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Production of Biosurfactants by Hydrocarbons Degrading Bacteria Isolated from Soummam Watershed Sediments of Bejaia in Algeria

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Hydrocarbons degrading bacteria were isolated from sediments of the Soummam watershed of Bejaia (Algeria). Eleven bacteria strains were isolated using an enrichment technique, method in mineral salt medium, with various hydrocarbons as the sole carbon source. The biodegradation confirmation of various hydrocarbons by these isolates was tested by hole-plate diffusion technique. Out of eleven cultures, nine had shown the growth around the holes. The isolates were screened for biosurfactant producing using oil spreading test and emulsification activity. The value of emulsification activity varied from 55.7 ± 1.1 to 78.5 ± 0.5%. The diameter of clear zone obtained varied between 20.7 ± 1.2 mm and 33.7 ± 1.2 mm, it was hence important compared to the negative control. Five bacterial strains were identified as Alcaligenes faecalis, Ochrobactrum, Cellulosimicrobium, Pseudomonas stutzeri, *and* Rhodococcus ruber *by using physiochemical characterization and MALDI-TOF mass spectrometry tools. The best production of crude biosurfactant by the identified bacterial strains was found in* R. ruber *which produced 6.7 ± 0.1 g/L of the crude biosurfactant after 168 h incubation in mineral salt medium (MSM) supplemented with 2% of glucose and 0.1 g/L of yeast extract. The biosurfactant produced by all bacterial strains showed a high emulsification index (E_{24}), where* P. stutzeri *revealed the highest one (92.2 ± 1.1%).* © 2017 American Institute of Chemical Engineers Environ Prog, 37: 189–195, 2018

Keywords: Biosurfactants, Biodegradation, Hydrocarbons, Sediments

NOVELTY OR SIGNIFICANCE

The Soummam Wadi is exposed to an organic-based pollution from a variety of sources that makes it an extreme ecosystem. Therefore, the main objective of this study was to select sediments for the first time from this Wadi to isolate bacterial strains implicated in the hydrocarbons degradation. The ability of the isolated bacteria to produce biosurfactants was also studied.

INTRODUCTION

Soummam Wadi is part of the watershed Soummam, it is formed from the confluence of two important Wadis: Boussellam Wadi which descends from the plate of Setif, and Sahel Wadi from Bouira. It runs the entire of the Soummam valley from Akbou to Bejaia where it flows into the Mediterranean Sea at Bejaia. It drains a large watershed whose surface area is about 9118 km^2 [1]. This watershed is subject to contamination by organic pollutants from a variety of sources. Organic contamination results from uncontrolled release of industrial pollutants, washing stations greasing, oil factories, and uncontrolled discharges. Besides, a significant volume of domestic wastewater was discharged by towns of the valley [2]. Crude oil is one of the most important organic pollutants. As a complex mixture, it contains a large number of distinctively different chemicals mainly composed of four fractions: saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes [3]. Mainly, these latter compounds are persisting in the environment due to their low water solubility and sequestration in particulate matter that is deposited in soils and sediments. They are categorized as mutagens and carcinogens and 15 of them have been identified as carcinogenic by the U.S. Department of Health and Human Services [4].

Faced with the risks represented by these pollutants, biological degradation processes of these compounds to nontoxic components are considered as the ultimate solution. It mainly focuses on a microbiological approach, in which species with the best potential effect for hydrocarbons' degradation were selected [5]. Besides, microorganisms were given more attention in bioremediation of polluted environments as well as the oil exploitation considering their adaptation to the extreme environment [6] capabilities to produce biosurfactants [7] and potential metabolic to degrade hydrocarbons [8].

Sediments from the Soummam watershed of Bejaia (Algeria) were selected for this study. This Wadi because of its increased pollution [2] it is considered as an extreme ecosystem. The microbiological study on biodegradation and bioremediation is carried out for the first time in this geographical

area. Therefore, the aims of this study are (1) to isolate hydrocarbon-degrading bacteria from Soummam sediments, (2) evaluate their capacity to produce of biosurfactants, and (3) identify them.

MATERIALS AND METHODS

Sampling of Soummam Sediments

For isolation of hydrocarbons-degrading bacteria, Soummam sediments were collected from five sampling points in Bridge Skala (36°43′58.89″ N, 4°04′04.47″ E, 4 m high) of Bejaia, Algeria. Sediment samples were taken from 10 to 25 cm below the surface using sterile scooping containers and transported on ice to the laboratory for analysis.

