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ORIGINAL ARTICLE

CHARACTERIZATION OF HYDROCARBONOCLASTES BACTERIA ISOLATED FROM MARINE WATERS WEST ALGERIA: EVOLUTION ANALYSIS IN PRESENCE OF CRUDE OIL

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ABSTRACT: This work aims at providing methodological approaches to characterize microbial communities in marine pollution by oil and to evaluate the influence of some abiotic factors on the biodegradation activity of oils by bacteria. We have isolated 09 bacteria strains from seawater contaminated by releases of the Arzew refinery (Littoral western Algeria). A phenotypic identification of these strains allowed bringing to *Staphylococcus aureus, Micrococcus lylae, Acinetobacter sp, Flavobactérium breve, Pseudomonas aerugenosa, Pseudomonas luteola, Burkholderia cepacia, Burkholderia gladioli* and *Providensia rettgeri*. Six of these strains were selected to measure their ability to grow in the presence of crude oil. To check the mechanism biodegradation of oil, an evolution study of certain parameters such as microbial concentration, pH, temperature and the follow-up of the targeted strains tolerance was approached. 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) and optimum temperature growth is observed at 25 °C. Our experiments revealed that the isolated marine hydrocarbonoclastes bacteria could tolerate up to 20% oil.

KEYWORDS: bacteria, hydrocarbonoclastes, characterization, biodegradation, contamination, crude oil, seawater, Littoral western Algeria.

INTRODUCTION

The contamination of the marine environment, in particular by oil, has a considerable ecological impact and a significant environmental repercussion not only because of the disruption it causes to the marine environment and organisms, but also for the definition criteria of quantitative and qualitative evaluation of substances involved. Oil is a source of pollution of marine environments that can affect the ecological balance and, by extension, economic activities in the polluted areas. Crude oil is a complex mixture contains a large number of distinctively different chemicals and is composed of four mains fractions: saturated hydrocarbons and aromatic hydrocarbons, resins and asphaltenes (Hasanuzzaman *et al.*, 2007). Hydrocarbon are relatively unereative due to lack of functional groups of low water solubility (Hassanshahian *et al.*, 2012).

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 (Gabet, 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.

Biodegradation by natural populations of microorganisms is the basic and the most reliable mechanism by which thousands of xenobiotic pollutants, including crude oil, are eliminated from the environment (Cappello *et al.*, 2007). The effects of environmental conditions on the microbial degradation of hydrocarbons and the effects of hydrocarbon contamination on microbial communities are areas of great interest (Rahman *et al.*, 2004; Cappello *et al.*, 2012). Bioremediation is the strategy to utilize biological activities as much as possible for quick elimination of environmental pollutants. Growth stimulation of indigenous microorganisms, biostimulation, along with inoculation of foreign oil-degrading bacteria is a promising means of accelerating detoxifying and degrading activities at a polluted site with minimum impact on the ecological systems (Cappello *et al.*, 2006).

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

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selected following the contamination (Head and Jones, 2006; Yakimov *et al.*, 2007). The elimination of oil from the marine environment requires the involvement of various biotic and abiotic factors (Soltani, 2004). 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; Bordenave *et al.*, 2007).

MATERIALS AND METHODS

This study was conducted at the laboratory of Environmental Monitoring Network (*LRSE*) at the University of Oran (Algeria) and whose primary purpose is: isolation, purification and identification of the microbial populations from seawater contaminated by fluids released from oil refinery and assess their capacity to degrade them.

2.1. Isolation and identification of strains

This step helps to highlight the existing microflora in seawater of industrial area contaminated by fluids releases. The samples originate from the sea water of industrial port of Arzew (Oran west Algeria). 1ml was taken and mixed with 9 ml of mineral medium (Bushnell-Haas 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-Mora 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 (OD_{600}) and a bacterial count on agar medium at regular intervals every 12 hours throughout the fermentation period (120 hours) (Akmousi-Toumi, 2009). The colonies are expressed as UFC/ml. We also calculated the following parameters:

- **μ** = N / t
- **G** = t / N
- **N** = (log UFC_0 - log UFC_1) / log2

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 24h 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 *et al.*, 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 50ml 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 150tours/min. Growth was checked by counting on nutrient agar (Akmousi-Toumi, 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 (OD_{600}) and by counting on solid GN.

