

Phenotypic characterization and tolerance test to crude oil of bacterial strains isolated from coasts of Oran

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Article history:

Received: 10 February, 2014

Accepted: 16 February, 2014

Available online: 15 March, 2014

Abbreviations:

BH: Bushnell-Haas medium

Keywords:

Biodegradation, oil, seawater, *Staphylococcus aureus*, *Micrococcus lylae*, *Pseudomonas sp.*, *Acinetobacter sp.*, *Providencia rettgeri*, *Flavobacterium breves*

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Abstract

Screening of hydrocarbon degrading microorganisms isolated from seawater contaminated by discharges of the Arzew refinery in west of Algeriaby selective enrichment technique, resulted in the collection of 06 distinct species. All strains were cultivated in liquid media with crude oil

as a sole carbon and energy source. Bacterial strains capable of degrading hydrocarbons belong to the genera *Staphylococcus aureus*, *Micrococcus lylae*, *Pseudomonas sp.*, *Acinetobacter sp.*, *Flavobacterium breve*, and *Providencia rettgeri*. Five of them have been targeted for their high ability to develop with oil. In order to test the capacity degradation of these strains to oil, a study of the evolution of certain parameters such as microbial concentration, pH, temperature and the monitoring of the tolerance of isolated strains tooil was realized. The results show that *Pseudomonas* has a broad spectrum of growth to a pH close to neutrality (6 to 8), while the optimum growth in the majority of species is observed at alkaline pH (10), the optimum temperature growth is observed at 25° C. We can see that the hydrocarbonclastesmarine bacteria isolated can tolerate up to 20% oil.

We must complete our study by the use of molecular biology techniques to understand the hydrocarbon degradation mechanisms and enzymes involved.

Citation:

Khelil-Radji F., Matallah-Boutiba A., Chemlal-Kherraz D., Boutiba Z., 2014. Phenotypic characterization and tolerance test to crude oil of bacterial strains isolated from coasts of Oran. The Journal of Biodiversity. Photon 113, 297-304.

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Photon Ignitor: ISJN83596193D658915032014

1. Introduction

The waters of all types and soil are essential to life resources in general (biodiversity) and human societies , especially (drinking water and other uses , agricultural and urban land) , which must protect the quality hygenic by preventing all kinds of pollution and restoring polluted compartments if necessary , without excessively distorting economic development (Bocard, 2006). There are necessary to

assess the acceptability of the products towards the environment, and to appreciate perspectives and possibilities of restoration of polluted sites.

Oil is a source of pollution of marine environments that can affect the ecological balance and, by extension, economic activities in the polluted areas. Many damages have been incurred in accidents, releases or deliberate spills, which can cause irreversible

ecological disasters. The consequences of this environmental pollution may have a direct or indirect impact on the ecosystems and human health (Gab et al., 2004). Knowledge of the effect of pollutants in the environment is essential to develop effective and realistic actions to complex problems. There are necessary to assess the acceptability of the products towards the environment, and to appreciate perspectives and possibilities of restoration of polluted sites.

The elimination of oil from the marine environment requires the involvement of various biotic and a biotic factors (Soltani, 2004). Biodegradation by microorganisms is undeniably the most successful in removing pollutants from oil process (Atlas, 1981). While it is relatively slow, this process allows almost complete degradation of hydrocarbons (transformation into CO₂). Nine kinds of cyanobacteria, 103 fungi, and 14 algae are currently known to be able to degrade hydrocarbons. But it has been proved that bacteria (79 genera identified in 2005) were qualitatively and quantitatively the most effective (Prince, 2005). Microorganisms play a crucial role in the evolution of pollutants, particularly in the degradation of petroleum hydrocarbons (Leahy and Colwell, 1990). Numerous studies show that hydrocarbonoclastes microorganisms were selected following the contamination (Head et al., 2006; Yakimov et al., 2007). The effect of oil on microbial communities involves metabolic capacity complex mechanisms, it is depended and influenced by environmental parameters and duration of exposure to pollutants (Yakimov et al., 2004; Bordenav et al., 2007).

The aim of our study is: isolation, purification and identification of the microbial populations from seawater contaminated by fluids released from refinery and assess their capacity to degrade them. This work has been in conducted in the laboratory Environmental Monitoring Network at the University of Oran.

2. Objective of Research

The primary objectives of this study were to isolate and characterize hydrocarbon-degrading microorganisms indigenous to aquatic sites contaminated by discharges of the Arzew refinery in west of Algeria by selective enrichment techniques, and evaluation of the effect of abiotic parameters on the growth of bacteria hydrocarbonoclastes

which requires us to be careful, patient and vigilant.

