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Biodegradation of Crude Oil in Marine Medium

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Abstract

In this work a laboratory study was carried out to investigate the efficiency of some microbial strains to degrade crude oil in a marine medium to be used in the bioremediation of oil polluted sea in Skikda, the North-East of Algeria. Two bacterial strains isolated from different hydrocarbon contaminated sites in Skikda, in addition to a consortium isolated from hydrocarbon contaminated site in Alexandria (Egypt) were tested for their capacity to degrade oil in a marine medium. The strains were able to degrade 81-90% of 1% of oil after 15 days of incubation. The two local strains were identified as *Pseudomonas* sp. S and *Rhodococcus* sp. S. The use of local urea as nitrogen source with local phosphorus fertiliser slightly stimulated oil biodegradation by *Rhodococcus* sp. S and *Pseudomonas* sp. S and slightly inhibited oil degradation by the consortium. The addition of chemical surfactants stimulated the rate of oil degradation. The concentration of oil was elevated from 1 to 6 % in presence of Triton X-100. The microorganisms were able to degrade 88-90% of 2% of oil after 15 days of incubation. The immobilisation of bacterial strains on wheat straw reduced the incubation time to 12 days. The results of this work revealed that bioremediation using bacterial strains is an effective technique for the decontamination of crude oil polluted marine media.

Keywords: *Pseudomonas* sp. S, *Rhodococcus* sp. S, Consortium, Oil, Immobilisation.

Introduction

Pollution of marine environments with oil products became a world wide problem on the tide of industrialization [1]. The sources of marine hydrocarbon pollution are mainly runoff from land and municipal/ industrial wastes, routine ship maintenance like bilge cleaning, air pollution from cars and industry, presence of 1% of natural seep, tanker accidents and offshore oil production [2,3]. It is well known that oil film contamination causes serious damage to aquatic life as oil film retards the penetration of oxygen into water. If the oil is not collected and removed within a certain time after the spill, evaporation of volatiles contained in the oil will enhance the concentration of non volatiles and thus increase the density and viscosity of the oil, causing it to sink. It will then associate irreversibly with sediments, including those which support populations of economic or ecological significance [4].

Among these pollution cases the town of Skikda [5-7], knows dangerous problems of pollution which affected the sea [8], the earth and the atmosphere. In fact, skikda is doted of a petrochemical industrial platform which constitutes the principal source of hydrocarbon pollution [9].

The elimination of hydrocarbons from marine environments needs the intervention of many biotic and abiotic factors. Among these factors the biodegradation using microorganisms and in particular the bacteria are the natural process the most important in the depollution of marine environments. As a result, the mechanisms of oil hydrocarbons degradation by bacteria and the parameters which could influence the degradation have been largely studied [10].

Microbial studies in laboratories, experimental field trials and clean up operations following actual marine oil spill incidents have demonstrated that bioremediation strategy based on the enhancement of oil biodegradation rates through nutrient addition was effective [11-15].

In biological treatment it is necessary to perform laboratory feasibilities tests to determine the microbial potential to degrade the pollutants and whether nutrients are required to increase the degradation rate [16]. This work is a laboratory study to investigate the efficiency of oil biodegradation by some microorganisms in marine medium to be used in the bioremediation of oil polluted sea in Skikda, the North-east of Algeria. The optimisation of some parameters which affect the utilisation of oil by the selected strains of bacteria was also done.

Materials and Methods

Isolation of microorganisms

The enrichment culture method was used for the isolation of the microorganisms. Oil 1% (v/v) was used as carbon source. 30 microorganisms (28 bacteria and two fungi) were isolated from hydrocarbon contaminated sites in Skikda in addition to an unknown consortium isolated from oil contaminated site in Alexandria, Egypt. A bacterial strain isolated from kerosene contaminated clay in Alexandria, Egypt was also tested for its ability to degrade crude oil in a marine medium.

