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Biosurfactant Producing Bacteria Capable of Hydrocarbon Biodegradation from the Soils of Hassi-Messaoud Region of Algeria

H. Guetarni*[1,2], and A. Kararmouch[1]

[1]*Department of Biology, Faculty of Nature and Life Sciences and Earth Sciences, University Bounaama Djilali of Khemis Miliana, 44225, Ain Defla, Algeria*

[2]*Natural Substances Valorization Laboratory, University Bounaama Djilali of Khemis Miliana, 44225, Ain Defla, Algeria*

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Abstract: Discharges from the petroleum industries are important pollutants in the environment and therefore major risks to human and animal health. The biological decontamination of soils and aquifers contaminated by petroleum hydrocarbons requires the intervention of competent microorganisms. "Hydrocarbonoclastic" bacteria produce biomolecules called "biosurfactants" which are of great interest in environmental and industrial applications. The aim of our study is a characterization of biosurfactant-producing bacterial strains associated with the oil industry of Hassi-Massaoud. Nineteen strains were isolated on synthetic mineral salts medium (MSM) containing 2% crude oil as a carbon source. Our isolates were grouped into six main genera: *Pseudomonas, Staphylococcus, Enterobacter, Citrobacter, Bacillus, Micrococcus* and two genera attached to the classes of *Bordetella/ Alcaligenes/Moraxella* spp. The species *Pseudomonas aeruginosa* showed significant degradation of hydrocarbons among all isolated bacterial species as determined in MSM supplemented with crude oil. This species can be used as a potent biosurfactant-producer.

Key words: Biosurfacants, Hassi-Massaoud oil industry, hydrocarbons, MSM, petroleum, soil.

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Praveen Kumar
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K.S. Jadon
S.C. Meena

***Correspondence**

H. Guetarni
kmhg2009@yahoo.fr

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Polycyclic aromatic hydrocarbons (PAHs) contained in petroleum products like naphthalene are one of the most common persistent organic pollutants (POPs) in the environment. Soil and groundwater contamination by PAHs is an emerging environmental threat due to their possible toxic impacts on different ecological receptors for global warming and pollution (Nguemté *et al.*, 2017). Furthermore, the major problem encountered in soils polluted by petroleum products is their movement to the water table, thus affecting the quality of water and hence necessitating its treatment (Perfumo *et al.*, 2010). Soil depollution techniques based on physico-chemical or biological processes are mostly utilized worldwide. There are techniques which completely modify the integrity of the soil and which are most often very expensive, such as excavation, vitrification, incineration, etc. (Akmouci,

2009). In other cases, organic co-solvents or surfactants are used. These are agents with surface activity (surfactants) synthesized chemically or biologically (biosurfactants) (Abouseoud *et al.,* 2008). Most commercially available surfactants are of chemical origin and are petroleum-derived products. They pose a risk to the environment as they are generally toxic and non-biodegradable (Vipulananda and Ren, 2000). This pollution therefore requires the intervention of other factors for its elimination. This is why, for several years, scientists are working towards biodegradation of PAH by microorganisms, in particular by bacteria (Tarayre, 2012).

Bioremediation primarily exploits biological agents to remove targeted hydrocarbons. Hydrocarbonoclastic bacteria is capable of degrading hydrocarbons and can use them as a sole source of carbon such as *Pseudomonas, Micrococcus, Xanthomonas, Arthrobacter, Acinetobacter, Rhodococcus, Flavobacterium, Flavobacterium*, *Agrobacterium*, etc. (Berthe-Corti and Höpner, 2005). They have the ability to synthesize biomolecules (biological surfactants or biosurfactants) viz., glycolipids, lipopeptides, phospholipids, lipo saccharides and neutral lipids (Abouseoud *et al.,* 2008). These have the same surfactant properties as their chemical counterparts but have the advantage of being biodegradable and non-toxic. Hence, it is the most important natural process in the depollution of the environment (Banat *et al.,* 2000; Gayathiri *et al.,* 2022).

Native microbes isolated from hydrocarbon-contaminated environments are expected to be more robust in degradation than non-native species. Native microbes can produce metabolites (biosurfactants) that can readily solubilize hydrocarbons or other similar pollutants to make them readily available for microbial conversion; therefore, they outperform non-native or maladaptive microbes. Alternatively, co-cultivation of native microbes with effective oil-degrading microbes is considered a good strategy to increase contaminant removal in a short time (Parthipan *et al.,* 2017).

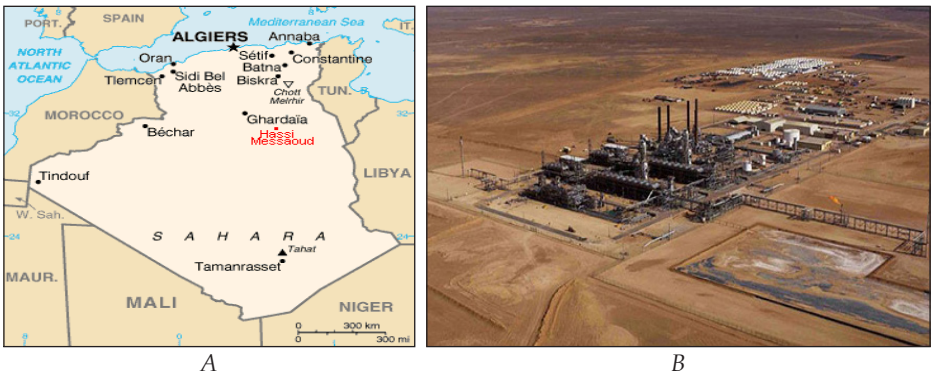
In this paper we have presented results on isolation, characterization and identification of the native hydrocarbonoclastic bacteria from the soil samples of oil contaminated sites in Hassi-Messaoud Region of Algeria.

Materials and Methods

Sampling

Soil sample was taken from a drilling site polluted by petroleum hydrocarbons, located in the Southeast industrial center of Algeria "Hassi Messaoud". The Hassi Messaoud field is one of the largest oil fields in the world. Its area is 2000 km² and it is 142 m above mean sea level (Anonymous, 2011) and is located 798 km southeast of Algiers (Fig. 1).

The study area was chosen on the basis of its degree of pollution in relation to the age of the quagmire. A quagmire is constructed in such a way that it occupies as small a surface area as possible and that the discharged drilling waste is contained there without generating risks of pollution. The operation of quagmires can become inefficient given that they receive a high volume of waste which can, as a direct consequence, damage of the covering plastics, hence the failure to maintain the spoil; and subsequently leading to the vertical migration



A *B*

Fig.1. Sampling site location A: Algeria map; B: Hassi Messaoud oil field (Source: The Maghreb Times, 2017).

of pollutants contaminating a large area of the subsoil and water reserves (Chabouni, 2008).

The soil samples collected were processed for their physicochemical characterization and hydrocarbonoclast strains present in soil were isolated, purified and characterized. The physicochemical characterization of the soil samples focused mainly on color, particle size composition (texture), pH value and pH-KCl, hygroscopic humidity, conductivity and organic matter content (NF ISO 11464, 2006). The bacteria present in soil were isolated on modified MSM (supplemented with 2% crud oil), purified and characterized using macroscopic, microscopic, biochemical and selective enrichment techniques and their biosurfactant production potential on liquid and solid medium and their efficacy was assessed through parafilm M test, oil displacement test, study of the emulsification index and surface tension were also studied.

Isolation and purification of hydrocarbonoclast strains on MSM medium

We carried out seeding on a synthetic solid culture medium based on "modified MSM" mineral salts supplemented with 2% crude oil as a sole carbon source (Margaritis *et al.,* 1979; Van decasteele 2008). Seeding was done on the surface from the stock solution and the first two dilutions and then incubated at 30°C for 24 to 48 hours.

