openheimer, C.H. and Miget, R.J. "Microbial Degradation of Louisianian Crude Oil in Closed Flasks and Under Simulated Field Conditions." *Int. Joint Conf. on Prevention and Control of Oil Spills*, API, Washington DC., 1971, 287-296.

[10] Peace, J.;, Daniel, D., Nirmalakhandan, N. and Egmen, E. "Predicating Microbial Toxicity of Nonuniform Multicomponent Mixtures of Organic Chemicals." *J. of Env. Eng.*, 123, No. 4 (April 1997), 329-334.

[11] Ministry of Irrigation and Water Resources of Saudi Arabia. *A Water Atlas in Saudi Arabia*, 1980.

[12] Atlas, R.M. "Effect of Temperature and Crude Oil Composition on Petroleum Biodegradation." *App. Microbiology.*, 30, No. 3 (1975), 396-403.

[13] Riesfeld, A., Rosenberg, E. and Gutnick, D. "Microbial Degradation of Crude Oil: Factors Affecting the Dispersion in Sea Water by Mixed and Pure Cultures." *App. Microbiology*, (Sept. 1972), 363-368.

عزلة بكتيرية من تربة شاطىء الخليج العربي في المملكة العربية السعودية قادرة على تكسير النفط العربي الخام

عبد العليم هاشم السيد و محمد بن سعود البليهد

قسم هندسة النفط ، كلية الهندسة ، جامعة الملك سعود ، ص.ب ٨٠٠ ، الرياض ١١٤٢١ ، المملكة العربية السعودية

(استلم في ١٥/١١/١٩٩٧ م ، وقبل للنشر في ١١ / ٥ /١٩٩٨ م)

ملخص البحث. تمّ فصل نوعان من الكائنات الدقيقة ، باستخدام تقنية الإخصاب لزراعة البكتيريا ، من عيّنات تربة ملوثة بالزيت جُمعت من شواطىء المملكة العربية السعودية على الخليج العربي . أثبتت عزلة بكتيرية منهما قدرتها على تكسير النفط العربي الخام المضاف إلي وسط مائي (محلول ملحي ومياه الخليج المعقّمة) ، بينما فشلت الأخرى في التأقلم مع المحلول الملحي . عُرفت العزلة النشطة بأنها نوع صيدومونص (Pseudomonas sp.) . وجد أن ٨٠٪ من خام النفط العربي المضاف إلى المحلول الملحي ومياه الخليج غير المعقّمة اختفت خلال ١٠ أيام (فترة الحضانة لهذه العزلة النشطة) . أنتجت مياه الخليج غير المعقّمة نسبة أكبر من مياه الخليج المعقّمة بسبب تنشيط الكائنات الدقيقة الموجودة في المياه ووفرة المواد المغذية العضوية وغير العضوية . أثبتت البكتريا أمثل نسبة تكسير عند درجة حرارة ٢٥ درجة مئوية وتركيز ٢,٥ ملجم/مل من خام النفط العربي . أدى إضافة مواد مغذية إلى تنشيط عملية التكسير ووجد أن المغذيات الفسفورية هي الأحسن بين المغذيات الأخرى . زيادة عدد البكتريا مع وجود مواد مغذية كافية يؤثر بطريقة مباشرة على نسبة التكسير لخام النفط العربي المضاف إلى وسط مائي .