Culture medium

The medium of Moran and co-workers [17]was used. It consisted of a filtered sea water supplemented with the following (per litter of sea water): NH_4NO_3, 1g ; yeast extract, 0.2g ; and 4 ml of a phosphate solution containing (g/l) Na_2HPO_4, 25 ; NaH_2PO_4, 3.6. Crude oil 1% (v/v) was added to each flask as carbon source after sterilisation of the medium. The flasks were inoculated with 2% (v/v) of bacterial

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suspension and incubated at 30ºC and 120 rpm. Uninoculated flasks were also prepared to test the effect of abiotic factors.

Identification of microorganisms

The bacterial strains able to degrade crude oil in a marine medium were gram stained. Different standard morphological, physiological and biochemical tests were performed using API 20 kits.

Fertilisers

Urea 46% was obtained from Abou Quir fertilisers and chemical industries Company, Alexandria, Egypt. Super Phosphate 15.5% was obtained from Suez Company, Egypt. Local fertilisers (nitrogen and phosphorus fertilisers) were obtained from ASMIDAL Company, Algeria.

Determination of residual oil

After the incubation periods oil was extracted from marine medium using acetone- hexane (1:1) solvent [18]. The extract was analysed by a gas chromatography with a mass spectrometry (GC-MS). The degradation percent was determined according to Bento et al. [19]. It was calculated by the following formula: % degradation = [(TO contrôle –TO traitement) / TO control] x100, where TO control is the total oil in the uninoculated marine medium , TO treatment is the total oil after treatment.

Immobilisation of cells by adsorption on wheat straw

Immobilised cells on wheat straw were prepared according to the method of Gouda and co-workers [20] with some modifications. Wheat straw (2 g) cut into small pieces (2 cm) and nutrient broth (50ml) were sterilized at 120°C for 20 min and then inoculated with 5.5ml of 24 h old seed culture (cultivated on nutrient broth medium). The flasks were incubated for 48 h. At the end of incubation time, the nutrient broth was decanted and the straw immobilized with cells was washed with sterile distilled water 2-3 times. The wheat straw immobilised cells were then used to inoculate 50 ml of marine medium containing 2% (v/v) of crude oil and 1% of the surfactant Triton X-100 and incubated for different incubation periods (3, 6, 9, 12 and 15 days). A control uninoculated flask was also prepared.

Immobilisation of cells by entrapment in alginate

3% of alginate was prepared and sterilised at 100-110° C for 10 minutes. 10 ml of the alginate was added to 100ml of sodium chlorite. The sodium chlorite was after that filtered and the alginate bills were washed by distilled water. 85 ml of the marine medium containing 15 % of the bacterial suspension, 2% of oil and 1% of Triton X-100 were added to the alginate bills. The flasks were incubated at different incubation periods (3, 6, 9, 12 and 15 days). A control uninoculated flask was also prepared.

Statistical analysis

Experiments were realised using independent replicates. Data were subjected to analysis of variance at $p \leq 0.01$ [21].

Results

Effect of incubation period on oil biodegradation

From the 30 isolates three local bacteria (O,R,V) in addition to an unknown consortium of bacteria (M) and a bacterial strain (VA) isolated from Alexandria (Egypt) had the capacity to degrade oil in the marine medium. The isolates able to degrade oil were cultured in a marine medium with 1% of oil and incubated for 7 and 15 days. A consortium of the three local bacterial isolates (C) was also cultured in the same marine medium. The degradation rate of oil reached its maximum using the unknown consortium after 15 days of incubation (Table 1). Figure 1 represents the chromatogram of crude oil using different bacterial strains after 15 days of incubation. The sharp peaks comprise the n- alkanes , whereas the peaks between them represent the aromatics and naphtenes having the similar molecular weight of n-alkanes. We observed that the n- alkanes are easily degraded in comparison with n-naphthenes and aromatics. The isolates chosen to continue this research are the two local isolates (O and V) in addition to the consortium isolated from Alexandria, Egypt.