Isolation of Hydrocarbon-Degrading Bacteria

Fresh sediments (10 g) were transferred to a flat bottle containing physiological saline (1% NaCl), they were then agitated 1–2 h and let stand 1 h. An aliquot of 5 mL of supernatant was used as inoculums in Erlenmeyer flasks containing 45 mL sterilized MSM. One liter of MSM medium contains, in g/L distilled water, 30 g of NaCl, 2.0 g of KH_2PO_4, 1.0 g of NH_4NO_3, 3.0 g of Na_2HPO_4, 0.7 g of $MgSO_4.7H_2O$, and 1.0 mL of trace element solution. The trace element solution was prepared as follows (mg/L): $ZnSO_4.7H_2O$, 10; $CuSO_4.5H_2O$, 0.50; $MnSO_4.H_2O$, 0.50; $CaCl_2$, 20; $FeCl_3$, 30 and the solution was adjusted to pH 7.2 ± 0.2 [9]. Culture medium was further supplemented with 1% model hydrocarbon compounds as sole carbon and energy source (diesel, petroleum, kerosene, and toluene) added separately in each flask and incubated in a rotary shaker at 30°C and 180 rpm for 10 days, and then transferred to fresh medium, incubated at the same conditions for another 10 days. After the four subcultures, the culture broth was spread on hydrocarbon-MSM agar plates, solid media were obtained by adding 18 g/L of agar in MSM (each plate contained one of the hydrocarbon; i.e. diesel, petrol, kerosene, or toluene). The plates were then incubated at 37°C for one week in an incubator. After that, pure and representative bacterial colonies were transferred to nutrient agar plates as well as to nutrient agar slants for preservation.

Confirmation the Biodegradation of Various Hydrocarbons

The biodegradation confirmation of various hydrocarbons by the strains isolates was tested by Hole-plate diffusion assay on MSM agar medium using different hydrocarbons as a carbon source (diesel, petroleum, kerosene, and toluene) [10]. Different pure colonies obtained from hole-plate diffusion test were preserved in 25% (v/v) sterile glycerol solution at −20°C. For day to day experimentation strains were maintained on nutrient agar slants at 4°C in refrigerator and subcultured at an interval of 30 days.

Screening of Bacteria for Biosurfactant Production

To screen the isolated bacteria for biosurfactant production, a single colony of each isolate was inoculated into 50 mL sterilized MSM with 1% of petroleum and 1% of glucose as carbon source in 250 mL Erlenmeyer flasks and incubated in a rotary shaker at 30°C and 180 rpm for 168 h to obtain the highest microbial growth and biosurfactant concentrations. After 7 days of fermentation, bacterial cells were removed by centrifugation (12,000 g at 4°C for 20 min) and accordingly the biosurfactants producing capacity was measured by oil-spreading method and emulsification index measurement (E_{24}) [11].

Emulsification Index Measurement (E_{24})

The emulsification activity was determined by the addition of crude oil to the same volume of cell free culture broth. The mixture was mixed for 2 min and left to stand for 24 h. The emulsification activity was given using the following expression:

$$\%E_{24} = \left(\frac{H_e(\text{mm})}{H_t(\text{mm})}\right) \times 100$$

where E_{24} is the emulsion index after 24 h, H_e is the height of emulsion layer, and H_t is the total height of the liquid [12]. The emulsions formed by the isolates were compared with those formed by a 0.35% (w/v) solution of synthetic surfactant Sodium Dodecyl Sulfate (SDS) in distilled water.

Oil Spreading Method

The oil spread technique was carried out according to Morikawa et al. [13] and Youssef et al. [14]. In total 50 mL of distilled water were added to Petri dishes followed by addition of 100 μL of crude oil to the surface of the water. Then, 10 μL of the culture supernatants were put on the crude oil surface. The diameter of the clear zone on the oil surface was measured.

Identification of Hydrocarbons Degrading Bacteria

Classical Physiochemical Identification

The pure hydrocarbons-degrading strains bacteria were subjected to a classical identification which was carried out using Gram staining, series of biochemical tests which included citrate utilization, oxidase production, catalase test, methyl red, Vogues Proskauer test, gelatine liquefaction test, triple sugar iron test, fermentation of carbohydrate, urease test, indole production test and nitrate reduction. Antibiotic susceptibility of isolated strains was tested on seven antibiotics with different family using disk-diffusion agar technique on Mueller Hinton medium.