2. Materials and Methods

The main objective is to determine the role of indigenous marine bacteria in the degradation of petroleum hydrocarbons, we tried to solve it by the isolation, purification and identification of hydrocarbonoclastes marine bacteria, and the effect of various parameter sabiotic their growth. The microorganisms were obtained originally by enrichment culture in B-H: MgSO₄ 0.2 g/l, CaCl₂ 0.02 g/l, KH₂PO₄ 1g/l (NH₄)₃PO₄ g/l, KNO₃ 1 g/l and FeCl₃ 0.05 g/l, pH 7.2. Peptone broth (0.1%, pH 7.2) was used for serial dilutions to determine bacterial viable cell counts. Each medium was sterilized for 20 minutes at 120° C.

2.1 Isolation and identification of strains

This step helps to highlight the existing micro flora in seawater of industrial area contaminated by fluids releases. The samples originate from the sea water of industrial port of Arzew (Oran). 1 ml was taken and mixed with 9 ml of mineral medium BH. This mixture was added of crude oil at a rate of 2% which is considered here as the sole carbon source. The mixture is then incubated at 30° C with stirring at 150 revolutions / min. After the pre - enrichment, we do the seeding marine agar. The incubation was at 30° C during 24 hours. Bacteriological identification of our isolates is mainly based on morphological, physiological and biochemical characterization (using API 20 NE) (Rossello and Amann, 2001).

2.2 Monitoring the kinetics growth of bacteria isolated

We have been tracking the growth isolated bacteria on mineral medium (BH) containing crude oil as the sole source of carbon and energy within the microbial concentration. We measure optical density with a spectrophotometer (OD₆₀₀) and a bacterial count on agar medium at regular intervals every 12 hours throughout the fermentation period (120 hours) (Akmousi, 2009). The colonies are expressed as UFC/ml. We also calculated the following parameters:

- $\mu = N / t$
- $G = t / N$
- $N = (\log \text{UFC}_0 - \log \text{UFC}_1) / \log_2$

With μ : growth rate

t: the doubling time of the number of bacteria

N: number of division

G: generation time

2.3 Study of the optimum temperature

The isolated strains (24 hours old) were inoculated into Erlenmeyer flasks of 250 ml filled with 50 ml of sterile BH medium + 2 % of crude oil. The pH is adjusted to 7. The incubation of 24 h is made with rotary stirring rate of 150 revolutions / min at different temperatures (10° C, 15° C, 20° C, 25° C and 30° C) (Rodrigo, 2005). To estimate the optimum temperature, the viability of the cultures was determined by counting on nutrient agar at a temperature of 30° C after 24 to 48 hours of incubation. The colonies are expressed as UFC/ml.

2.4 Study of optimum pH

This study was performed with use of 50 ml of BH medium in Erlenmeyer flasks of 250 ml supplemented by crude oil as the sole carbon source at a rate of 2 %. The different values of pH (5, 6, 7, 8, 9 and 10) are adjusted by adding NaOH 1M or KCl 1M. The cultures were incubated at 30° C with stirring 150 tours/min.

Growth was checked by counting on nutrient agar (Akmousi, 2009).

2.5 Tolerance stem oil

According to the method of Fukumaki et al. (1994) were inoculated 2 ml of inoculums in 100 ml sterile BH medium in Erlenmeyer flasks of 500 ml. Then 0 % (control), 2 %, 5 %, 10 %, 15 %, and 20 % of oil (v / v) the media was covered. Incubation is at 30° C under agitation (150 rpm / min) for 2 days. The counting was done by measuring the optical density (OD₆₀₀) and by counting on solid GN.

3. Results

3.1 Isolation and identification of bacterial species

Macroscopic study has differentiated 6 strains, distinct by the following characteristics: color, size, shape, contour elevation. To identify the different isolates, the biochemical tests were used and the results are summarized in Table 1:

Table 1: Results of biochemical testing of isolates strains

Biochemical characters		Strains	S1	S2	S3	S4	S5	S6	
metabolism energy	Type respiratory (VF)		AS	AS	AAF	AS	AAF	AAF	
	Oxidase		+	+	-	-	-	+	
	Catalase		+	+	+	+	+	+	
	Nitrate réductase		+	-	+	+	+	-	
metabolism carbohydrate	Milieu Mevag		O	O	OF	O	OF	OF	
	Milieu TSI	Glu	+	+	+	+	+	+	
		Lac./Sac.	-	-	-	-	-	-	
		H ₂ S	-	-	-	-	-	-	
		Gas	-	-	-	-	-	-	
	β-galactosidase (ONPG)		-	-	-	-	-	-	
	Citrate perméase		-	+	+	+	+	+	
	Milieu Mannitol/ mobilité	Mannitol		+	-	-	-	+	+
		Mobilité		+	+	-	-	+	-
	Milieu Clarck et Lubs	VP		-	-	+	-	-	-
		RM		+	+	+	+	+	+
metabolism protein	Milieu Moeller	ADH	-	+	+	-	-	-	
		LDC	-	-	-	-	-	-	
		ODC	-	-	-	-	-	-	
	Milieu Urée/ Indole	Urée	+	-	+	+	+	+	
		Indole	-	-	-	-	+	+	
TDA	+	-	-	+	-	-			
Milieu King	King A		-	+	-	-	-	-	
	King B		-	+	-	-	-	-	

AS: strictly aerobic. O: oxidative. AF: Aerobic optional. OF: oxidative and fermentative

S1 strain was straight to the genus *Micrococcus* because of its cells shaped cocci Gram and catalase positive. (Holt et al., 1994; Atlas, 1995; Leyral Guy et al., 1998). Madigan et al., 2000). This strain is strictly aerobic, oxydase positive and negative mobility based on the results of biochemical tests (Table 1)

and colonies that have no pigment and in comparison with those established by Bergey (1984) and Delarras (2007), this strain correspond to *Micrococcus lylae*.

S2 strain shaped bacilli, Gram-negative strictly aerobic oxydase, ADH, catalase, citrate

permease and ONPG positive, negative LDC (Table 1), may be linked to the species *Pseudomonas aeruginosa* since it is characterized by the production pigments pyocyanin and pyoverdine (Delarras, 2007; Lotfabad et al., 2000).

S3 strain as Gram-positive cocci in clusters presents irregular "bunch of grapes", which characterizes the species *Staphylococcus aureus* with a predominant aerobic metabolism and facultatively anaerobic, producing coagulase, thermolabile nuclease and catalase, but no oxidase (Ananthanarayan and Paniker, 2006).

S4 strain was straight to the genus *Acinetobacter sp.* due to the shape of these cells which are coccobacilli, Gram negative or sometimes grouped by two variable length strings, catalase positive. This strain has a negative response to the test LDC, ODC, ADH, producing hydrogen sulfide, indole, and beta-galactosidase (Bergey, 1994).

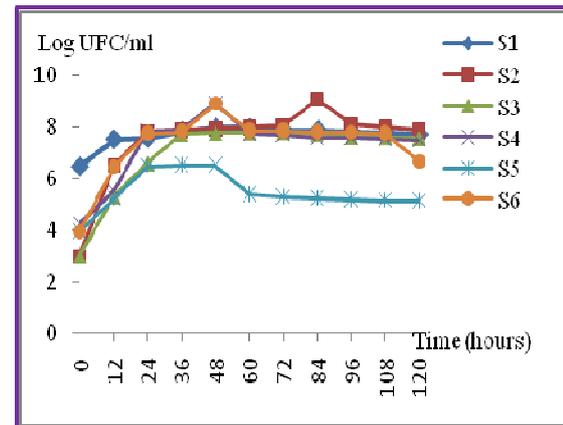
S5 strain is related to the family *Enterobacteriaceae* and has the following characteristics: a bacillary form, Gram-negative, aero-anaerobic facultative, catalase positive and fermentative metabolism (Madigan and Martinko, 2007). Based on the results of biochemical tests: oxidase, ONPG, Lactose, H₂S negative and TDA, citrate permease and urease positive, we assumed that the strain belonged to the species *Providenciarettgeri* (Bergey, 1994; Delarras, 2007).

S6 strain was connected to the genus *Flavobacterium* because of these pigmented yellow on the middle colonies, as well as Gram-negative bacilli short, strict aerobic and immobile (Bergey, 1994; Delarras, 2007). Disasters caused by oil spillshas motivated us to look for more reliable and less expensive solutions bioremediation by indigenous bacteria hydrocarbonoclastes.

3.2 Monitoring the kinetics of growth of bacteria isolated

The growth kinetics of the isolated strains on medium BH supplemented with 2% of crude oil as the sole carbon source was followed by measuring the microbial concentration versus time which allowed us to draw the curves shown in Figure 1. The Log UFC / ml = f (t) curves have the appearance of a classical bacterial growth curve in a medium not renewed.