After 24 to 48 hours of incubation many colonies varying in color, shape, diameter, opacity, etc. had grown. A sample from each type of colony was taken and was purified by successive subcultures using the streak method (Martineau, 1996). The strains, were conserved both for short and long period. For short conservation, the isolated hydrocarbonoclast strains were preserved on nutrient agar slants. The strains were seeded in streaks on the slope of the tubes, then incubated at 30°C for 24 hours. The tubes in which there was growth were stored at 4°C for a period of 4 to 6 weeks (Marchal and Bourdon, 1982; Martineau, 1996). In long conservation, the isolated strains were preserved in a nutrient medium supplemented with 1:1 v/v glycerol and 0.005% filtered carbon source. The mixture was vortexed and stored at -20°C (Obayori *et al.,* 2009).

Characterization and identification of purified strains

Bacteria were identified based on their macroscopic, microscopic and biochemical characters (Thoma *et al.,* 1970; Singleton, 1999; Meyer *et al.,* 2004; Joffin and Leyral, 2006).

Macroscopic identification: The colonies were examined for their shape (round, wavy, filamentous, etc.) the outline appearance (irregular, star-shaped, etc.), the size of the colonies (punctiform; diameter <1 mm, non-punctiform >1 mm), the color of the colony, elevation (convex, concave or flat colonies), opacity (opaque, translucent or transparent), the surface (smooth, rough, dry, shiny, or dull), the consistency (viscous or non-viscous) and the smell (presence and its nature or absence) as per Joffin and Leyral (2006).

Microscopic identification: Microscopic identification consisted of observing the bacterial cells in their fresh state under a light microscope, and after Gram staining (Marchal and Bourdon, 1982).

Biochemical characterization: The identification of bacteria is largely based on the biochemical characters, the demonstration of which constitutes the essential part of the identification gallery. These characters are the translation of bacterial metabolism, therefore making it possible to predict biochemical characters and vice versa (Meyer *et al.*, 2004). Biochemical tests generally make it possible to distinguish even closely related species (Tortora *et al.*, 2003).

Catalase activity: One or two drops of 10 volume hydrogen peroxide were placed on a glass slide and then a colony (after 24 h) was put into it. Observation on effervescence was recorded immediately (effervescence or not) (Tortora *et al.,* 2003).

Oxidase detection: Using a forcep, placed an "OX" disk containing N dimethyl paranitrophenylene diamine oxalate in a petri dish which had been previously soaked with a drop of sterile distilled water. Using an ose, took out a well-isolated colony representative of the fresh culture to be tested (avoided use a metal ose; with the exception of platinum). Gently rubbed the colony on the disk and observed the appearance of purple color within 30 seconds (Denis *et al.,* 2011).

Starch hydrolysis test: Starch is a polysaccharide, polymer of α glucose. This polyglucose contains two constituents: amylose (20% of the total mass) or poly-α (1-4)-D-glucopyranoside, and amylopectin (80% of the total mass). The microbial amylases that degrade starch are exoenzymes. Starch hydrolysis is expressed by clearing around each colony, a clear observation was obtained by flooding the starch agar [5 g pepton, 10 g Starch (wheat or potato), 20 g Agar, 1000 mL distilled water, pH = 6.8] surface with lugol (Wisdomkofi *et al*., 2006; Delarras, 2014).

Lecithin hydrolysis and lipoproteinase test: The culture medium was prepared by adding 2ml of an emulsion of egg yolk and sterile physiological water to 20 ml of nutrient agar, melted and brought back to 47±2°C, poured into petri dishes and left to solidify. It was inoculated in patches with the strains to be studied and incubated for 1 to 5 days at 37°C depending on the bacterial strains to be tested (Delarras, 2007). According to Delarras (2014), the colonies exhibit certain characteristics. When a colony forms, it initially appears with a halo. If this halo is opaque and yellowish-white, it tests positive for lipoproteinase. If there is a clear edge under the colony or at its periphery, it is indicative of lecithinase activity (lecithinase +). Conversely, if there is no halo present, it suggests a lack of lecithinase activity (lecithinase -). In cases where lecithinase is absent (lecithinase -), any fuzzy opacity observed is limited to the colony's surface, indicating the presence of lipase.

Casein hydrolysis: Proteolytic micro-organisms are the agents of putrefaction and owe their activity to the secretion of exocellular proteinases. In practice, only one proteinase is sought, it is caseinase, which hydrolyzes casein (Meyer *et al.,* 2004). Its identification consists of inoculating the strain to be tested in milk-based agar medium for 1 to 8 days and incubating at 37°C (Delarras, 2007). The presence of caseinolysis is reflected by the appearance of clear zones around the colonies**.**

Media based bacterial identification: *Identification of the genus Staphylococcus using Chapman and Baird Parker agar*: Chapman agar is an old culture medium, intended for the isolation and differentiation of staphylococci. Sodium chloride at high concentration inhibits many germs, except halophilic or halotolerant germs. The fermentation of mannitol by certain species of staphylococci releases acids which cause phenol red to turn yellow in an acidic environment: otherwise, the medium retains its initial red color. (Delarras, 2007). Chapman agar is a traditional culture medium designed for the purpose of isolating and distinguishing staphylococci. The presence of high concentrations of sodium chloride inhibits the growth of many microorganisms, except for those that are halophilic or halotolerant. In certain staphylococci species, the fermentation of mannitol produces acids, resulting in the phenol red indicator turning yellow in an acidic environment. Otherwise, the medium retains its original red color (Delarras, 2007). To utilize Chapman's agar, it is typically streaked with strains that are suspected to belong to the Staphylococcus genus and then incubated at 37°C for 24 hours.

Identification of the genus Pseudomonas using cetrimide-based medium: After purification of the strains which could be attached to the *Pseudomonas* genus on solid nutrient medium (Gram Negative), the strains were streaked on cetrimide-based medium which allows the enumeration and selective isolation of *Pseudomonas*, more precisely *Pseudomonas aeruginosa* (Delarras, 2007).

To isolate bacterial colonies with morphological characteristics matching the sought-after bacteria, they were initially cultured on nutrient agar. These colonies were then transferred into pure culture in the inclined tubes containing King's A and King's B media. This transfer was carried out by creating median streaks on the surface of the sloped medium, followed by sealing the tube caps without tightening them, and subsequently incubating them at 30°C for a period of 4 days (Marchal and Bourdon, 1982; Delarras, 2007).

Identification by the Analytical Profile Index gallery (Micro-gallery): The use of API galleries in our study serves the purpose of verifying the accuracy of macroscopic identification by comparing it with the relevant API galleries. We employed two types of API galleries: the API 20E Gallery for identifying Gram-negative bacilli, including Enterobacteriaceae and Pseudomonas, and the API STAPH Gallery for identifying

staphylococci, Micrococcus species, and Kocuria (Delarras, 2007).

Selection of the best biosurfactant-producing strains

In this study, we utilized highly efficient oil-degrading bacterial strains, which were chosen from the preceding methods and were designated as S1 (*Pseudomonas*), S4 (*Staphylococcus*), S5 (Enterobacteriaceae), S10 (Enterobacteriaceae), S14 (Enterobacteriaceae), S15 (Enterobacteriaceae), S17 (*Staphylococcus*) and S19 (*Bacillus*). We employed these selected strains for studying the biodegradation of petroleum in both liquid and solid media.