Identification of microorganisms

The bacterial strain VA was identified by physiological and biochemical methods as *Pseudomonas* sp. CK (DSMZ, Braunschweig, Allemagne). The chosen isolates (O and V) were identified using gram staining and API 20 kits as *Rhodococcus* sp. S and *Pseudomonas* sp. S. The consortium is composed of *Bacillus sp. S, Acinetobacter sp. S and Aerobacter sp. S* (Table 2).

Effect of different nitrogen sources on oil biodegradation

In this experiment the ammonium nitrate used in the basal medium was substituted by different organic and inorganic nitrogen sources. The results mentioned in Table 3 revealed that urea 46% enhanced oil degradation by *Rhodococcus* sp. S, *Pseudomonas* sp. S and the consortium. The local urea stimulated oil biodegradation by the consortium and *Pseudomonas* sp. S and it had no significant effect on *Rhodococcus* sp.S. The nitrogen fertilizer had a negative effect on *Pseudomonas* sp.S and *Rhodococcus* sp. S while it had a stimulating effect on oil biodegradation by the consortium. Manure stimulated oil degradation by all the chosen microorganisms. Local urea was used as nitrogen source in the rest of the work due to its low price.

Effect of some phosphorus sources on oil biodegradation

We tested the effect of some phosphorus sources on oil biodegradation by the chosen microorganisms. According to the results mentioned in Table 4, the use of super phosphate 15.5% as phosphorus source increased the oil biodegradation by all the isolates under test. The local phosphorus fertiliser slightly inhibited oil biodegradation by the consortium. The ammonium phosphate increased the effect of *Rhodococcus* sp. S., whereas it slightly inhibited the effects of *Pseudomonas* sp. S and the consortium. The phosphorus fertiliser was chosen to continue this work due to its low cost and to its availability.

Effect of nitrogen/ phosphorus ratio on oil biodegradation

We tested the effect of different nitrogen phosphorus ratios (1/1,

Incubation time	7 days	15 days
Organisme	% oil degradation	
VA	45.00% ± 1.00 **D**	60.00% ± 2.00 **E**
R	35.00% ± 1.00 **E**	53.50% ± 0.50 **F**
O	50.00% ± 1.00 **B**	86.75% ± 1.25 **AB**
M	60.75% ± 1.25 **A**	90.00% ± 1.00 **A**
V	48.25% ± 0.75 **C**	81.00% ± 0.50 **CD**
C	46.25% ± 0.75 **CD**	79.50% ± 0.75 **BC**

Same capital letters are statistically not different among treatments at p<0.01± standard error (n=2)

Table 1: Effect of incubation periods on crude oil biodegradation using different bacterial Strains.