Figure 1: Growth kinetics of isolated bacterial strains cultivated on BH medium supplemented with 2% of crude oil



In comparing the results of the growth parameters of the different strains (Table 2), isolates S2 and S6 are the only ones to reach a number of live cells to 10⁹ UFC/ml 10⁸ with a very high growth rate of 0.97 and 0.63 h⁻¹, respectively for the two strains and corresponding to a generation time of 1.02 h and 1.44 respectively.

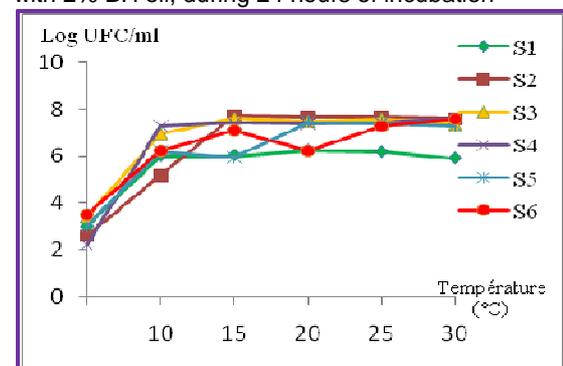
Table 2: Parameters of the kinetics of growth of isolates

Strains	S1	S2	S3	S4	S5	S6
N	3,52	11,66	7,64	4,25	4,18	8,30
μ (h ⁻¹)	0,29	0,97	0,63	0,35	0,34	0,69
G (h)	3,40	1,02	1,57	2,82	2,87	1,44

3.3 Study of the optimum temperature

To evaluate the influence of temperature on the growth of strains isolated, different values of temperatures ranging from 10° C to 30° C have been tested. The results obtained are shown in Figure 2.

Figure 2: Effect of different temperatures on the growth of the isolates on medium supplemented with 2% BH oil, during 24 hours of incubation



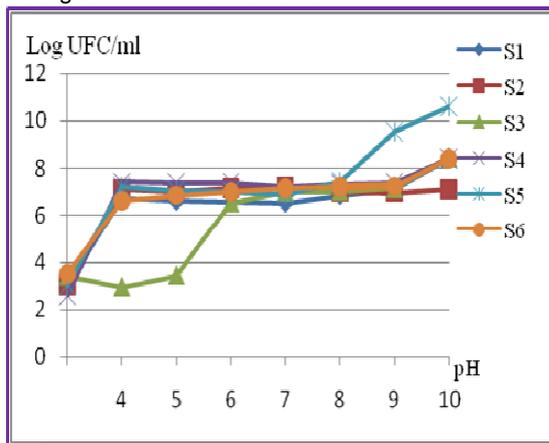
From the curves in Figure 3, it can be seen that the majority of isolates support changes in pH, with a maximum of reported growth at pH between 9 and 10, except for the S2 that gives

a maximum strain of growth a pH between 6 and 8.

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3.4 Study of optimum pH

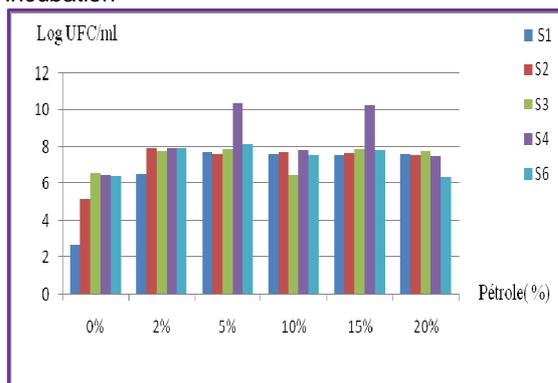
Figure 3: Effect of different pH on the growth of the isolates on BH medium supplemented with 2% of oil during 24 hours of incubation.



3.5 Tolerance stem oil

The results of the tolerance of the strains to different concentrations of the oil are summarized in Figure 4.

Figure 4: Effect of different concentrations of oil on the growth of isolate son BH medium for 48 h incubation



4. Discussion

The presence of different strains namely *Staphylococcus*, *Micrococcus*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium* isolated from seawater contaminated by discharges of the Arzew refinery was predictable since they are among the predominant hydrocarbonoclastes bacterial genus cited in studies of Leahy and Colwell (1990) and floodgate (1995).

