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Effect of Salinity, Temperature, pH and Crude Oil Concentration on Biodegradation of Crude Oil by *Pseudomonas aeruginosa*

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ABSTRACT

The present study was carried out to evaluate the effect of culture conditions on biodegradation of crude oil by *Pseudomonas aeruginosa*. *P. aeruginosa* was isolated from sea water using Bushnell Haas agar supplemented with 0.1% crude oil. The strain was cultured at different temperature, pH a salinity and crude oil concentrations. Maximum growth and biodegradation occurred at 38ºC, pH 8.0, 35‰ and 2.0% of crude oil. Addition of fertilizer at 0.1% concentration resulted increase in growth and biodegradation rate. Emulsification experiment revealed that *P. aeruginosa* showed good emulsification activity against waste motor lubricant oil, crude oil, peanut oil, diesel, kerosene, naphthalene, anthracene and xylene. Results obtained from the biodegradation experiments at different culture condition revealed that, each factor is having significant impact on biodegradation. Biodegradation of crude oil and emulsification of eight different hydrocarbons by *P. aeruginosa* indicated the potentiality of the strain.

Key words: Bioremediation, biodegradation, biosurfactant, emulsification, hydrocarbons.

INTRODUCTION

Oil spills, industrial waste and run-off, refuse from coastal oil refineries, off-shore oil production, shipping activities and accidental spillage of fuels and other petroleum products (Yakimov et al 1998), all contribute to marine oil pollution, and amount to approximately 1.3 million tons per annum (National Research Council, 2002). Microbial degradation plays an important role in the removal of oil, besides evaporation and weathering process. However, natural biodegradation is a slow process as a result of environmental factors and the limited availability of nitrogen and phosphorous salts in the seawater (Passeri et al 1992) and is not well characterized. There are few reports on the effect of environmental factors on biodegradation such as, degradation of hydrocarbons and alcohols at different temperatures and salinities (de Carvalho and da Fonseca 2005), oxygen, nitrogen and temperature (Crawford and Zhou 1995), pH, nutrients and electron acceptors (Mukherji and Vijay 2002) substrate enrichment and dissolved organic carbon concentration (Bengtsson and Zerhouni 2003). But none of the reports contains cumulative information of effect of temperature, pH, salinity and crude oil concentration on biodegradation. Hence, the present investigation was carried out to explicate the effects of salinity, temperature, pH and crude oil concentration on biodegradation of crude oil by *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Isolation and identification of strain

P. aeruginosa was isolated from sea water collected from Tuticorin harbor (Lat. 08º 45'N; Long.78º 13'E) using Bushnell Haas agar with 0.1% of crude oil and identified to the species level by following Bergey's manual of Determinative Bacteriology (Holt et al 1994).

Bacterial adhesion to hydrocarbons (BATH)

Cell hydrophobicity was measured by bacterial adherence to hydrocarbons (BATH) according to a method similar to that described by Rosenberg *et al* (1980). The cells were washed twice and resuspended in a buffer salt solution (g l^{-1} 16.9 K_2HPO_4, 7.3 KH_2PO_4) to give an O.D at 600nm of 0.5. The cell suspension (2 ml) with 100 µl crude oil added was vortex-shaken for 3 min in test tubes (10 x 100mm). After shaking, crude oil and aqueous phase were allowed to separate for 1h. The O.D of the aqueous phase was then measured at 600nm. Hydrophobicity is expressed as the percentage of cell adherence to crude oil calculated as follows: 100 x (1-O.D of the aqueous phase/O.D of the initial cell suspension). For a given sample, three independent determinations were made and the mean value was accounted. Few drops of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution was added to the BATH assay solution and

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observed under microscope. The INT turned red, if it is reduced inside the cells, indicating the viability and adherence of cells with crude oil droplets (Betts et al 1989).

