Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(10):413-422

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Isolation and biosurfactants production by *Pseudomonas aeruginosa* S7PS5

Mohammed EL Amine Bendaha[1]*, Boumedien Meddah[1,2], Hadj Ahmed Belaouni[3] and Aicha Tirtouil[1,2]

[1]*Laboratoire de Bioconversion, Génie Microbiologique et Sécurité Sanitaire (LBGMSS), Faculty of Natural and Life Sciences, MASCARA University, Algeria*
[2]*Laboratoire de Recherche sur les Systèmes biologique et Géomatique (LRSBG), Faculty of Natural and Life Sciences, MASCARA University, Algeria*
[3]*Laboratoire de Biologie des Systèmes Microbiens (LBSM), ENS Kouba, Algeria*

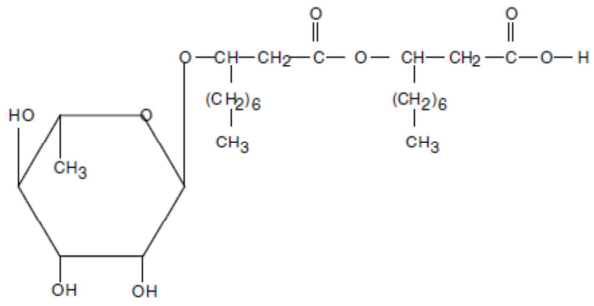
ABSTRACT

Thirty bacterial strains were isolated from soils contaminated with hydrocarbons in the north west of Algeria, and tested for biosurfactants production in a nutrient broth supplemented with olive oil using drop collapsing, oil displacement, emulsification activity (E_{24}) and measurement of the surface activity. S1PS2, S7PS5 and S9PS2 strains were the best three producers and were selected to optimize the production (S1PS2 : E_{24} = 56.32 ± 1.95%, Ø = 5.733 ± 0.35 cm and % $_{Reduction}$ = 30.17 ± 0.12, S7PS5 : E_{24} = 43.38 ± 0.07%, Ø = 8.3 ± 0.10 cm and % $_{Reduction}$ = 31.23 ± 0.01, S9PS2 : E_{24} = 44.27 ± 0.26%, Ø = 8.66 ± 0.05 cm and % $_{Reduction}$ = 32.00 ± 0.13). After optimization, S7PS5 shows the optimal of rhamnolipids production with the following composition: 100 ml of nutrient broth, 1% of olive oil and 1% of inoculums, with shaking at 75 rpm/min at room temperature, for an incubation period of 22 h, and it was selected for an identification using API 20NE system and 16S rDNA sequencing which allowed the link S7PS5 to the genus Pseudomonas and showed 98.84% of similarity with Pseudomonas aeruginosa LMG 1242^T/Z76651 as the closest specie.

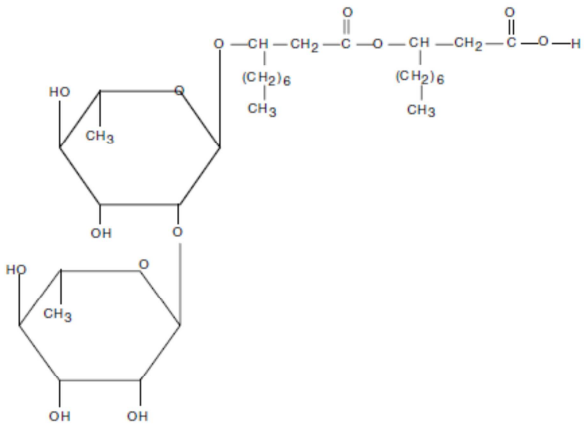
Keywords: Hydrocarbons, biosurfactants, emulsification activity, rhamnolipids *Pseudomonas aeruginosa*.