These organisms are hydrocarbons degrading and ubiquitous (Atlas, 1995; Olivera et al., 1997). We identified *Providencia rettgeri* which can be isolated from the marine environment according to studies of Hassen et al. (1998) and Foti et al. (2009). For the six bacterial strains tested, the observed lag phases are very short. This result demonstrates a more or less rapid adaptation of these strains which possess an appropriate enzymatic equipment to attack different types of petroleum hydrocarbons used as the sole source of carbon and energy (Akmousi, 2009).

From the curves (Figure 1), we can observe that the microbial concentration increases from the first fermentation time until reaching maximum values after 24 hours for the S5 strain, after 36 hours for S1 and S3 strains, after 48 hours for S4 and S6 strains and after 84 hours for the S2 strain. This increase corresponds to the exponential phase during which the dissolution of the substrate (oil) satisfied the metabolic cells needs. This solution is based on the enzymatic equipment of bacteria and is specific for each type of petroleum hydrocarbons, which explains the different peaks of microbial loads marked in different time for the six strains studied (Akmousi, 2009).

After the exponential phases that are specific for the various strains, growth became stable. This can be explained by the level of nutritional requirements that surpasses the rate of dissolution of the substrate, then the bioavailability becomes limiting. During this stationary phase, the most complex petroleum fractions are degraded (Rocha et al., 2007).

Beyond 108 hours, there is a drop in the microbial load, which is due to the depletion of the medium or secretion of secondary metabolites or the inability of bacteria to degrade the most complex oil fractions. From the curves of Figure 2, we note that our isolates grow in a wide range of temperatures from 10° C to 30° C.

Our results are consistent with those found by Rodriguez -Blanco et al. (2010) who reported that the degradation of crude oil or diesel might as well be done at 25, 10 or 4° C in Mediterranean water with controlled conditions. This tolerance to different temperatures can be explained by marine origin of these bacteria and the temperature changes in the interval between seasons. We also find that the highest growth rates were recorded at 25° C for all strains studied, which

allows us to deduce that this temperature value can be considered optimal growth temperature. The Research Work of Sauret (2011) show that the optimal hydrocarbon biodegradation was observed in pure culture around 25-37° C, which corresponds to the temperature optimum of a large number of bacterial enzymes. Therefore, even if biodegradation is a process that can be done all year, some authors suggest that it is more efficient in summer than winter (Atlas et al., 1973).

According to Vandecasteele (2005), extreme pH inhibits the biodegradation of the oil. However, in the marine environment as fresh water, it is within a very favorable pH ranges (between 7 and 8). However, we noted a significant increase in growth of the strains: S1, S3, S4, S5 and S6 to pH 10, this result can be explained by the acid produced by the aerobic metabolism of hydrocarbons which will acidify the medium culture that is alkaline from the outset in our experimental cases.

To study the tolerance of our strains to different concentrations of oil, we note that all isolates support up to 20 % oil, these results are consistent with those reported by Suzuki et al. (2001); Priefert et al. (2004) who argument that the bacteria isolated from a chronic contaminated area by oil (port of Arzew in this study) tolerate significant concentrations of oil because the polluted areas, mechanisms of induction of enzymes of interest were found in the presence of oil. This adaptation results in high stability which gives bacteria the ability to respond more quickly to new source of hydrocarbons that bacteria without spoilage (Head et al., 2006; Maila et al., 2006; Bordenave et al., 2007).

Acknowledgements

Authors gratefully acknowledge the financial support of Laboratory: Environmental Monitoring Network, Department of Biology, Faculty of Science University of Oran - Ministry of Higher Education and Scientific Research, Government of Algeria.

Research Highlights

- Marine bacteria isolated from chronic polluted Oil areas;
- Purification and identification of hydrocarbonoclastes marine bacteria;
- Test the influence of biotic parameters on the growth of hydrocarbonoclastes marine bacteria.

Limitations

We could not do the genetic identification of our isolates and the use of molecular biology techniques to understand what happens to the enzyme level and metabolism of different classes of hydrocarbons, because of lacks the means.

Recommendations

To minimize limitation above call sare made of molecular biology techniques for a better accuracy in a short duration.

Funding and Policy Aspects

We cannot recommend anything on current policies, but that is for the improvement of public government.

Justification of Research

Disasters caused by oil spills have motivated us to look for more reliable and less expensive solutions bioremediation by indigenous hydrocarbonoclastes bacteria.

Conclusion

Our study aims to use indigenous strains to solve the problem of marine pollution by hydrocarbons. To achieve this goal we must deepen our current research in two main areas, namely the phylogenetic and physiological analysis of the most tolerant strains.

Author's Contribution and Competing Interests

Auteur de contribution et des intérêts divergents:

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