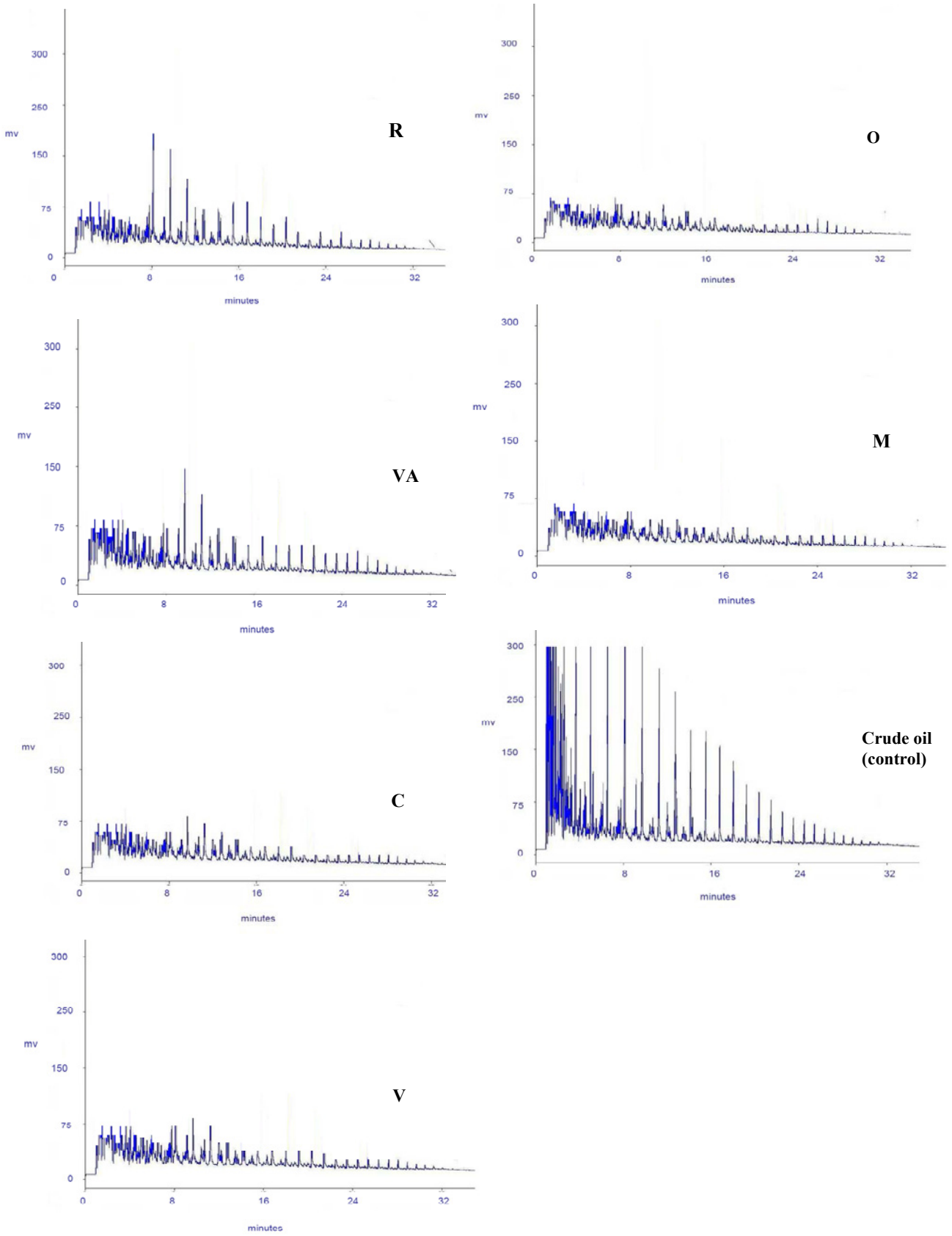


Figure 1: Gas chromatograms of the residual crude oil in marine medium using different bacterial strains after 15 days of incubation.

Isolate characteristics	*Pseudomonas* sp.S	*Rhodococcus* sp. S	Consortium		
			Bacillus sp. S	*Acinetobacter* sp.S	*Aerobacter* sp. S
Morphology	Rods	Coccobacilli	Rods	Rods	Rods
Colony	Green	Orange	White	White	Creamy
Gram	-	+	+	-	-
Urea	-	+	-	-	-
Indol	-	-	-	-	-
TDA	-	-	-	-	-
Mannitol	-	+	-	-	-
Nitrite	-	+	+	-	-
Glucose	-	+	-	+	+
Lactose	-	+	-	-	-
Saccharose	-	+	-	-	-
H_2S	-	+	-	-	-
Gas	-	-	-	-	-
Citrate	+	-	-	+	+
RM	-	-	+	-	-
VP	-	-	-	-	-
LDC	-	-	-	-	-
ODC	-	-	+	-	-
Oxydase	+	-	-	-	-
Catalase	+	+	+	+	+
Gelatine	+	-	-	-	-
ONPG	-	-	-	-	-
ADH	+	-	-	-	-
Mobility	+	-	+	-	-

+ : a positive reaction
- : a negative reaction

Table 2: morphological, physiological and biochemical characteristics of the chosen isolates.