RESULTS

3.1. Isolation and identification of bacterial species

Macroscopic study has differentiated 9 strains, distinct by the following characteristics: color, size, shape, contour elevation. To identify the different isolates, the biochemical test was used and the results are summarized in Table (1).

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 *et al.*, 1998; Madigan and Martinko, 2007). This strain is strictly aerobic, oxydase positive and to 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 Delarra, (2007) this strain correspond to *Micrococcus lylae*.

S2 strain shaped bacilli, Gram-negative strictly aerobic oxidase, 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 (Lotfabad *et al.*, 2000; Delarra 2007).

Table 1: Results of biochemical testing of isolates strains

Biochemical characters \ Souches			S1	S2	S3	S4	S5	S6	S7	S8	S9
Metabolism energy	Type respiratory (VF)		AS	AS	AAF	AS	AAF	AS	AS	AS	AAF
	Oxydase		+	+	-	-	-	+	+	+	-
	Catalase		+	+	+	+	+	+	+	+	+
	Nitrate réductase		+	_	+	+	+	+	-	+	-
Metabolism carbohydrate	Milieu Mevag		O	O	OF	O	OF	O	O	O	OF
	Milieu TSI	Glu	+	+	+	+	+	+	+	+	+
		Lac./Sac.	-	-	-	-	-	-	-	-	-
		H_2S	-	-	-	-	-	-	-	-	-
		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.

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, thermostable nuclease and catalase, but no oxidase (Ananthanarayan, 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, 1984).

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, H_2S negative and TDA, citrate permease and urease positive, we assumed that the strain belonged to the species *Providencia rettgeri* (Bergey, 1984; Delarra, 2007).

S6 strain was connected to the genus *Flavobacterium* because of these pigmented yolk on hecktoen middle colonies, as well as Gram-negative bacilli short , strict aerobic and immobile (Bergey, 1984; Delarra, 2007).

S7 strain was straight to the genus *Pseudomonas* due to the shape of these cells which are bacilli, Gram negative, catalase positive, oxidase negative. This strain has a positive response to the test catalase, ADH, citrate permease and beta –galactosidase, assumed that the strain belonged to the species *Pseudomonas luteola* (Singleton, 1999).

S8 strain free bacilli, Gram-negative, generally accumulating granules of poly-beta-hydroxybutyrate, moving through one or more polar flagella, strict aerobic catalase positive, oxidase positive, capable of growth using as a sole carbon source, glucose, glycerol, inositol, galactose, sorbitol, mannose, and mannitol, this strain correspond to *Burkholderia cepacia*.

S9 strain is related to the species *Burkholderia gladioli* because it has the following characteristics: aerobic, catalase positive, urease positive, non spore formers. *Burkholderia gladioli* can be distinguished from the other *Burkholderia* because it has a negative response to the test oxidase, indole, nitrate and lysine decorboxylation (Coenye and Vandamme, 2007).

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 = ƒ(t) curves have the appearance of a classical bacterial growth curve in a medium not renewed.

In comparing the results of the growth parameters of the different strains (Table 2), isolates S1, S4 and S6 are the only ones to reach a lesser number of live cells to 10^6UFC/ml with a very small growth rate of 0.29, 0.27 and 0.06 h^{-1}, respectively for the three strains and corresponding to a generation time of 3.40, 3.68 and 15.7 h respectively.

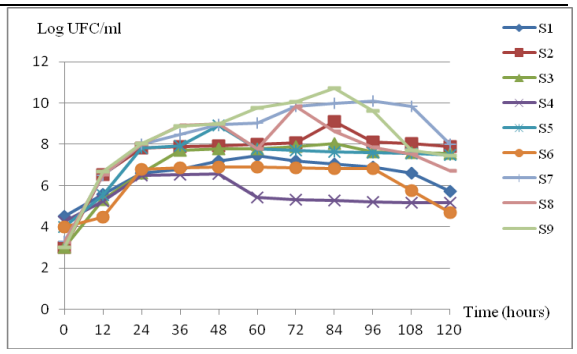

Figure 1: Growth kinetics of isolated bacterial strains cultivated on BH medium supplemented with 2% of crude oil.