Growth and biodegradation of crude oil

Biodegradation experiment was carried out at various temperatures (28, 30, 32, 34, 36, 38, 40, 42, 44 and 46^{o}C), substrate concentrations (crude oil 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5%) and pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5). The impact of salinity on oil degradation was evaluated using various concentration of NaCl (0, 5, 10, 15, 20, 25, 30, 35 and 40‰). The effect of fertilizer on biodegradation was done with urea and K_2HPO_4 which were used at a ratio of 1:1 (w/w) at 0.1, 0.5, 1.0, 1.5 and 2.0% concentrations (w/v). All the experiments were performed in 250 ml conical flasks with 50 ml of mineral salt medium as described by Juwarker and Khirsagar (1991). The percentage of crude oil degradation was estimated fluoremetrically as described in IOC Manuals and Guide No. 13 (1982). Growth was estimated gravimetrically, for gravimetric estimation of biomass the broth culture was filtered through a 0.45µm sized Millipore filter paper and dried at 80^{o}C in a hot air oven and weighed. Biomass was expressed in dry weight (mg ml^{-1}) and a control was used to exclude the weight of crude oil.

Gas chromatographic analysis of crude oil degradation

Crude oil residues from the culture were extracted using hexane and the extract was made up to 10 ml using hexane. A capillary column (30 m Fused Silica column, Restek Corporation, USA) and GC (Perkin-Elmer 8310) with flame ionisation detector were used for analysis. The injection temperature was 250^{o}C, detector temperature was 250^{o}C and column temperature was programmed as 50^{o}C/4minutes which was then increased at the rate of 10^{o}C/minute to attain 330^{o}C and maintained at that temperature for 20 minutes. Florida Total Recoverable Petroleum Hydrocarbon (TRPH) standard (Restek-USA) was used to identify the n-alkanes (nC8-nC40). The analysis was carried out at School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK.

Estimation of emulsification activity

Five hundred µl of cell free culture broth was added to 5 ml of Tris buffer (pH 8.0) in a 30 ml screw capped test tube. Hydrocarbons like waste motor lubricant oil, crude oil, peanut oil, diesel, kerosene, naphthalene, anthracene and xylene were tested for emulsification activity. Five mg of hydrocarbon was added to the above solution and vortexed for 20 minutes and the mixture was allowed to stand for 20 minutes. The optical density of the mixture was measured at 610 nm and the results were expressed as D_{610} (Rosenberg et al 1979).

RESULTS

BATH, growth and biodegradation of crude oil

Table 1 shows biochemical and physiological characteristics of *P. aeruginosa*. BATH assay results revealed that *P. aeruginosa* showed high affinity (95.3%) with crude oil. Visualization of bacterial cells adhered with crude oil using INT staining (Figure 1) also confirmed the BATH results. The INT turned red and reduced inside the cells, indicating the viability and adherence of cells with crude oil droplets (Betts et al 1989).

Biodegradation experiments were conducted in two sets, i. with fertilizer and ii. without fertilizer. Crude oil residues in culture broth were extracted with hexane and the quantity of crude oil in the hexane extract was estimated using fluorescence spectrophotometer (Varian, Cary Eclipse, Fluorescence Spectrophotometer, Australia). OD value of the sample was measured at 310nm excitation and at 374nm emission wave length. Standard graph was prepared with different concentration of crude oil to calculate the percentage of degradation. When the strain was cultured at different temperature, maximum growth and crude oil degradation (91.5%) occurred at 38^{o}C with fertilizer and 81.3% without fertilizer (Figure 2 and Table 2). Regarding pH, increasing trend in degradation and growth was observed from pH 5.5 onwards, which attained its maximum at pH 8.0 with 85% of crude oil degradation with fertilizer and 73% without fertilizer (Figure 3 and Table 2). *P. aeruginosa* showed maximum growth and degradation at 35‰ of salinity and the rate of crude oil degradation was found as 78% in fertilizer added set and 60% in the set without fertilizer (Figure 4 and Table 2). Maximum growth and degradation activity was observed at 2.0% substrate concentration (Figure 5 and Table 2). Experiments with different concentration of fertilizer inferred that *P. aeruginosa* required 0.1% of fertilizer concentration for biodegradation crude oil (Figure 6).

Gas chromatographic analysis of crude oil degradation (Figure 7) revealed that, *P. aeruginosa* has utilized 86% of nC23 compounds followed by nC22 (66%), nC21 (88%), nC19 (64%), nC15 (43%), nC14

(75%), nC13 (80%), nC12 (63%), nC11 (37%) and nC10 (17%). These results confirmed the fluorescence spectrophotometric analysis of crude oil degradation in the present study.