Nitrogen source Organism	Basal medium[1]	Ammonium sulfate	urea 46%	Local urea	Sodium nitrate	Nitrogen fertiliser	manure
	% dégradation						
Rhodococcus sp. S[ab]	86.75% ± 0.75 **AB** (5.78) [2]	75.00% ± 1.00 **D** (5.00)	88.25% ± 0.75 **ABC** (5.48)	85.50% ± 0.50 **ABC** (5.70)	79.75% ± 1.25 **CD** (5.32)	77.75% ± 1.25 **CD** (5.18)	95.00% ±1.00 **B** (6.33)
Consortium[a]	90.00% ± 1.00 **ABC** (6.00)	78.50% ± 1.50 **CD** (5.23)	90.00% ± 1.00 **AB** (6.00)	94.50% ± 1.50 **AB** (6.33)	85.75% ± 1.00 **ABC** (5.72)	93.75% ± 0.75 **AB** (6.25)	94.25%±0.75 **AB** (6.28)
Pseudomonas sp. S[b]	81.00% ± 2.00 **C** (5.40)	68.25% ± 1.25 **E** (4.88)	86.75% ± 1.25 **ABC** (5.78)	84.00% ± 1.00 **ABC v**(5.60)	83.00% ± 2.00 **C** (5.33)	79.00% ± 1.00 **CD** (5.27)	97.00% ± 1.00 **A** (6.47)

[1]Ammonium nitrate was used as nitrogen source
[2] Degradation rate (% degradation/ incubation time)
Same capital letters are not statistically different among nitrogen sources at p<0.01± standard error (n=2)
Same small letters are not statistically different among organisms at p<0.01± standard error (n=2)

Table 3: Effect of some nitrogen sources on crude oil biodegradation by the chosen bacterial strains after 15 days of incubation.

Phosphorus source organism	basal medium [1]	super phosphate 15.5% [2]	Local phosphorus fertilise P_2O_5 [2]	ammonium phosphate[2]
	% dégradation			
Rhodococcus sp.S [ab]	85.50% ± 1.50 **BC** (5.70)[3]	91.75% ± 1.50 **AB** (6.12)	87.00% ±1.00 **ABC** (7.25)	93.00% ± 1.00 **AB** (6.20)
Consortium [a]	94.50% ± 1.50 **AB** (6.36)	97.00% ± 1.00 **A** (6.47)	90.00% ± 2.00 **B** (6.00)	87.25% ± 0.75 **ABC** (5.82)
Pseudomonas sp. S [b]	84.00% ± 1.00 **C** (5.60)	84.50% ± 1.50 **C** (5.63)	88.25% ± 1.25 **ABC** (5.88)	70.25% ± 0.75 **D** (4.68)

[1] Local urea was used as nitrogen source
[2] Phosphorus sources in the basal medium were substituted by the sources mentioned in table 3
[3] Degradation rate
Same capital letters are not statistically different among phosphorus sources at p<0.01± standard error (n=2)
Same small letters are not statistically different among microorganisms at p<0.01± standard error (n=2)

Table 4: effect of some phosphorus sources on crudeoil biodegradation by the chosen bacterial strains after 15 days of incubation.

3/1, 7/1, 10/1 and 20/1) on oil biodegradation. Local urea and local phosphorus fertiliser were used as nitrogen and phosphorus sources respectively. According to the results in Figure 2 the ratio10/1 had a positive effect on oil biodegradation by *Rhodococcus* sp. S and the consortium and it had no significant effect on *Pseudomonas* sp. S. The ratio 20/1 inhibited the action of the chosen microorganisms. The ratio 10/1 was chosen to continue the work.