Identification by MALDI-TOF Mass Spectrometry

All isolated bacteria were identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry at Laboratory of Bacteriology-Virology-Hospital Hygiene, Robert Debre Hospital of Reims, France. A MALDI-TOF instrument was used to create spectra of isolates for identification by MALDI-TOF. Resulting spectra were compared to reference spectra using Bruker MALDI-TOF Biotyper software to obtain identification with a confidence score. The confidence levels for MALDI-TOF identifications were taken as that assigned by MALDI-TOF instrument software, for example, 1.700–1.999: probable genus identification, 2.000–2.299: secure genus identification and probable species identification, and 2.300–3.000: highly probable species identification.

Biosurfactant Synthesis

Isolates that showed high values of emulsification activity and oil spreading activity were screened for biosurfactant production. The MSM was supplemented with 2% of glucose and 0.1 g/L of yeast extract. Biosurfactant production was performed in flasks (2 L capacity), containing 500 mL of production medium. The culture medium was inoculated with 1% of 24 h inoculums. The flasks were kept under 150 rpm orbital agitation at 30°C for 7 days.

Precipitation and Extraction of Biosurfactants

Bacterial cells were removed by centrifugation (12,000 g at 4°C for 20 min) after achievement of the period of production (98 h). The biosurfactant was precipitated from the cell free supernatant of the culture by adjusting the pH to 3.0 using 6 N HCl and keeping it at 4°C overnight [15,16]. After