Table 1. Physiological and biochemical characteristics of *P. aeruginosa*.

Name of the Test	Results
Gram reaction	-
Shape of the cell	Rod
Motility	+
Utilization of carbohydrates	
Adonitol	-
Glucose	+
Lactose	-
Trehalose	+
Xylose	+
Hydrolysis	
Starch	+
Gelatin	+
Fat	+
Casein	+
Catalase	+
Oxidase	+
Nitrate reduction	+
Pigmentation	Green

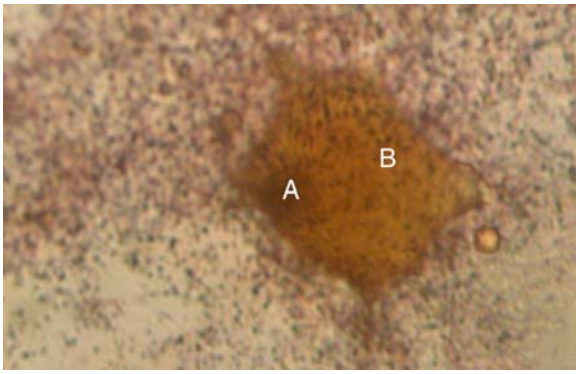

Figure 1. BATH assay A- INT stained cells, B-Oil droplet.

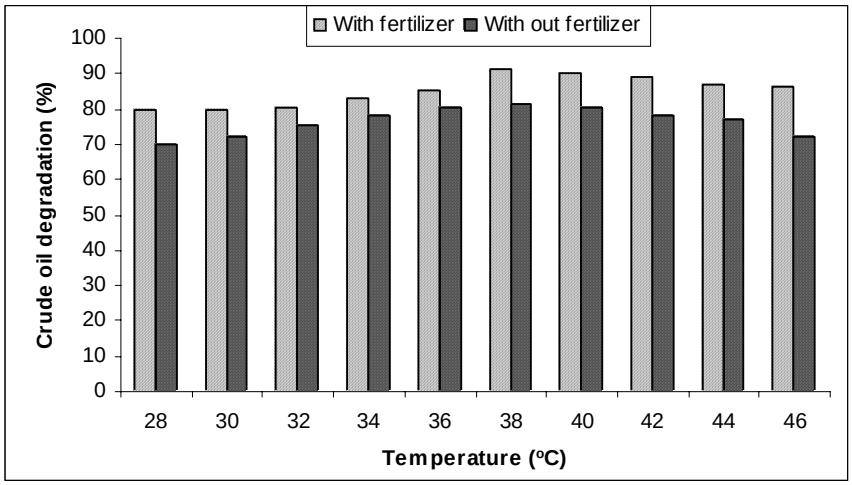

Figure 2. Biodegradation of crude oil at different temperature.

Table 2. Growth characteristics of *P. aeruginosa.*

T	G		pH	G		S (‰)	G		S (%)	G	
	WIF	WOF		WIF	WOF		WIF	WOF		WIF	WOF
28°C	4.21	3.5	5.5	2.76	2.11	0	2.28	2.11	0.1	4.11	3.65
30°C	4.35	3.8	6.0	2.81	2.45	5.0	2.31	2.15	0.5	4.25	3.75
32°C	4.41	4.1	6.5	2.92	2.56	10.0	2.57	2.32	1.0	4.38	3.83
34°C	4.48	4.21	7.0	2.97	2.61	15.0	2.81	2.42	1.5	4.55	3.92
36°C	4.51	4.36	7.5	3.12	2.85	20.0	2.92	2.65	2.0	4.66	4.12
38°C	4.54	4.4	8.0	4.51	3.16	25.0	3.16	2.76	2.5	4.1	3.84
40°C	4.54	3.8	8.5	3.46	3.11	30.0	2.25	2.15	3.0	3.52	3.25
42°C	4.41	3.2	9.0	3.58	2.25	35.0	2.15	2.0	3.5	3.21	2.84
44°C	4.35	3.1	9.5	3.21	2.12	40.0	1.80	1.12	4.0	3.05	2.50
46°C	3.12	2.9							4.5	2.58	2.12

T- Temperature, S(‰) - Salinity, S(%) - Substrate concentration, G - Growth rate (mg ml-1), WIF - With fertilizer, WOF - Without fertilizer.