Effect of P^H

The p^H of the medium was adjusted to 4,6,7,8 and 9. We observed that the acidic and alkaline p^H (4 and 9) inhibited the action of the two bacteria and the consortium. The p^H 6, 7 and 8 had the best effect on oil biodegradation (Data not mentioned).

Effect of surfactants on oil biodegradation

The surfactants Igepal, Tergitol, Triton X-100, Tween 20 and Tween 80 were used to emulsify oil with the liquid medium. Each surfactant was added at 1% and the p^H was adjusted to 7. The percentage of oil biodegradation reached 90-96.5%. Statistical analysis showed that there was no significant difference between the surfactants (Data not mentioned). Triton X-100 was chosen to complete the work due to its low price.

Effect of oil concentration on oil biodegradation

The concentration of oil was elevated from 1% to 2, 3, 4, 5 and 6%. Triton X-100 was added at 1%. In presence of 2% of oil *Rhodococcus* sp .S could degrade 89% of oil in comparison with 94% in presence of 1% of oil. The consortium was able to degrade 90.5% of 2% of oil and *Pseudomonas* sp. S 87.5%. The capacity of the chosen microgranisms to degrade oil decreased when the oil concentration increased to 3.4,5 and 6% (Figure 3).

Biodegradation of oil using free and immobilised cells on wheat straw and by entrapment in alginate

Oil biodegradation using free and immobilised cells on wheat straw and by entrapment in alginate was performed in liquid medium containing local urea as nitrogen source and local phosphorus source as phosphorus source. The surfactant Triton X-100 was supplied at 1%. The experiment was realised in presence of 2% of oil. We found that the immobilised cells by entrapment in alginate stimulated oil biodegradation in comparison with free cells and immobilised cells on wheat straw. The immobilised cells by entrapment in alginate could degrade 87.75-92% from 2% of oil after 9 days (Figure 4). The

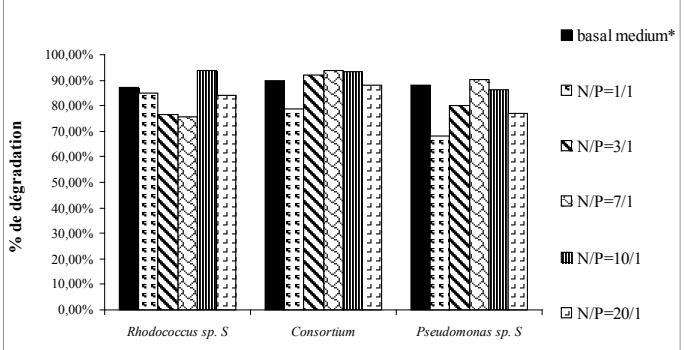


Figure 2: Effect of N/P ration on oil biodegradation in marine medium using bacterial strains after 15 days of incubation

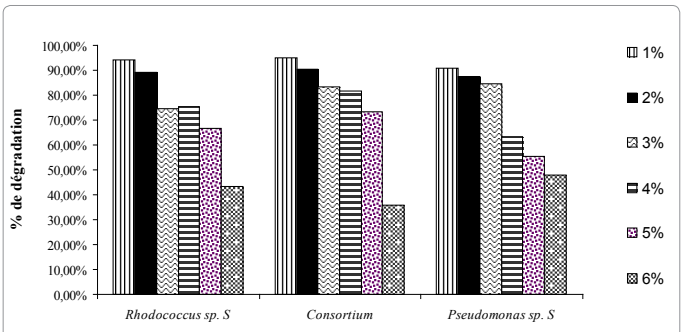
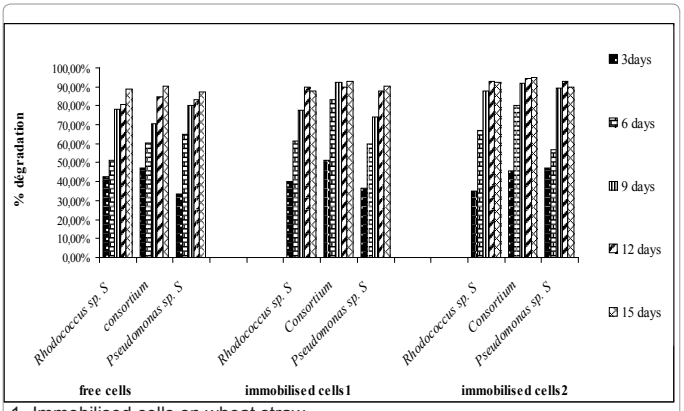