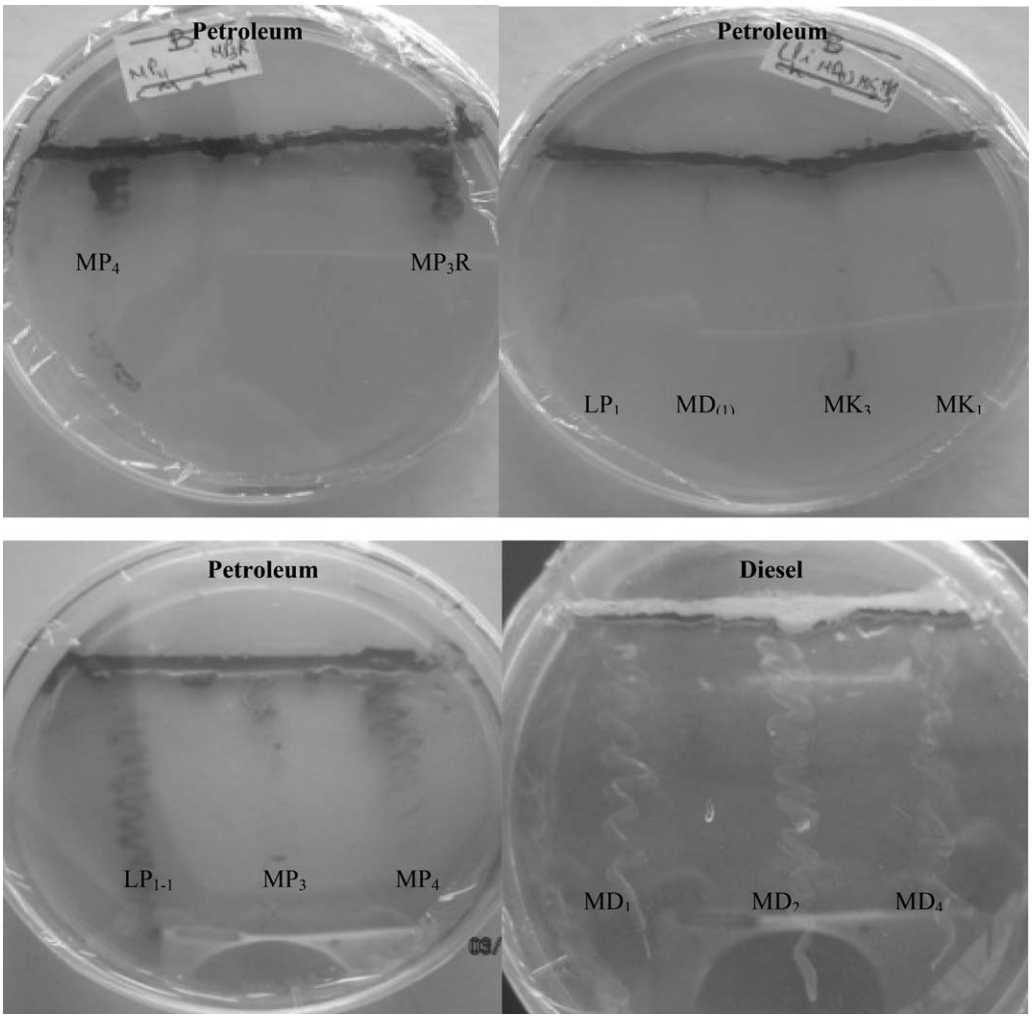

Figure 1. Growth response of bacterial isolates in presence of different hydrocarbons using Agar Ditch method. MP_4, MP_3, MP_3R, LP_1, LP_{1-1}, $MD_{(1)}$, MD_1, MD_2, MD_4, MK_3, and MK_1: are bacteria strains isolated from Soummam Watershed sediments.

that, precipitated material was collected by centrifugation at 20,000 g for 20 min at 4°C. The crude biosurfactant was then lyophilized and weighed for quantification and measurement of tensoactive properties.

Dried Weight Measurement of Crude Biosurfactants and Their Tensoactive Properties

The weights of biosurfactants were determined using the method of Chandran et al. [17]. The cell-free culture broth was centrifuged at 12,000 rpm for 20 min and extracted with chloroform/methanol (2:1 v/v), the solvents were then removed by rotary evaporation. The residue was placed into the sterile Petri dish, was weighed and placed in the hot air oven for drying at 100°C for 30 min, after that the weight of biosurfactants were calculated using the following formula:

$$W_b = W_{pb} - W_p$$

where W_b, dry weights of biosurfactants; W_{pb}, Weights of the Petri dish and biosurfactant residue after drying; W_P, weight of the empty Petri dish.

The weights of the biosurfactants were expressed in terms of grams per liter (dry weight).

The crude biosurfactant emulsification activity was evaluated using the prepared solutions of 0.35% (w/v) of crude biosurfactant using the method cited in section earlier.

Statistical Analysis

The experimental data were presented as averages of three replicates, means and standard errors were calculated for results of all experiments for each isolate. Standard deviations were represented with error bars. Analysis of variance [least significant difference (LSD)-test with $P < 0.05$] was performed on emulsification activity and oil spreading data. All analyses were done using Statistical Analysis Software Statistica (version 5.5).

RESULTS AND DISCUSSION

Isolation of Hydrocarbon Degrading Bacteria

Hydrocarbon degrading bacteria were enriched and isolated from the Soummam sediments. These cultures yielded 11 isolates that were also differentiated based on colony morphology on nutrient agar plates and selected for further characterization. In the hole-plate diffusion method, biodegradation confirmation of various hydrocarbons used as carbon source (diesel, petroleum, kerosene, and toluene) showed bacterial growth around the hole. Out of 11 cultures, 9 had shown the growth around the holes, the bacterial growth was observed at perpendicular of the ditches containing different hydrocarbons as shown in Figure 1. Therefore, microorganisms in those samples were adapted to live with hydrocarbons as carbon source. The majority of microorganisms were able to utilize hydrocarbons as the sole carbon and energy sources [18,19]. Other studies

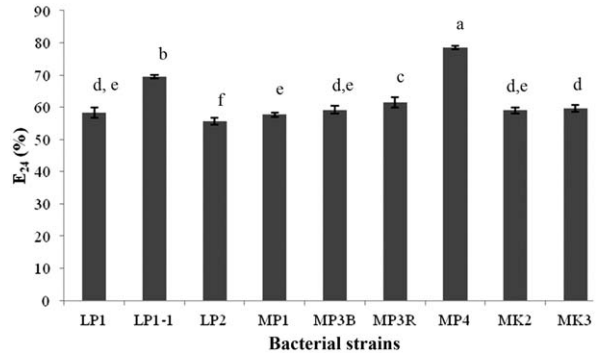

Figure 2. E_{24} for supernatants of bacteria cultivated for 7 days. Statistics data are given as mean ± SD ($n = 3$–5). The data marked with the different letters of each sample category share significant differences at $P < 0.05$.