INTRODUCTION

Surfactants are amphiphilic molecules that consist of a hydrophilic head and a hydrophobic tail [1]. Biosurfactants are environmentally friendly, biodegradable, less toxic and non hazardous, they have better foaming properties and higher selectivity and due to their amphiphilic structure, they increase the surface area of hydrophobic water insoluble substances, increase the water bioavailability of such substances and change the properties of the bacterial cell surface [2]. *Pseudomonas* is a large group of bacteria colonizing the soil, plants and water. These Gram negative bacteria, non spore-forming, are obligating aerobic, with the exception of some that can use the NO_3 as the final electron acceptor. Their mobility is ensured by several polar flagella; they have a mesophilic and chemo-organothorphe metabolism, mostly saprophytic [3].



RLL or R1 (α-L-Rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate)



RRLL or R2 (2-O-α-L-Rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate)

Fig. 1 Chemical structure of rhamnolipids [1]

Pseudomonas aeruginosa produces an extracellular glycolipid surfactant called rhamnolipids [4] and composed of β-D-(β-D-hydroxyalkanoyloxy) alkanoic acids (HAA) (primarily β-hydroxydecanoyl-β-hydroxydecanoate) derivatized with one or two rhamnose sugars [5] (Fig. 1).

Potential applications of biosurfactants in cosmetic, food, healthcare, pulp and paper processing, coal, ceramic, and metal industries have been conceived. Even more promising are applications considering the cleaning of oil contaminated tankers, oil spill removal, transportation of crude oil, recovery of crude oil from soil, facilitated oil recovery as well as bioremediation of sites contaminated with hydrocarbons, heavy metals and other pollutants [6].

EXPERIMENTAL SECTION

2.1. Sampling

12 soil samples were collected at three sampling sites polluted by hydrocarbons in the north west of Algeria (Mascara, Saida and Al-Bayadh); about 50 g of soil was collected in sterile bags for each one.

2.2. Isolation strategy

2.2.1. Isolation of *Pseudomonas aeruginosa* strains

The isolation of *Pseudomonas aeruginosa* begins with enrichment, environmental samples were inoculated at a rate of 1 g of soil in tubes containing 9 ml of sterile nutrient broth [7]. Inoculated tubes were incubated at 42 °C for 24 h. 0.1 ml from enrichment tube was placed on Cetrimide agar and incubated at 42 °C for 24 h [8].

Colonies producing blue-green or yellow-green pigment, revealed under UV at 366 nm were picked and purified three times on Cetrimide medium at 42 °C for 24 h.

2.2.2. Screening assays for potential biosurfactant producing strains

2.2.2.1. Inoculums preparation

After purification, bacterial suspensions were prepared for each isolate in sterile nutrient broth, their O.D was adjusted at 0.5 Mc.Farland at 600 nm to bring them under the same conditions of initial biomass (10^6 cell / ml), in order to select the most effective strains.

2.2.2.2. Culture medium inoculation

All isolates were grown in sterile nutrient broth [9] containing 2% of olive oil as the best carbon substrate [10].

Each 250 ml Erlenmeyer flask containing 100 ml of the culture medium is inoculated with 1 ml of inoculum prepared in advance, and stirred at 75 rpm at room temperature for 48 h, biosurfactants are recuperated in the supernatant after centrifugation at 9000 g for 15 min [11].

2.3. Biosurfactants producing tests

2.3.1. Drop collapsing

The screening for biosurfactants production was conducted using the test of collapse [12], a supernatant drop of each isolate was placed on the surface of a glass slide covered with a thin layer of oil. After 1 min, supernatant of the culture that led to the collapse of the drop is shown as a positive result, and the drops remaining with the beads are marked as negative results.

2.3.2. Oil displacement

In a Petri dish, 15 µL of crude oil are placed on the surface of 40 ml of sterile distilled water, then; 10 µL of supernatant of each culture was slightly put on the oil film surface. After 30 s, diameter of the clear halo is measured under visible light [13].

2.3.3. Emulsification activity

4 ml of supernatant was added to 4 ml of soybean oil [14] and vortexed vigorously for 2 min. The emulsification index (E_{24}) was estimated after 24 h, as follows:

$$E_{24}(\%) = \frac{H_{EL}}{H_S} \times 100$$

H_{EL}: Height of emulsion layer, H_S: Height of total liquid column.