Figure 3: Effect of oil concentration on oil biodegradation in marine medium using bacterial trains after 15 days of incubation.



1- Immobilised cells on wheat straw
2- Immobilised cells by entrapment in alginate

Figure 4: Effect of oil concentration on oil biodegradation in marine medium using bacterial trains after 15 days of incubation.

immobilised cells on wheat straw had the capacity to degrade 88-90% of oil after 12 days. Wheat straw absorbed 8-10% of oil.

Discussion

This study showed that *Rhodococcus* sp. S could degrade 87% of oil after 15 days of incubation. The consortium was able to degrade 90% of oil while *Pseudomonas* sp. S degraded 81% of oil. Quek and co-workers [22] reported the capacity of *Rhodococcus* sp. F92 to degrade petroleum hydrocarbons. A consortium of *Pseudomonas* sp. K12-5, *Moraxella* sp. K12-7 and *Yarrowia lipolytica* 180 was used to degrade oil in an intertidal environment [23]. Moran and co-workers [17] isolated a strain of *Bacillus subtilus* O9 which could degrade hydrocarbons.

Chromatographic analysis showed that the n- alkanes fraction are easily metabolised in comparison with aromatic fractions. The same results were obtained by Gouda and co-workers [24]. Oh and co-workers [23] demonstrated that short chain n- alkanes are rapidly degraded whereas long chain n- alkanes are slowly degraded.

Microorganisms need nutrients for their multiplication [20]. It was noted that the limiting factors in microbial biodegradation of hydrocarbons are nitrogen and phosphorus [25]. The carbon, nitrogen and phosphorus ratio vary with the microorganism type, the carbon source and the habitat [26]. We found that the use of urea 46% increased the biodegradation of oil by *Pseudomonas* sp. S and *Rhodococcus* sp. S and had no significant effect on the consortium. Gouda and co-workers [24] reported that urea 46% slightly decreased the degradation

of kerosene by *Pseudomonas* sp. AP and *Gordonia* sp. DM. The local urea used in this work had a positive effect on *Pseudomonas* sp. S and the consortium and it had no effect on *Rhodococcus* sp. S. The use of sodium nitrate decreased the degradation of oil by *Rhodococcus* sp. S and the consortium. Gouda and co-workers [24] found that the sodium nitrate decreased the degradation of kerosene by *Pseudomonas* sp. Ap and enhanced the effect of *Pseudomonas* sp. CK. The use of local urea with super phosphate 15.5% increased the rate of oil biodegradation by *Pseudomonas* sp. S, *Rhodococcus* sp. S and the consortium. The substitution of phosphorus sources in the basal medium by a local phosphorus fertiliser slightly inhibited the degradation of oil by the consortium and enhanced the action of *Pseudomonas* sp. S and *Rhodococcus* sp. S. Sharma and Pant [27] isolated a strain of *Rhodococcus* able to degrade more than 50% of Arabian Assam in marine waters in presence of urea (35 mM of nitrogène) and dipotassium orthophosphate (0.1 mM of phosphorus) after 72h at 30ºC.

The ratios carbon/ nitrogen/ phosphorus vary with the authors, but they are not too different [28]. Some ratios reported in the literature are 100 :15 :3, 33 :5 :1 [28,29]. In this study we used the ratio N/P (10/1). This ratio stimulated the oil degradation rate by *Rhodococcus* sp. S and le consortium and slightly decreased the action of *Pseudomonas* sp. S. Gouda and co-workers [24] observed that the N/P ratio had no effect on kerosene biodegradation.