Biodegradation of petroleum in liquid MSM medium: Bacterial species were tested individually (eight strains: S1, S4, S5, S10, S14, S15, S17 and S19) or in a mixed population of these eight strains in the presence of crude oil. A culture of the isolated strains was made in nutrient broth and incubated at 30°C for 24 to 48 hours with rotary shaking at 160 rpm. Culture broths were centrifuged (3000 rpm for 15 mins at 4°C) and washed five times with liquid MSM (1.2 g NH_4Cl, 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.1 g NaCl, 1 g KNO_3, 20 g $MgSO_4.7H_2O$, 10 g $CaCl_2.2H_2O$, 0,05 g $FeCl_3$, 2 ml crude oil, 1000 distilled water, pH=7). Around 10 ml of MSM was then added (Van De Casteele *et al.,* 2001; Sifour *et al.,* 2007) and the bacterial biomass was assessed by its optical density at a wavelength of 600 nm at 24 h time intervals (Shweta *et al.,* 2014). This measurement is considered a direct biological indicator of biodegradation (Tian *et al.,* 2018).

Petroleum biodegradation on MSM agar: A procedure has been established on MSM agar for the detection of microorganisms capable of degrading non-volatile hydrocarbons on a solid medium. We took the eight strains previously selected and followed a modification of the procedure described by Hohzoh *et al.* (1982). The nine cultures (eight strains in single culture and mixed) were grown in nutrient broth for 24 hours at 30°C, which were then centrifuged at 3000 rpm for 10 min. The pellet was washed five times with liquid MSM. From the different pellets, cultures were taken using sterile loop and deposited in the form of spots on solid MSM medium supplemented with crude oil. They were incubated away from light at 30°C for 7 days and were observed daily for the appearance of clear zones.

Selection of the best strains producing biosurfactants: The selection of the best strain producing biosurfactants was carried out according to the method of Patel and Desai (1997). Erlenmeyer flasks of the strains cultured on liquid MSM added with crude oil as the sole carbon source during the exponential phase of the samples was used to perform the following tests:

Parafilm M Test: The parafilm test is used as a preliminary test for the detection of biosurfactant production. Approximately, 25 µl of the bacterial culture was added to the hydrophobic surface of the parafilm. The shape of the drop on the surface was assessed after one minute and its diameter was measured with reference to the same volume of distilled water as a negative control. According to Walter *et al.* (2010) the stability of the droplet depends on the biosurfactant concentration.

Oil displacement test: In a petri dish of 10 cm diameter, 20 ml of distilled water was placed and then 10 µl of crude oil was added using a micropipette to the surface of the water. Then, 10 µl of the bacterial suspension was gently placed at the center. A petroleum displacement halo was observed and the diameter of the clear zones was measured after 30 s. Each experiment was repeated three times to determine a mean clear zone diameter (Morikawa *et al.,* 1993).

Emulsification index (E24): Analyzing the emulsification index (E24) involves a qualitative test to evaluate a strain's emulsification capacity, specifically its ability to disperse a hydrophobic phase (e.g., diesel or petroleum) within a hydrophilic phase. This test provides insights into whether the isolates are capable of biosurfactant production. The calculated value is called emulsion index or E24, using diesel (Cooper and Goldenberg, 1987; Bodour *et al.,* 2004; Rodrigo *et al.,* 2005). The test consists of mixing 4 ml of the liquid culture medium with 4 ml of diesel in sterile tubes, then vortexing at high speed for 2 min to homogenize the two phases. The stability of the emulsion was determined after 24 h of standing at room temperature (Abu-Ruwida *et al.,* 1991). According to Bodour *et al.* (2004), the emulsion index represents the height of the emulsion

Table 1. Results of study of the macroscopic characteristics of isolated colonies

Strains / Criteria	Shape	Size	Color	Contour	Elevation	Surface	Opacity	Consistency	Smell
S1	Round	Small	Yellow green	Regular	Convex	Smooth	Translucent	Creamy	+
S2	Round	Small	Yellow green	Regular	Convex	Smooth	Translucent	Creamy	+
S3	Round	Small	Yellow green	Regular	Convex	Smooth	Translucent	Creamy	+
S4	Round	Medium	White	Regular	Convex	Smooth	Opaque	Creamy	-
S5	Round	Medium	Dark green	Regular	Convex	Smooth	Opaque	Creamy	+
S6	Circular	Medium	White	Irregular	Flat	Rugeuse	Opaque	Dried	-
S7	Round	Small	Yellow green	Regular	Convex	Smooth	Translucent	Creamy	+
S8	Round	Small	White	Regular	Flat	Smooth	Opaque	Creamy	-
S9	Round	Small	Yellow	Regular	Convex	Smooth	Opaque	Creamy	-
S10	Round	Small	Green	Regular	Convex	Smooth	Opaque	Creamy	+
S11	Circular	Small	Yellow orange	Regular	Convex	Smooth	Opaque	Creamy	-
S12	Round	Small	Yellow green	Regular	Convex	Smooth	Translucent	Creamy	+
S13	Round	Small	White	Regular	Convex	Smooth	Opaque	Creamy	-
S14	Round	Medium	Yellow orange	In peak	Convex	Smooth	Opaque	Creamy	+
S15	Round	Small	Yellow orange	Regular	Convex	Smooth	Opaque	Creamy	+
S16	Round	Small	Golden yellow	Regular	Convex	Smooth	Opaque	Creamy	-
S17	Round	Small	White	Regular	Convex	Smooth	Opaque	Creamy	-
S18	Round	Small	Yellow green	régulier	Convexe	lisse	Translucent	Creamy	+
S19	Circular	Medium /Big	White	irrégulier	Plat	Rugueuse	Opaque	Dried	-

formed (He) divided by the total height of the liquid in the tube (Ht) multiplied by 100:

E24= (He/Ht) 100

Surface tension study: Surface tension is defined as a state resulting from interactions between molecules on the surface of a liquid at rest. This tension comes from the intermolecular forces which exert a downward pull on the different surface molecules (Abu-Ruwida *et al.*, 1991). The method used consisted of taking a sample of culture for monitoring the surface tension using a tensiometre. After calibration of the device, the sample (10 ml of the supernatant collected aseptically in a watch glass) was introduced on the tensiometer sampling table. The table was adjusted until the ring emerged in the supernatant (formation of a film) and led to breakage of the film. The value on the dial at the moment the film breaks corresponded to the surface tension of the liquid. This process was carried out for each bacterial strain studied.

Results and Discussion

Physico-chemical analysis of the soil

Upon inspecting the soil samples, it was evident that the sample retrieved from the "Hassi Messaoud" quagmire exhibited a deep blue to black hue and possessed an aggregated texture. The soil appeared compact and moist upon sampling, indicating its saturation with oil. According to the "U.S.D.A" textural triangle classification, this soil sample falls under the category of sandy loam. Its composition consisted of 37.21% coarse silt, 34.4% fine silt, 15.32% fine sand, 12% clays, and 1.07% coarse sand. The soil sample was characterized as slightly alkaline pH (7.90 ± 0.1) with an electrical conductivity equal to 0.0282 dS m^{-1}, a humidity rate of 6.7% and having very low organic matter (1.058 mg g^{-1}).