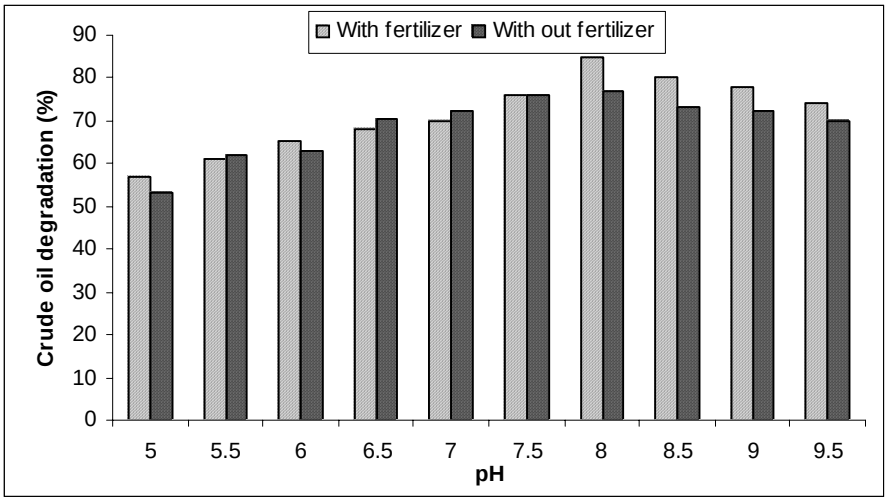

Figure 3. Biodegradation of crude oil at different pH.

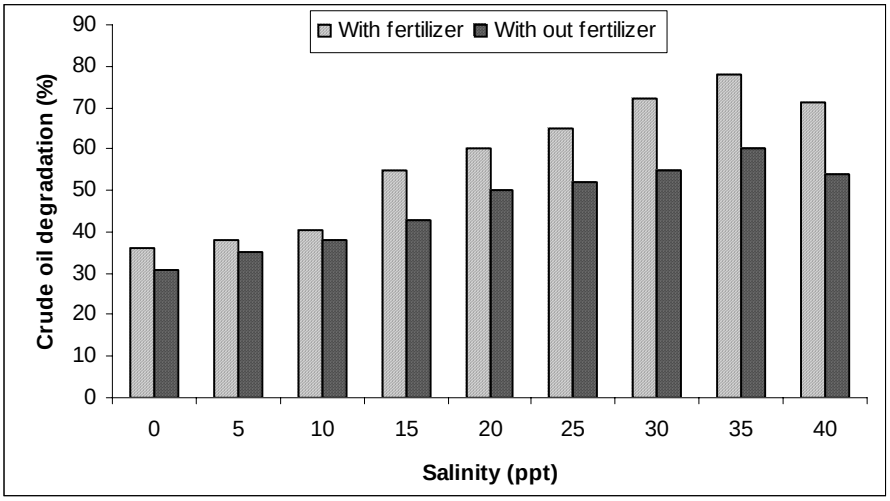

Figure 4. Biodegradation of crude oil at different salinity.

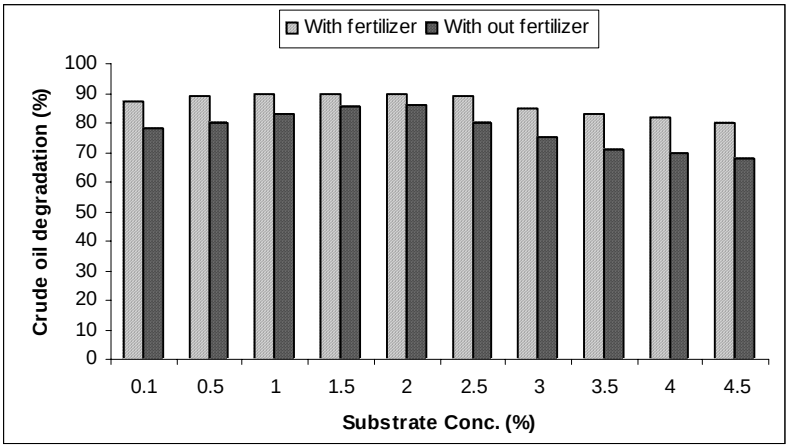

Figure 5. Biodegradation of crude oil with different substrate (crude oil) concentration.