The effect of p^H on oil biodegradation was tested in this study. We observed that the degradation of oil by the consortium and *Rhodococcus*. S reached its maximum at p^H 7 whereas *Pseudomonas* sp. S was slightly affected by the p^H 7. The p^H 4 and 9 inhibited oil degradation by all the chosen microorganisms. Baszczyk-Maleszak and co-workers [30] observed that at p^H 5- 7 the rate of oil reduction oil was 60-70% however it reached 80-90% at p^H 7-9 after 21 days of incubation. Shin and co-workers [31] reported that the p^H had no dramatic effect on the bacterial growth in presence of phenanthrene as carbon source.

The use chemical surfactants may accelerate the degradation rate [24]. The biodegradation proceeds more rapidly when the oil is emulsified into small droplets [32]. In this study we found that the percentage of oil degradation reached 88.5-96% in presence of 1% of surfactant. Lee and co-workers [33] reported that the use of mycolic acid as a surfactant at low concentrations helps in accelerating the rate of oil biodegradation by *Rhodococcus baikonurensis* EN3.

The effect of oil concentrations was investigated in this work. The obtained results revealed that the microorganisms under test were able to degrade 87.5-90% of 2% of oil in comparison with 90.75-95% in presence of 1% of oil. The augmentation of bacterial capacity to degrade oil is due to the addition of 1% of Triton X-100. The surfactants increase the contact between the microorganism and the contaminant [34]. The results are in agreement with those found by Okuda and co-workers [35] who reported that the use of Triton X- 100 enhanced the dodecane degradation. According to Gouda and co-workers [24] the addition of Triton X-100 increased the kerosene biodegradation by *Pseudomonas* sp. AP, *Pseudomonas* sp. CK and *Gordonia* sp. DM.

The time needed for oil biodegradation was decreased from 15 days to 12 days when we used immobilised cells on wheat straw and it was reduced to 9 days in presence of immobilised cells by entrapment in alginate. The immobilised cells on wheat straw had the capacity to degrade 88-90% of oil after 12 days, whereas the immobilised cells by entrapment in alginate could degrade 87.75-92% from 2% of oil after 9 days. These results are in agreement with those found by Diaz and co-workers [36] who reported that the bacterial consortium shows good stability in immobilised systems. Apparently the increased stability of intracellular activities can be attributed to the protective effect of the biofilm against physicochemical stress. The use of alginate as a carrier accelerated the degradation of oil in comparison with the wheat straw; this might be due to the high immobilisation efficiency of the cells on the immobilisation material and the high affinity between the hydrophobic immobilisation material and the substrate [22]. This makes the substrate more available for the bacterial cells. The findings obtained in this study indicate that immobilised cells on wheat straw showed faster and better oil degradation than free cells. This is due to immobilisation material which protects the bacterial cells from the contaminants [20]. On the other hand, the oil –absorbing capacity of wheat straw (8-10%) can be used to prevent migration of floating petroleum products from an oil to spill to beaches and shorelines. The petroleum adsorbed products may be then degraded in-situ or ex-situ [22]. The wheat straw immobilised cells could be easily collected from marine waters and can keep their capacity to degrade oil for a certain period. This finding can be applied to the polluted sea waters of the industrial zone of skikda, Algeria, where the Mediterranean waters suffer from the petroleum refinery activities.

Conclusion

- Bioremediation using bacterial strains is an effective technique for the decontamination of oil polluted marine media.

- Local fertilisers (local phosphorus fertiliser and local urea) may be used as phosphorus and nitrogen sources.

- The use of wheat straw immobilised cells is a cost effective technique, it accelerates the rate of oil biodegradation and could be employed as a practical technology for oil biodegradation in oil polluted sea waters.

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