Soil microbiological analysis

Enumeration of hydrocarbonoclast strains*:* In collected soil sample polluted with hydrocarbons the total microflora on liquid medium (Most Probable Number) and on agar medium were 1.4 x 1013 CFU g^{-1} and $2.08x10^{13}$ CFU g^{-1} of soil respectively. According to Margesin and Schinner (2001) a count $\geq 10^6$ CFU $\mathbf{g^{-1}}$ of soil is minimum standard and is generally sufficient to carry out the biodegradation of hydrocarbon compounds in soil. Occurrence of much higher population in the polluted soil showed that the microbial population has adapted to oil and feed on petroleum derivatives. The growth of bacteria from the samples on the MSM culture medium containing oil as the

Table 2. Results of the microscopic study of the isolated strains

Isolates	Gram	Cell shape	Grouping mode	Presence of spores
S1	-	Coccobacille	Isolated	-
S2	-	Coccobacille	Isolated	-
S3	-	Coccobacille	Isolated	-
S4	+	Shell	Isolated/in pair	-
S5	-	Bacillus	Isolated/in pair	-
S6	+	Shell	Isolated/in clusters	-
S7	-	Coccobacille	Isolated	-
S8	+	Shell	Isolated/in pair	-
S9	+	Shell	Isolated/in pair/in clusters	-
S10	-	Bacillus	Isolated/in pair	-
S11	+	Shell	Isolated/in pair	-
S12	-	Coccobacille	Isolated	-
S13	+	Shell	Isolated/in pair	-
S14	-	Bacillus	Isolated/in pair	-
S15	-	Bacillus	Isolated/in pair	-
S16	+	Shell	Isolated/ bunch of grapes	-
S17	+	Shell	Isolated/in pair	-
S18	-	Coccobacille	Isolated	-
S19	+	Bacillus	Isolated /in chains	+

sole carbon source indicated the ability of the isolates to metabolize the latter as well as their derivatives.

Identification of the hydrocarbonoclast strains: Out of the cultured bacteria nineteen strains distinct in appearance, size, color, Gram reaction, catalase and oxidase, were isolated (Table 1). The adaptation time between isolates to access this carbon source however varied. From our observations, it appeared that all isolates possessed catalase except two strains: S5 and S15. However, catalase is usually present in Gram-positive bacteria (Meyer *et al.*, 2004). About the oxidase results, this reaction is particularly useful in Gram-negative bacteria, it is positive with strict aerobic bacteria (*Pseudomonas*, etc.) (Meyer *et al.*, 2004). We detected an oxidase in the nine isolates viz., S1, S2, S3, S5, S7, S9, S10, S12 and S18, indicating the presence of a respiratory chain.

On Chapman medium after 48 hours of incubation at 37°C, we observed colonies presenting a macroscopic appearance characteristic of the *Staphylococcus* genus. Indeed, on this medium, *Staphylococcus* colonies often appear pigmented and surrounded by a yellow halo in the case where mannitol is fermented. Other colonies were white in color, i.e., strains S4, S8, S13, S16, S11 and S17. The colonies of isolate S9 appear rounded with regular edges of 1 to 2 mm in diameter.

On Baird-Parker medium, the coagulase-positive staphylococci (*Staphylococcus aureus*) produced dark gray to black glossy convex colonies with a complete outline and transparent areas which was observed for strain S16. These colonies may or may not be surrounded by an opaque zone. Coagulase-negative staphylococci often grow poorly, and produce gray to black colonies without transparent or opaque areas, such as S4, S8, S13 and S17. Microorganisms other than staphylococci are often inhibited, seen in strain S9 and S11. This shows that out of nineteen bacteria initially selected five (S4, S8, S13, S17 and S16) were staphylococci.

During the isolation, purification and identification of our isolates, the strains which produced pigments that made the agar medium or the broth green, were considered as belonging to the genus *Pseudomonas*. But for further confirmation they were seeded on the medium specific to *Pseudomonas* (Cetrimide Agar) to isolate single type of bacterial colony distinct in appearance, size, color and smell. The macroscopic observation of the colonies of the six isolated strains (S1, S2, S3, S7, S12 and S18) showed that they had a round shape, 2 mm in diameter and a regular outline with a granular surface. These white, translucent, flat colonies had a creamy consistency. The revelation of a blue-green color on King's A and greenish-yellow on King's B confirmed

Table 3. Results of identification of bacteria isolated by the API (Analytical Profile Index) 20 E gallery

Strains \ Test	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
S1	-	+	-	-	+	-	+	-	-	-	+	+	-	-	-	-	-	+	-	±
S2	-	+	-	-	+	-	+	-	-	-	+	+	-	-	-	-	-	±	-	+
S3	-	+	-	-	+	-	+	-	-	-	+	+	-	-	-	-	-	+	-	+
S5	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S6	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
S7	-	+	+	+	+	-	+	-	-	-	+	+	-	-	-	-	-	+	-	+
S10	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S12	-	+	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	±	-	+
S14	+	+	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+
S15	+	+	-	+	-	+	-	-	-	-	-	+	+	-	+	+	+	+	+	+
S18	-	+	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+
S19	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-

S1: *Pseudomonas aeruginosa*; S2: *Pseudomonas aeruginosa*; S3: *Pseudomonas aeruginosa*; S5: *Bordetella/Alcaligenes/ Moraxella* spp.; S6: *Brevibacillus agri*; S7: *Pseudomonas aeruginosa*; S10: *Bordetella/Alcaligenes/Moraxella* spp.; S12: *Pseudomonas aeruginosa*; S14: *Enterobacter sakazakii*; S15: *Citrobacter braakii*; S18: *Pseudomonas aeruginosa*; S19: *Bacillus mycoides*
ONPG: O-Nitrophenyl-β-D-galactopyranoside; ADH: Arginine dihydrolase; LDC: Lysine decarboxylase; ODC: Ornithine decarboxylase; CIT: Citrate; URE: Urease; TDA: Tryptophan deaminase; IND: Indole; VP: Voges-Proskauer; GEL: Gelatinase; GLU: Glucose; MAN: Mannose; INO : Inositol;SOR : Sorbitol; RHA : Rhamnose; SAC : Saccharose; MEL : Melibiosis; AMY: Amygdalin; ARA: Arabinose.

the species *Pseudomonas aeruginosa* which synthesizes fluorescent pigments.

The different results of the identification of bacterial species presented in Tables 1, 2, 3, 4 and 5 allowed us to identify at least 12 different species represented by seven genera belonging to four families: Pseusomonadaceae, Enterobacteriaceae, Micrococaccaceae and Bacillaceae. From a diversity point of view, the Pseusomonadaceae family is the most represented with six species of *Pseudomonas aeruginosa*: S1, S2, S3, S7, S12 and S18. The two strains S6 and S19 are attached to the genus *Bacillus* (*Brevibacillus agri*, *Bacillus mycoides*). Strains S5, S10, S14 and S15 are related to the Enterobacteriaceae family. The two strains S5 and S10 are attached to the following genera: *Bordetella/Alcaligenes/Moraxella* spp. S14 attached to the genus *Enterobacter* and identified as *E. sakazakii*. S15 attached to the genus *Citrobacter* and identified as *C. braakii*. Strains S4, S8, S11, S13, S16 and S17 are related to the *Staphyloccocus* genus: S4 and S17: *S. xylous*, S16: *S. aureus,* S8: *S. scuiri*, S13: *S. caprae*,

Table 4. Results of identification of bacteria isolated by the API (Analytical Profile Index)20 Staph gallery

Strains \ Test	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL	NIT	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE
S4	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	-	+
S8	+	+	+	+	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-
S9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S11	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-
S13	+	+	+	+	+	+	-	-	-	+	+	+	-	+	-	-	-	+	-
S16	+	+	+	+	+	+	+	-	-	+	+	+	-	-	+	-	+	+	+
S17	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	+

S4: *Staphylococcus xylosus*; S8: *Staphylococcus sciuri*; S9: *Micrococcus* sp.; S11: *Staphylococcus lentus*; S16 : *Staphylococcus aureus*; S17: *Staphylococcus xylosus*.
GLU: Glucose; FRU: Fructose; MNE: Mannose; MAL: Maltose; LAC:Lactose; TRE : Trehalose; MAN: Mannitol; XLT: Xylitole; MEL: Melibiose; NIT: Nitrate reductase; PAL : Phosphatase alcaline; VP : Voges-Proskauer; RAF : Raffinose; XYL: Xylose; SAC: Sccharose; MDG: Methyl a-D-glucopyranoside; NAG: N-acetylglucosamine; ADH: Arginine Dihydrolase; URE: Urease.