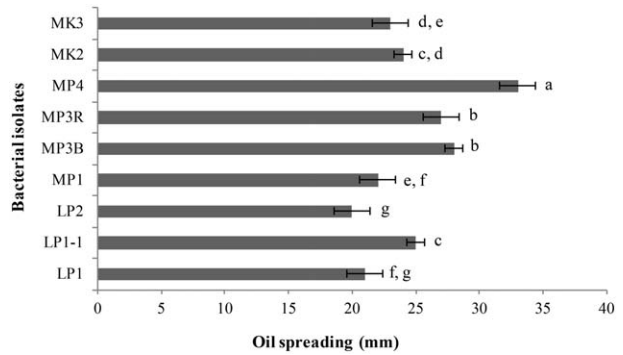

Figure 3. Oil displacement by supernatants from 7 day old bacterial cultures. Statistics data are given as mean ± SD ($n = 3$–5). The data marked with the different letters of each sample category share significant differences at $P < 0.05$.

Table 1. Some Biochemical characteristics of selected hydrocarbon degrading bacteria.

Isolate	LP_1	$LP_{1–1}$	LP_2	MP_1	MP_3B	MP_3R	MP_4	MK_2	MK_3
Gram	N	P	N	N	N	N	P	N	N
OXY	+	–	+	+	+	+	–	+	+
CATA	+	+	+	+	+	+	+	+	+
ONPG	–	+	–	–	–	–	–	–	–
ADH	+	–	–	+	–	–	–	–	–
LDC	–	–	–	–	–	–	–	–	–
ODC	–	–	–	–	–	–	–	–	–
CIT	–	–	+	–	+	+	–	+	+
H2S	–	–	–	–	–	–	–	–	–
URE	+	–	+	+	–	–	–	+	+
TDA	–	–	–	–	–	–	–	–	–
IND	–	–	–	–	–	–	–	–	–
VP	+	+	+	–	+	+	+	+	+
GEL	–	+	–	–	–	–	–	–	–
GLU	–	+	–	–	–	–	–	–	–
MAN	+	+	–	+	+	+	+	+	+

Abbreviations: N, Gram negative; P, Gram positive; +, positive response; –, negative response.

have reported that some microbial species, for instance, *Pseudomonas* sp., *Rhodococcus* sp., *Mycobacterium* sp.,and *Alcanivorax* sp. could degrade hydrocarbons [20–23].

Table 2. Resistance profile of hydrocarbon degrading bacteria: Measurement of clear zone (Ø: mm).

Isolate	LP_1	$LP_{1–1}$	LP_2	MP_1	MP_3B	MP_3R	MP_4	MK_2	MK_3
Ofloxacin OF_5	33.33 ± 0.57	24.33 ± 0.57	31.66 ± 1.52	30.33 ± 0.57	29.66 ± 0.57	31.33 ± 0.57	36.33 ± 0.57	35.66 ± 0.57	34.66 ± 0.57
Chloramphenicol C_{30}	18.66 ± 0.57	36.33 ± 0.57	31.66 ± 0.57	30.33 ± 0.57	28.33 ± 0.57	24.33 ± 0.57	36.66 ± 0.57	17.00 ± 1.00	15.00 ± 1.00
Streptomycin S_{10}	20.66 ± 0.577	37.66 ± 0.57	22.66 ± 2.30	30.33 ± 0.57	28.33 ± 0.57	30.33 ± 0.57	36.66 ± 0.57	15.33 ± 0.57	16.3 ± 0.57
Penicillin G P_{10}	7.00 ± 0.00	20.66 ± 0.57	7.00 ± 0.00	30.33 ± 0.57	28.66 ± 0.57	7.00 ± 0.00	36.33 ± 0.57	7.00 ± 0.00	7.00 ± 0.00
Novobiocin NV_{30}	29.66 ± 0.57	30.33 ± 0.57	20.33 ± 0.57	25.33 ± 0.57	11.66 ± 0.57	12.00 ± 1.73	37.33 ± 1.15	30.66 ± 1.15	31.33 ± 1.15
Doxycyclinhydro chloride DO_{30}	32.66 ± 0.57	36.33 ± 0.57	22.66 ± 0.57	30.33 ± 0.57	29.66 ± 0.57	29.33 ± 0.57	36.66 ± 0.57	36.66 ± 1.15	34.00 ± 1.73
Ciprofloxacin CIP_5	37.00 ± 1.00	23.33 ± 1.15	34.33 ± 0.57	30.66 ± 0.57	29.33 ± 0.57	37.66 ± 0.57	37.33 ± 1.15	37.66 ± 0.57	36.33 ± 1.52

Ø, Diameter of antibiotics testing (mm).
Values reported are averages of 03 replicates ± the standard error.