Table 2: Parameters of the kinetics of growth of isolates

Strains	S1	S2	S3	S4	S5	S6	S7	S8	S9
N	3.52	11.66	7.64	3.25	5.18	0.76	8.30	10.89	12.22
μ (h^{-1})	0.29	0.97	0.63	0.27	0.43	0.06	0.69	0.90	1.01
G (h)	3.40	1.02	1.57	3.68	2.31	15.7	1.44	1.10	0.98

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.

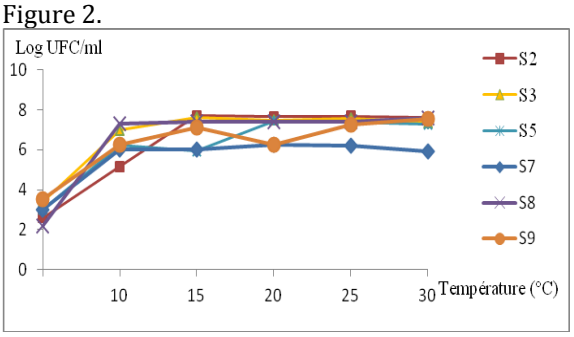

Figure 2: Effect of different temperatures on the growth of the isolates on medium supplemented with 2% BH oil, during 24 hours of incubation.

3.4. Study of optimum pH

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.

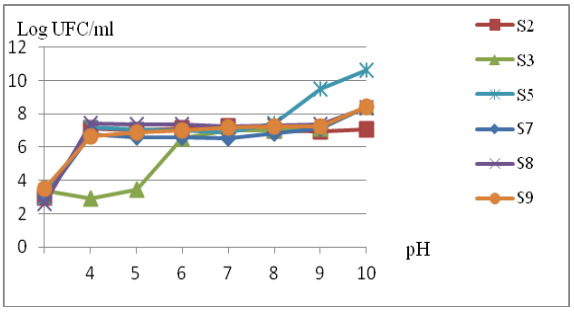

Figure 3: Effect of different pH on the growth of the isolates on BH medium supplemented with 2% of oil during 24 hours of incubation.

3.5. Tolerance stem oil

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

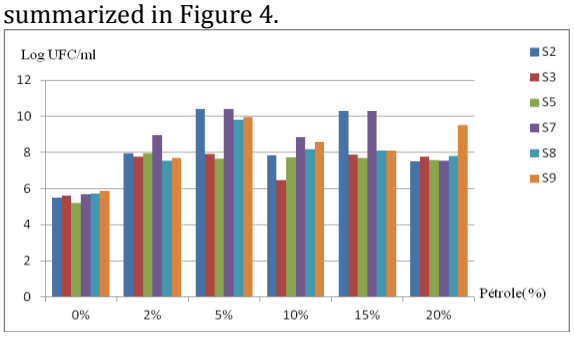

Figure 4: Effect of different concentrations of oil on the growth of isolates on BH medium for 48 h incubation.

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 degrading hydrocarbons are ubiquitous (Atlas, 1995; Olivera Nelda *et al.*, 2009). 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), and two species of the genus *Burkholderia* which can be isolated from marine waters.

For all 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-Toumi, 2009).

From the curves (Figure 1), we can observe that the microbial concentration increases from the first fermentation time until reaching maximum values after 36 hours for the S4 strain, beyond 48 hours for S5 strain, after 60 hours for S1 and S6 strains, after 72 hours for the S8 strain, after 84 hours for the S2, S3 and S9 strains and after 96 hours for the S7 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-Toumi, 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. these results are consistent with those found by Rodríguez-Blanco and Antoine, (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 and Bartha, 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 range (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. Some environmental parameters such as temperature and pH will affect the properties of the oil (oil viscosity, volatility and solubility of the molecules) and activity of microbial communities (Chang *et al.*, 2006; Boszczyk-Maleszak *et al.*, 2006).

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 and O'Brien, (2004) and Taketani *et al.*, (2010) 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 and Jones, 2006; Maila and Randima, 2006; Bordenave *et al.*, 2007).

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.

The results described in this paper show that efficient crude oil degrading bacteria present in the Arzew gulf at Algeria. Also a direct relationship found between biosurfactant production and crude oil biodegradation in *C. variabile* strain PG-Z.

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