Table 5. Final identification of isolates

Bacillus Gram-	*Enterobacteriaceae*	*Enterobacter sakazakii*	S14
		Citrobacter braakii	S15
		Bordetella/Alcaligenes/ Moraxella spp.	S5, S10
	Pseudomonas	*Pseudomonas aeruginosa*	S1, S2, S3, S7, S12, S18
Cocci Gram +	*Staphylococcus*	*Staphylococcus xylosus*	S4, S17
		Staphylococcus aureus	S16
		Staphylococcus sciuri	S8
		Staphylococcus caprae	S13
		Staphylococcus lentus	S11
	Micrococcus	*Micrococcus* sp.	S9
Bacillus Gram+	*Bacillus*	*Brevibacillus agri*	S6
		Bacillus mycoides	S19

and S11: *S. lentus.* The S9 strain attached to the genus *Micrococcus* spp.

Most of the knowledge concerning the biodegradation of hydrocarbons in the environment has been acquired from studies based on the isolation of hydrocabonoclastic microorganisms (Watanabe and Hamamura, 2003; Guetarni *et al.,* 2023).

Isolation has the essential advantage of being able to work on pure strains and thus to precisely study a microorganism and some of its functions. For example, it is possible to characterize *in vitro* the biochemical mechanisms involved in the degradation of a hydrocarbon. However, the isolated hydrocarbonclastic strains are not strongly representative of the metabolic capacities of the naturally present community (Thompson *et al.,* 2005).

A few of the strains isolated in this work could belong to the species *Pseudomonas aeruginosa* since they are characterized by the production of pyocyanin and pyoverdin pigments. These pigments diffuse into the agar cultures and the colonies stain the medium greenish blue or greenish yellow (Singleton, 1999; Lotfabad *et al.,* 2009). The work of Delarras (2007) and Lotfabad *et al.* (2009) reveals that King's A and B agars are two media favoring the pigmentation of *Pseudomonas* cultures, in particular *Pseudomonas aeruginosa*. These two media are used as a confirmation test, following the search for *P. aeruginosa*. In the work of Khlil-Radji (2015), two species *S. aureus* and *P. aeruginosa* were isolated from soil contaminated by discharges from the Arzew refinery (Oran, Algeria). Cetrimide agar allows the detection and selective isolation of *Pseudomonas aeruginosa*. Cetrimide or hexadecyltrimethyl ammonium bromide is an antiseptic that inhibits many germs other than *Pseudomonas aeruginosa*. Potassium sulphate and magnesium chloride promote the production of the two pigments: pyocianin and pyoverdin (Delarras, 2007).

Other hydrocarbonoclastic bacterial species less dominant than *Pseudomonas* are also

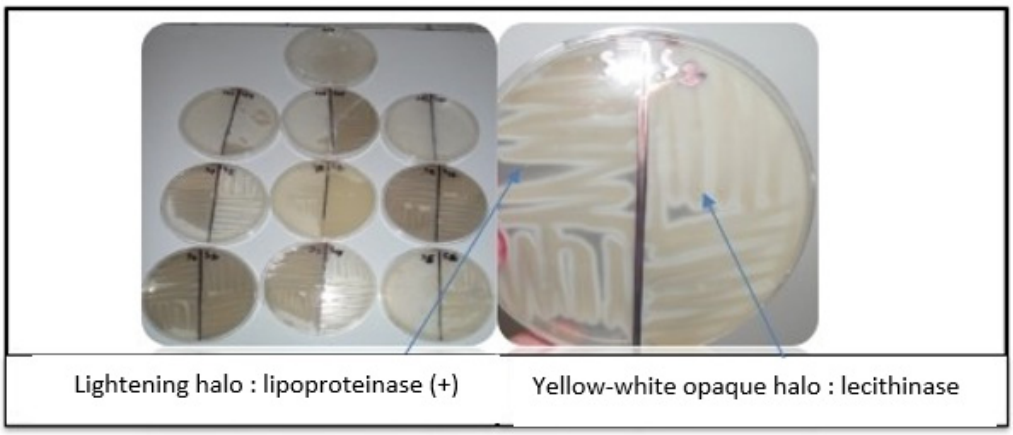


Fig. 2. Results of the search for lecithinase and lipoproteinase.

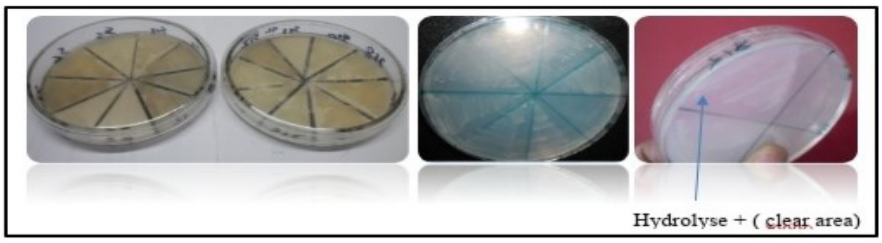


Fig 3. Results of the casein hydrolysis test.

isolated. These species belong to the following bacterial genera: *Staphylococcus*, *Micrococcus* and *Enterobacter*. The same genera were identified by Floodgate (1995); Khlil *et al.* (2014).

Protein metabolism

According to the results observed for lecithinase and lipoproteinase tests (Fig. 2), we obtained Lecithinase positive in S1, S2, S3, S7, S10, S11, S12, S14 and S18; Lipoproteinase positive in S1, S2, S3, S4, S7, S8, S11, S12, S13, S14, S18 and S19. Lecithinase is an enzyme tested on egg yolk agar poured into a Petri dish; it produces opacification by breaking the egg yolk emulsion. Egg yolk, a complex substrate, is composed of lecithin, triglyceride and a lipoprotein. It therefore makes it possible to search for 3 enzymes: lecithinase, lipase, and lipoproteinase for the identification of different genera such as: *Pseudomonas*, *Bacillus* and *Clostridium* (Delarras, 2007). Bacterial lecithinases are of special interest because of the possible role of these enzymes in pathogenicity. Lecithinases or phospholipases are enzymes released by bacteria that have the ability to destroy animal tissues. Phospholipid complexes are usually emulsifying agents occurring in tissues, serum and egg yolk (https://vlab.amrita.edu/?sub=3&brch=73&sim=974&cnt=1).

From the results obtained on the milk agar, we noticed the presence of a zone of degradation (light) for the isolates: S1, S2, S3, S6, S7, S12 and S18, while the two isolates S4 and S14 gave variable results (Fig. 3).

However, many strict aerobic bacteria (*Pseudomonas*, *Bacillus*) can hydrolysis casein (Meyer et *al.,* 2004). Casein, a milk protein, can be hydrolyzed by certain bacterial species: the search for caseolytic activity is particularly useful in the identification of *Bacillus* and sometimes *Pseudomonas*. The areas of lightening of the medium around the colonies reflect a positive result of caseinolysis (*B. subtilis, P. aeruginosa*); the absence of such zones indicates a negative caseinolysis test (**Delarras**, 2007). *Pseudomonas aeruginosa* hydrolyzes casein and can produce a yellow to green diffusible pigment (Eaton *et al.,* 1995). Casein Hydrolysis Test is the biochemical test used to determine the ability of bacteria to synthesize caseinase enzyme (https://microbenotes.com/casein-hydrolysis-test/).