Table 3. Results of identification by MALDI-TOF for hydrocarbon degrading bacteria strains.

Strain reference	Bacteria 1	Max score	Propos nb of the ident	C interpretation of field
$MK(_2)MC$	*Flavobacterium*	1.222	2	impossible to conclude
$MD(_2)$	*A. faecalis*	2.2	10	very good identification
MP_1	*A. faecalis*	2.176	10	very good identification
MK_3	*Ochrobactrum*	2.033	6	good identification to genre
LP_2	*A. faecalis*	1.997	10	very good identification
LP_{1-1}	*Cellulosimicrobium*	2.174	2	good identification
MK_2	*Ochrobactrum*	2.179	6	good identification to genre
MP_3R	*P. stutzeri*	1.854	6	good identification
MP_3B	*P. stutzeri*	2.297	7	very good identification
MP_4	*R. ruber*	2.31	10	excellent identification
MP_{1-1}	*A. faecalis*	2.264	10	very good identification

Table 4. Dry weight of biosurfactants produced by bacterial isolates.

Bacteria isolate	Dry weight of crude biosurfactants (g/L)
A. faecalis	4 ± 0.001
Ochrobactrum	4.86 ± 0.002
Cellulosimicrobium	4.74 ± 0.004
P. stutzeri	5.3 ± 0.002
R. ruber	6.56 ± 0.006

Values reported are averages of 03 replicates ± the standard error.

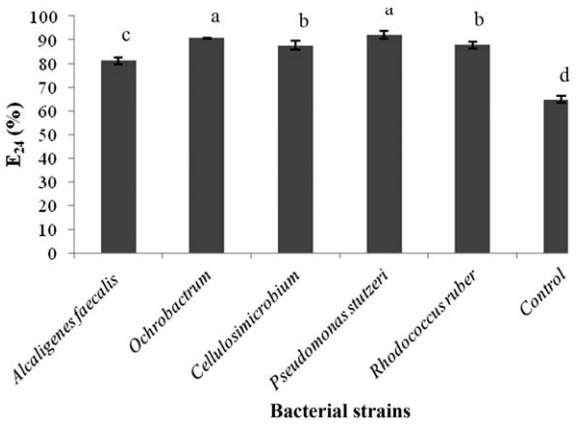

Figure 4. E_{24} for crude biosurfactants of bacteria strains identified. Statistics data are given as mean ± SD ($n = 3$–5). The data marked with the different letters of each sample category share significant differences at $P < 0.05$.

Screening of Bacteria for Biosurfactants Production

Biosurfactants producing microbes can be found in various ecosystems, although the environments that are impacted with hydrophobic contaminants such as refinery wastes and petroleum are more yielding than undisturbed/uncontaminated ones [24]. The biosurfactants detection of the bacterial isolates was made using an oil spreading technique and emulsification test. These techniques were employed by different authors to screening microorganisms with potential to produce biosurfactants [14,25,26]. All nine isolates were determined to be biosurfactant producing bacteria using two screening tests: emulsification activity and oil spreading method. However, the responses of these isolates to screening methods were different. Significantly ($P \leq 0.05$) highest emulsification index (E_{24}) was observed in strains MP_4 which has $78.50 \pm 0.50\%$ activity followed by LP_{1-1} and MP_3R that have 69.5 ± 0.5 and $61.5\% \pm 1.50\%$, respectively. The least emulsification index was observed in LP_2 with $56 \pm 1.15\%$ (Figure 2).The cultures that exhibited weak emulsification showed scarce or no turbidity in the aqueous phase. In those cultures the cells might be adhering to the emulsified oil slicks [27]. Emulsification of the crude oil in water is a prerequisite that paves the way for biodegradation of this environmental pollutant by many bacteria. It enhances the bioavailability of the oil and thus increases the biodegradation rate [18,27,28].

The oil spreading method has the positive response as shown in Figure 3. The clear zone diameters on the oil surface obtained using oil spreading method were larger for the cultures of the strains MP_4, LP_{1-1}, MP_3B, MP_3R, MK_2, and MK_3. However, the largest diameters were observed for strain MP_4 (Ø 33.66 ± 1.15 mm) indicating significant variations ($P \leq 0.05$) between the biosurfactants produced by the isolates. Thus, indicating the presence of higher concentrations of surface active compounds. Microbial production of the surface-active compounds by microorganisms growing on crude oil and other hydrophobic substrates has frequently been reported [29,30].