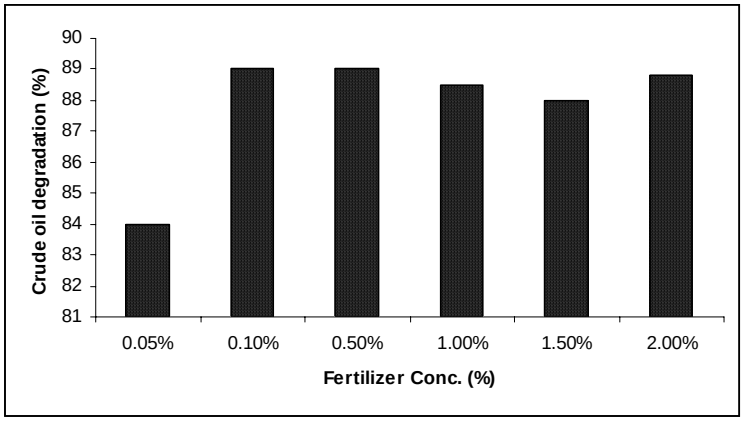

Figure 6. Biodegradation of crude oil with different fertilizer concentrations.

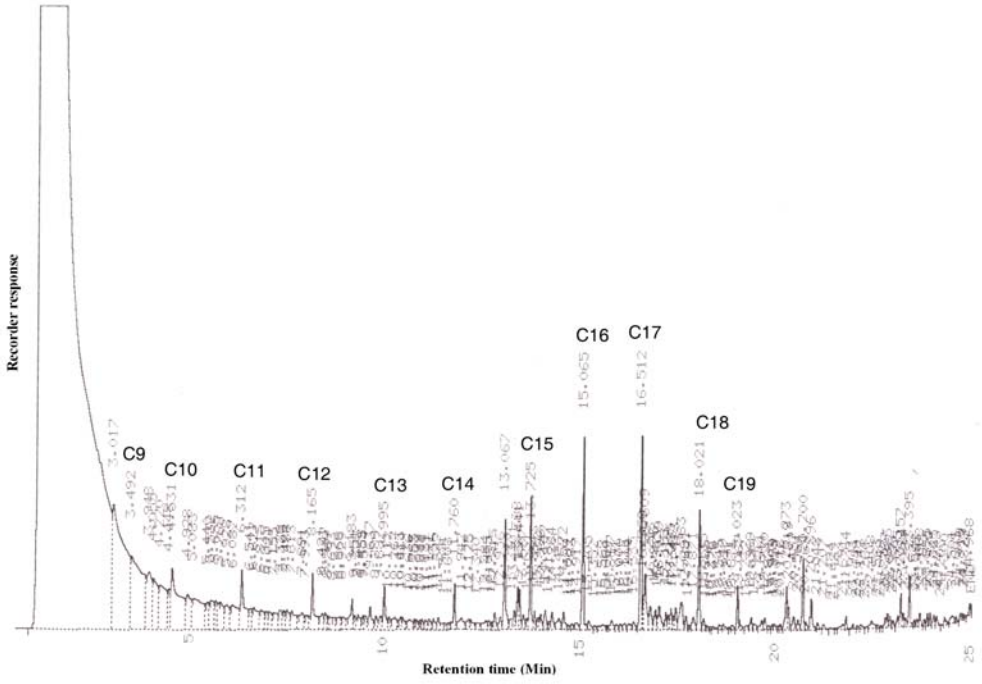

Figure 7. Gas chromatogram of degraded crude oil

The statistical analysis (ANOVA) of biodegradation at different culture conditions showed a high significant value ($P<0.05$) for all the factors.

Emulsification activity

Results obtained the present study on emulsification of hydrocarbons by the cell free culture broth indicated the presence of biosurfactants in the medium. In the present study, *P. aeruginosa* exhibited good emulsification activity with waste motor lubricant oil, crude oil, pea nut oil, kerosene, xylene, naphthalene and anthracene. The emulsification activity (D_{610}) was found as 2.01, 1.95, 1.58, 1.12, 0.61, 0.51 and 0.45 respectively to the above order of hydrocarbons.