Starch hydrolysis test

We detected starch degradation in isolates S6, S10 and S19, however the other isolates (S1, S2, S3, S4, S5, S7, S8, S9, S11, S12, S13, S14, S15, S16, S17 and S18) were unable to use this polymer (Fig. 4).

Starch is a complex carbohydrate whose degradation requires an amylase. Starch agar makes it possible to demonstrate the

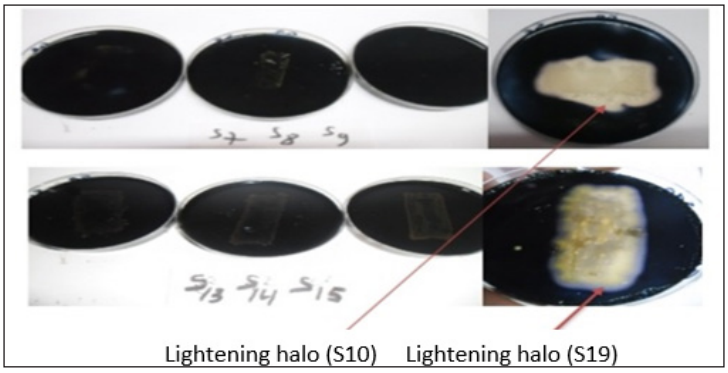
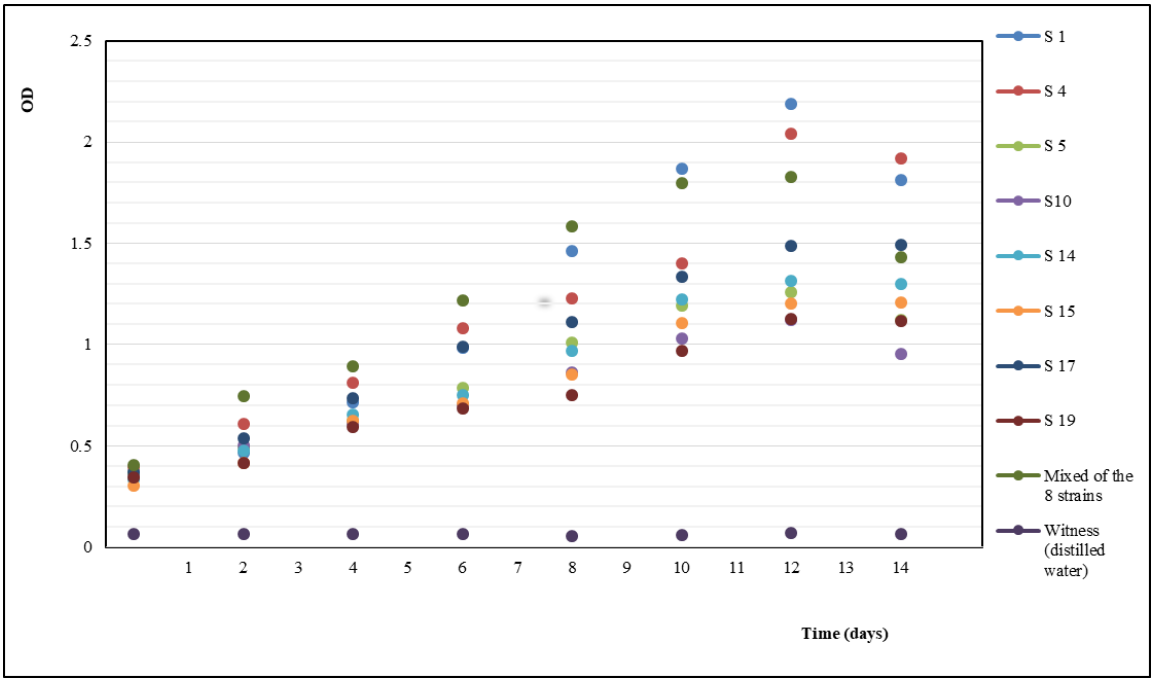


Fig. 4. Starch hydrolysis test results.



S1: Pseudomonas aeruginosa; S4: Staphylococcus xylosus; S5: Bordetella/Alcaligenes/ Moraxella spp.; S10: Bordetella/Alcaligenes/Moraxella spp.; S14: Enterobacter sakazakii; S15: Citrobacter braakii; S17: Staphylococcus xylosus; S19: Bacillus mycoides

Fig. 5. Bacterial biomass estimation curve by OD (optical density) of the strains tested.

degradation of this polysaccharide by certain species belonging in particular to the genus *Bacillus* and *Pseudomonas* (Delarras**,** 2014). According to Meyer *et al.* (2004), Amylases are found mainly in *Bacillus*, their detection is an identification criteria.

Petroleum biodegradation and selection of potential biosurfactant-producing strains

Biodegradation of petroleum on liquid MSM: The growth of the strains isolated on MSM medium supplemented with 2% (v/v) crude oil as the sole carbon source was monitored by measuring the OD at 600 nm as a function of time; which enabled us to plot the curves represented by Figure 5. For the bacterial strains studied, the latency phases observed were very short. From these curves, we could observe a similarity in the growth path for the strains tested.

The growth of the strains began with an OD of 0.3 to 0.4 then an increase in the OD was observed from the first hours of incubation until reaching maximum values after 12 days: OD S1=2.19, OD S4=2.04, OD S5=1.26, OD S10 =1.121 OD S14=1.313, OD S15=1.204, OD S17=1.489, OD S19 =1.124, OD Mix= 1.826. Thus, this OD decreased beyond 12 days until the end of incubation for all isolates except that S15 and S17 the OD value stabilized at its maximum values. This reduction shows that the level of nutritional requirements exceeds the dissolution speed of the substrate, bioavailability will then become limiting. Also, the biodegradation kinetics of a pollutant *in vitro* are very different from those observed in the environment (Watanabe and Baker, 2000). Additional tests are necessary to confirm the presumptive identification obtained on MSM medium (Lancette and Bennett, 2001). The variations in optical densities (OD) observed in the culture media of the different bacterial strains studied may be due to the different distribution of these bacteria between the two phases of our culture, or by their speed of adaptation to the substrate used (Akmouci, 2009). According to Atlas *et al.* (2011), the lighter the petroleum products, the faster their diffusion and the faster they will be bioavailable. Indeed, the short latency or slowdown phases demonstrate a more or less rapid adaptation of the strains studied to the carbon source used, "crude oil" (Akmouci, 2009).