Identification of Hydrocarbon-Degrading Bacteria

Some preliminary identification tests were carried out to discriminate between isolated strains. Table 1 shows the results of these diagnostic tests which were results of classical identification. They showed that the majority (eighty one percent of the bacterial isolates) of the hydrocarbon degrading bacteria in this study (LP_1, LP_2,MP_1, MP_3R, MP_3B, MK_2, and MK_3) belonged to Gram negative coccobacilli strains, followed by Gram positive (LP_{1-1}) cocci and only MP4 was Gram positive rod-shaped or coccoid. Furthermore, all isolated strains were catalase positive. However, biochemical patterns between isolated bacteria are different (Table 1). The antibiotics resistance profile of the all isolated strains showed some differences among the isolates (Table 2). All isolated strains were sensitive to the antibiotics: Ofloxacin (OF_5) and Ciprofloxacin (CIP_5). Strains LP_{1-1}, MP_1, and MP_4 were sensitive to all used antibiotics, but the isolated strains LP_1, LP_2, MP_3R, MP_3B, MK_2, and MK_3 showed varying resistance to Penicillin G (P_{10}).

Table 3 shows the results for 11 isolates tested using identification by MALDI- TOF with a score of at last 1.22 agreed to at least the genera-level. Combined analysis of morphological, biochemical, and physiological characterization and identification by MALDI-TOF results showed that the five strains were: *Alcaligenes faecalis*, *Ochrobactrum*, *Cellulosimicrobium*, *Pseudomonas stutzeri* and *Rhodococcus ruber*.

Biosurfactants Synthesis

Results for dry weights of crude biosurfactants were presented in Table 4. The highest biosurfactant producer was *R. ruber* which produced 6.56 ± 0.01 g/L of the crude biosurfactant, followed by *P. stutzeri* (5.3 ± 0.01 g/L). *Cellulosimicrobium* and *Ochrobactrum* produced 4.74 ± 0.004 and 4.86 ± 0.01 g/L, respectively. The least production was observed for *A. faecalis* with only 4.0 ± 0.01 g/L.

All crude biosurfactants showed the emulsification capacity of crude oil (Figure 4). The result of emulcification index revealed that *P. stutzeri* emulsified by $92.11 \pm 1.57\%$, *Ochrobactrum* by $90.61 \pm 0.41\%$, *R. ruber* by $87.77 \pm 1.57\%$, *Cellulosimicrobium* by $87.62 \pm 1.78\%$ and *A. faecalis* by $81.11 \pm 1.57\%$. *P. stutzeri* had the highest emulsification index indicating significant variations ($P \leq 0.05$) between the biosurfactants produced by the isolates. These results were higher than the positive control used ($64.77 \pm 1.6\%$). However, it is quite an appreciable quantity compared with that observed by Maalej et al. [31]. They found index values varied from $42.5 \pm 3.5\%$ to $89 \pm 4.2\%$ using different hydrocarbons for *P. stutzeri*. Emulsifying activity for *R. ruber* was also high compared with values obtained by Kuyukina and Ivshina [32] where emulsion index with n-hexadecane was 62%

CONCLUSION

Eleven bacterial strains were isolated from sediment samples of Soummam watershed from Bejaia (Algeria) with a high different ability to degrade hydrocarbons using an enrichment technique in MSM and Hole-plate diffusion assay with various hydrocarbons as the sole carbon source. Using oil spreading test and emulsification assay, the most of isolated bacteria have also different capacity to produce biosurfactants. Five out these isolated bacterial strains were identified by classical and MALDI-TOF identification. The weight values obtained of crude biosurfactants produced differed between these identified strains. These last had greater emulsification capacity than the synthetic surfactant SDS. Hence, the results of this study showed the presence of diverse bacteria in the sediment of Soummam watershed with interesting hydrocarbon degradation potentiality mainly for the most biosurfactant produced strains. Two of these five strains released the biosurfactants to the culture medium, and should be considered as potential candidates for large scale biosurfactant production and this work completed by characterization and optimization studies aimed at application in bioremediation and enhanced oil recovery processes and application in various fields of biotechnology.

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