DISCUSSION

The prerequisite access to the hydrocarbons is affected by membrane modifications in the lipid composition altering cell surface hydrophobicity and by production of extracellular products such as polysaccharides or surfactants (Solano-Serena et al 2000). This finding strongly supported the BATH assay results of the present study, where high affinity of cells with crude oil was found. Culture conditions had significantly affected the biodegradation process. Temperature profoundly influences all biological processes and can be expected to have a significant effect on oil degradation. Higher rate of biodegradation with strains incubated at 37^{o}C than at 20^{o}C and 28^{o}C in an oil sludge sample was observed by Dibble and Bartha (1979). An optimum temperature of 40^{o}C for the phenatheren degradation was reported by Stringfellow and Aitken (1994) in *Pseudomonas stutzeri* and *P. saccharophila.* Whereas in the present study *P. aeruginosa* preferred 38^{o}C as the optimum temperature for the degradation of crude oil.

Dibble and Bartha (1979) also observed maximum degradation rate at pH 7.8 in oil sludge samples, beyond which rate of degradation decreased. *Rhodococcus rhodochrous* KUCC 8801 and KUCC 8802 isolates showed their maximum growth at pH 8.0 in a crude oil containing medium kept at 40^{o}C (Sorkhoh et al 1990). In the present study also similar results were obtained, where maximum crude oil degradation occurred at pH 8.0 beyond that level a decreasing trend was observed. This indicated that extreme pH is inhibitory to majority of the microbial degradation processes.

Salinity is a major factor which significantly affects biological activity in the marine environment. In this study *P. aeruginosa* showed maximum growth and biodegradation activity at 35‰. Similar results were observed by de Carvalho and da Fonseca (2005), they reported better growth and hydrophobicity by *Rhodococcus erythropolis* at 2.5% of NaCl concentration. Mukherji et al (2004) observed significant degradation of diesel at 3.5% of salt concentration and above 3.5% and below 0.5%, it was very low. These observations strongly supported the present study.

de Carvalho and da Fonseca (2005), observed growth and degradation of alcohol and hydrocarbons at different concentrations ranged from 0.13% to 2.0% and found better growth rate at 0.5%. Zinjarde and Pant (2002) reported 92 ± 1.5% degradation of aliphatic fractions of crude oil at a substrate concentration of 0.5% by the bacteria and yeast strains obtained from tropical marine environments. Where as in the present study maximum degradation activity found at 2.0% indicated the potentiality of the strains to abide the higher concentration of crude oil.

The most discussed thing in bioremediation of oil spills is addition of fertilizer. According to Gibbs et al (1975) approximately 4mM of nitrogen was required to break down 1mg of crude oil and phosphorus was not become limiting down to a minimum P/N ratio of 0.02. It was clear from the present study that fertilizer addition increased the biodegradation process to greater extent and better results can be obtained by adding fertilizers in oil polluted sites.

Emulsification of the insoluble substrate (oil) is an important step before its degradation. Emulsification of hydrocarbons was used as a criterion for biosurfactant production by many workers (Rosenberg et al 1979; Juwarkar and Khirsagar 1991; Linares et al 1996). Emulsification of hydrocarbons needs the production of extracellular polymers or biosurfactants. Emulsification of eight different hydrocarbons by *P. aeruginosa* in the present study indicated the ability of the strain to use various hydrocarbons and possibility of the application of the strain or its products like biosurfactants against different hydrocarbon pollution. Juwarkar and Khirsagar (1991) also reported the emulsification of crude oil, xylene, pristine, n-octane, n-decane, n-hexadecane and n-dodecane by an unidentified marine bacteria isolated from Goa waters, west coast of India. The present study on crude oil emulsification was complemented by the work done by Fernandez-Linares et al (1996) in two marine strains, *Pseudomonas nautical* and *Marinobacter hydrocarboclasticus*.

This paper aims to contribute to asses the biodegradation potential of *P. aeruginosa* and the effect of culture conditions on growth and biodegradation process. Satisfactory results obtained in biodegradation experiments, BATH assay and emulsification of hydrocarbons indicated the possibility of the use of this strain or its products like biosurfactant against oil pollution.

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