Petroleum biodegradation on MSM agar: The strains selected and cultured on MSM agar supplemented with crude oil as the only carbon

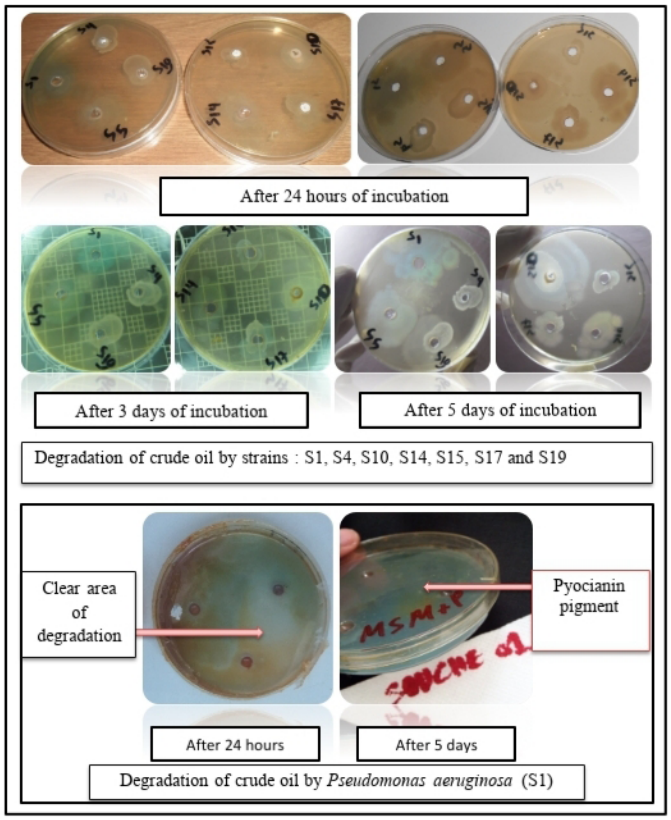


Fig. 6. Degradation of crude oil by microorganisms.

source (eight strains in sole culture and mixed), showed their ability to digest oil, by producing clear zones of variable sizes, which increased over time (Fig. 6).

The S1 strain (*P. aeruginosa*) strongly favored the pigmentation of the MSM agar medium added with crude oil. The degradation of the oil caused the reduction of the medium as a result of the increase in the pigmented zone (the product of a pyocyanic pigmentation characteristic of the S1 strain). Bacteria are the most numerous microorganisms, typically reaching about 10^8 to 10^{12} cells in 1 g sample of rhizosphere soil and up to 10^{26} cells in the phyllosphere (Elyamine *et al.*, 2021). As an important part of the global ecosystem, bacteria play a major role in the microbial degradation of organic pollutants. The bacteria that have a strong ability to degrade PAHs mainly include *Rhodococcus*, *Pseudomonas*, *Sphingomonas*, *Bacillus*, *Flavobacterium*, *Mycobacterium*, *Nocardia*, *Vibrio*, *Micrococcus*, and *Acinetobacter* (Elyamine *et al.*, 2021).

The consortium of strains studied showed a large concentration of cells. This can be explained from the fact that microorganisms in single culture can metabolize a limited range of substrates, while in mixed culture,

Table 6. Parafilm M test result

Culture	Droplet diameter (mm)		Culture	Droplet diameter (mm)	
	After 24 hours	After 7 days		After 24 hours	After 7 days
S1	7	10	S15	5.5	6.5
S4	6.5	9	S17	6	8
S5	6	7.5	S19	5.5	6.5
S10	6	7	Mixed of the 8 srains	7	10.5
S14	6	7	Witness (distilled water)	5	5

S1: Pseudomonas aeruginosa; S4: Staphylococcus xylosus; S5: Bordetella/Alcaligenes/ Moraxella spp.;S10: Bordetella/ Alcaligenes/ Moraxella spp.; S14: Enterobacter sakazakii; S15: Citrobacter braakii; S17: Staphylococcus xylosus; S19: Bacillus mycoides

Table 7. Oil displacement test result

Culture	Oil displacement test results		Culture	Oil displacement test results	
	After 24 hours	After 7 days		After 24 hours	After 7 days
S1	+	+++	S15	+	+
S4	+	+++	S17	+	+++
S5	+	+	S19	-	+
S10	+	+	Mixed of the 8 strains	++	+++
S14	+	+	Witness (distilled water)	-	-

(-) : Negative result / (+):Low production / (++) : High production / (+++) : Very high production

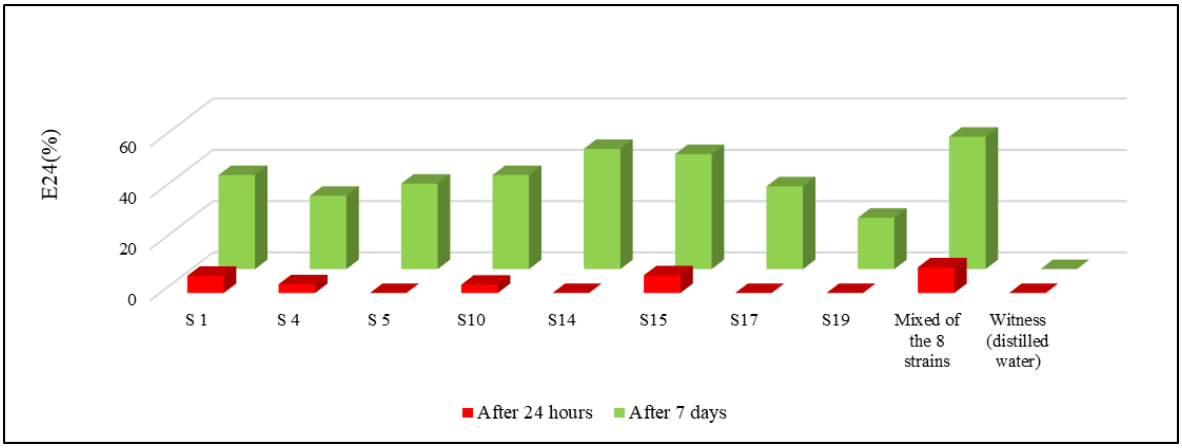
S1: Pseudomonas aeruginosa; S4: Staphylococcus xylosus; S5: Bordetella/ Alcaligenes/ Moraxella spp.; S10: Bordetella/ Alcaligenes/ Moraxella spp.; S14: Enterobacter sakazakii; S15: Citrobacter braakii; S17: Staphylococcus xylosus; S19: Bacillus mycoides.

microbial strains have broader co-metabolism and attribute greater ability to degrade complex mixtures of hydrocarbons (Mueller *et al.*, 1997). With the decrease in labile carbon sources, nutrients were most likely limited in supporting microbial growth (Bento *et al.*, 2005).

Selection tests for potential biosurfactant-producing strains

Parafilm M test: The results after collapse of the culture on parafilm paper showed that the diameters of the droplets obtained ranged between 5.5 and 10.5 mm after 24 hours and 7 days of incubation. The S1 strain and the S4 strain were the most productive of biosurfactants in which a collapse of 10 mm for the S1 strain and 9 mm for the S7 strain was observed after 7 days of incubation. However, by comparing the results of the strains tested with a drop of distilled water (5mm) as a stable control, we were able to say that *Pseudomonas aeruginosa* (S1) and *Staphylococcus xylosis* (S4) were the potential producers of biosurfactants (Table 6). For the screening of biosurfactant producing microbes, enrichment cultures utilizing hydrophobic compounds as the sole carbon source are applied. This is an indirect screening method as the growth on hydrophobic compounds indicates the production of biosurfactants (Walter *et al.*, 2010).

Oil displacement test: It is a fast and effective technique for detection of biosurfactant production. It was observed in our isolates by the appearance of a clear zone on the oil-water surface, such that the clear zone was proportional to the concentration of biosurfactant produced. Indeed, the results obtained showed that the supernatant of most of the strains generated zones of oil displacement as an indication of the production of biosurfactant. Strains S1, S4, S17 and the consortium (mix of the strains) showed the highest displacement halo, which allowed us to say that these are the potent



S1: Pseudomonas aeruginosa; S4: Staphylococcus xylosus; S5: Bordetella/Alcaligenes/Moraxella spp.; S10: Bordetella/Alcaligenes/Moraxella spp.; S14: Enterobacter sakazakii; S15: Citrobacter braakii; S17: Staphylococcus xylosus; S19: Bacillus mycoides

Fig. 7. Histogram representing the estimation of emulsion quality (E24).

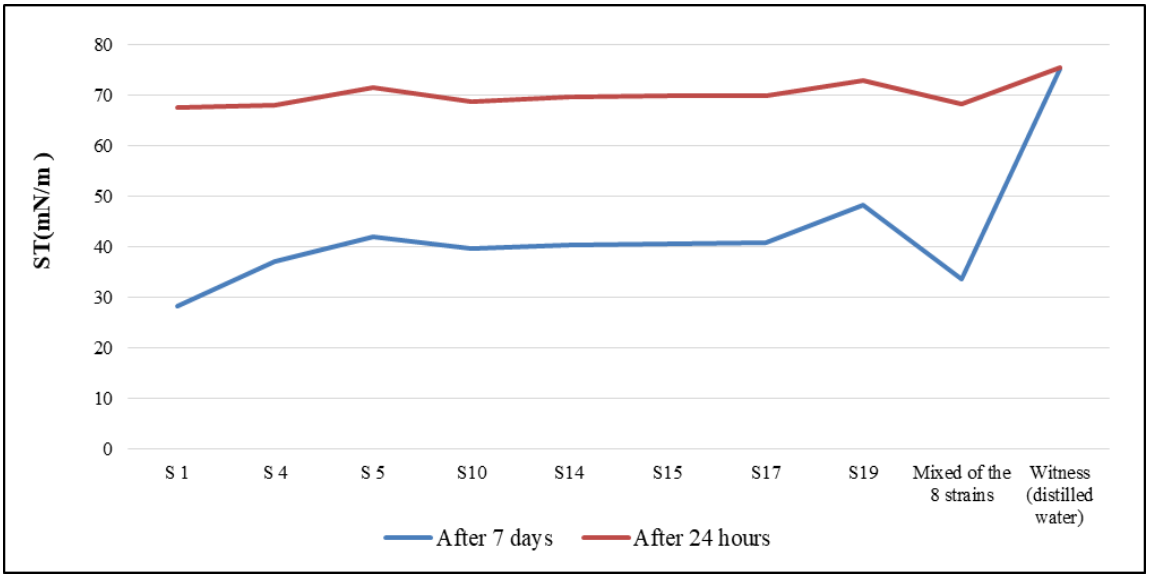
biosurfactant-producing strains. The S19 strain showed the weakest result among the strains tested (Table 7). Efficient biodegradation depends on various parameters, in particular on the composition of the oil, on the ability of bacteria to adhere and on the potential for degrading the hydrocarbons. It is often difficult to find organisms that individually degrade all crude oil fractions (Das and Chandran, 2011).

The results obtained in this present study are consistent with the observations of Rahman *et al.* (2002); Kayode-Isola *et al.* (2008); John *et al.* (2011) that *Pseudomonas* sp. strains exhibit the highest bacterial concentrations and the greatest abilities to degrade petroleum and diesel. Some scavenger microorganisms also have the ability to improve the solubilization of hydrocarbons, through the production of biosurfactants (formation of micelles), or through the production of biofilms (adhesive and protective extracellular matrices) (Johnsen and Karlson, 2004; Leglize *et al.*, 2008). Guermouche (2014) showed that *Pseudomonas* sp. has more degradation ability than *Bacillus* sp. and *Enterobacter* sp. Other works done by Chithra and Hema-Shenpagam (2014) using *Pseudomonas* sp. showed that it degraded oil better than other isolated species (*Bacillus* and *Micrococcus*). Cipinyte *et al.* (2011) showed that the production rate of biosurfactants differs from one isolate to another.

Emulsification index (E24): In order to confirm and estimate the production of biosurfactants by the isolated strains, we carried out the emulsification test which makes it possible to calculate the emulsion index (E24), and therefore the quantitative estimation of the rate of emulsification by the different isolates. The mixture of strains completely emulsify the amount of diesel added, while the other isolates form partial emulsions.

Figure 7 showed that the Gram-negative strains (S1, S5, S10, S14 and S15) were more emulsifying than the Gram-positive strains (S4, S17 and S19). The most productive strain was found to be S15 with a rate of 46.88. The best results achieved values of 51.61%, 46.88% and 44.83% for the consortium, S14 and S15 respectively after seven days of incubation.

The strains S14, S15, and the mixture were able to produce considerable amounts of biosurfactants after seven days of incubation. It can be seen on the histogram that the rate of production of biosurfactants was higher after one week of incubation. The biological strategy that can improve contact between bacteria and water-insoluble hydrocarbons is emulsification. Therefore, bacteria growing on hydrocarbons usually produce emulsifiers which stimulate their growth and accelerate bioremediation (Ron and Rosenberg, 2002). Similar results were found in *Pseudomonas aeruginosa* which



S1: Pseudomonas aeruginosa; S4: Staphylococcus xylosus; S5: Bordetella/Alcaligenes/ Moraxella spp.; S10: Bordetella/Alcaligenes/ Moraxella spp.; S14: Enterobacter sakazakii; S15: Citrobacter braakii; S17: Staphylococcus xylosus; S19: Bacillus mycoides

Fig. 8. Linear curve representing the estimation of emulsion quality by the surface tension (ST).

can reach an emulsion index of 50% (Benincasa and Accorsini, 2008).

Surface tension (ST): The surfactant property of the biosurfactant mainly depends on its ability to reduce surface tension and the formation of stable emulsions with different water-immiscible substrates (Raza *et al.,* 2007). Figure 8 shows that the S1 strain and the consortium could reduce the maximum surface tension from 75.5 mN/m down to 28.4 mN/m and 33.8 mN/m, respectively. This result allows us to suggest that there is production of specific biomolecules with surfactant properties or biosurfactants, known to cause the lowering of surface tension.

Walter *et al.* (2010) demonstrated that the drop stability is dependent on biosurfactant concentration. According to our results, the strain *Pseudomonas aeruginosa* (S1) has a better surface tension value of 28.4 mN/m. The same result was obtained by Raza *et al.* (2007) who used olive oil as the sole carbon source in the production of the biosurfactant by *Pseudomonas aeruginosa* which reduced surface tension upto 29 mN/m, after incubation for 5 days. Robert *et al.* (1989), were also able to produce a biosurfactant by *Pseudomonas aeruginosa* using crude oil, with a surface tension of 38 mN/m.

This confirms that biosurfactant production occurs during the exponential phase of growth and suggests that it is produced as a primary metabolite (Amiriyan *et al.,* 2004). Bacterial growth associated with biosurfactant production could facilitate the adhesion of bacterial cells to hydrophobic substrate molecules and metabolize them (Barathi and Vasudevan, 2001).

Conclusions

Soils polluted by petroleum hydrocarbons have a detrimental effect on the environment in general. Each polluted soil contains an autochthonous microflora that adapts to degrade these pollutants and use them as food. They produce natural surfactants that stimulate access to the complex carbon source for a good process of bioremediation of the polluted ecosystem. In this work, from a soil sample polluted by petroleum, we were able to isolate 19 hydrocarbonoclastic strains which belong to the following bacterial genera: *Pseudomonas*, *Staphylococcus*, *Bacillus*, *Enterobacter*, *Micrococcus*, *Citrobacter* and *Bordetella/Alcaligenes/Moraxella* spp. The S1 strain (*Pseudomonas aeruginosa*) gave the most OD growth in medium containing crude oil. Also, the performance of four tests: parafilm M, oil displacement, Emulsification E24 and surface tension allowed us to suggest that there is production of specific biomolecules with surfactant properties. The most biosurfactant-producing strain from petroleum is the S1 strain (*Pseudomonas aeruginosa*) with an E24= 36.67%, parafilm M= 10 mm collapse and surface tension of 28.4 mN/m. We conclude that this strains could be used for bioremediation process to degrade hydrocarbon compounds like diesel oil.

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