2.3.4. Surface activity measurements

To study the surface activity of the biosurfactants produced by the different isolated strains, supernatants of each sample were measured for surface tension using a tensiometer. Measurement of surface tension was carried out according to the Du Nouy ring method [15] at room temperature after dipping the ring plate in the solution for a certain time to reach the equilibrium conditions. For the instrument calibration, the surface tension of pure water was measured. The measurement is repeated at least three times, and an average value was used to express the surface activity of each sample.

Biosurfactant surface activity is expressed as a reduction percentage of the surface tension reduction by the following equation:

$$\%\ \text{surface tension reduction} = \frac{(\gamma_m - \gamma_c)}{\gamma_m} \times 100$$

γ_m is the surface tension of the medium as prepared, γ_c is the surface tension of the supernatant.

2.4. Culture medium and growth conditions for biosurfactants production

Selection of the performing three strains was done by comparing the oil displacement diameters (cm), emulsions indexes (E_{24}) and percentages of surface tension reduction for each strain.

2.4.1. Optimizing the olive oil amount

The culture medium used for biosurfactants production was optimized by varying the olive oil amount from 1 to 5% (v / v) in the broth culture. Then, the culture medium was incubated at room temperature in a shaking incubator at 75 rpm. After 48 h, bacteria were removed by centrifugation at 9000 g for 15 min [11], reduction percentages measures of surface tension are performed for each used olive oil concentration. The optimum amount of olive oil for biosurfactants production is used for further optimization.

2.4.2. Inoculums optimization

2.4.2.1. Growth kinetics

A preculture of each strain was performed on nutrient broth and incubated at 30 °C for 24 h, then a loop fool each culture is used to inoculate an Erlenmeyer flask containing 100 ml of nutrient broth, each flask is incubated at room temperature with shaking at 75 rpm. Microbial growth was studied as a function of culture time by measuring the culture medium absorbance, after each 2 h using a 600 nm UV spectrophotometer. From graphic layout of the microbial concentration versus time, the best culture time for the inoculum preparation was determined.

2.4.2.2. Optimizing the inoculums amount

In order to find the appropriate amount of inoculum for biosurfactants production, an inoculum of each strain was prepared using the best culture time obtained in advance, and by varying the inoculum amount from 1 to 5% (v/v), and transferred into 100 ml of culture medium, containing the optimum amount of olive oil, then,

the culture was incubated at room temperature, with shaking at 75 rpm for 48 h. After that, reduction percentages measures of surface tension are performed for each used inoculum concentration.

The optimum inoculum amount for biosurfactants production is used for further optimization.

2.5. Identifying the most efficient strain

After isolation, colonies producing blue-green diffuse pigment aspects and gram colorations were done, the obtained rods gram negative bacteria were tested for oxidase production, this positive test due to this enzyme presence related the colonies to the genus *Pseudomonas.*

2.5.1. API 20NE (Non Enterobacteriaceae)

Based on biochemical substrates degradation, API 20NE (Biomérieux, France) system is a rapid frequently used method for identifying. This method is suitable to identify Gram-negative bacilli at the species level by evaluating the profile of 21 different biochemical reactions. API 20NE stripes were incubated at 30 °C for 24 h and the results were performed by the Bacterial identification Program [16].

2.5.2. Molecular characterization and phylogenetic analysis

2.5.2.1. Extraction and PCR amplification of 16S rDNA

The most powerful strain was cultured at 30 °C for 24 h with stirring (250 rpm) in a 500 ml Erlenmeyer containing 100 ml of TSB medium. DNA was extracted according to the method described by Liu et al. (2000) [17].

16S rDNA fragment was amplified by PCR (Polymerase Chain Reaction) using an invitrogen kit and two primers: 10-30F (5'-GAG TTT GAT CCT GGC TCA-3 ') and 1500R (5'-AGA AAG GAG GTG CAG ATC CC-3 ').

A reaction volume of 50 µl was prepared by mixing 5 µl of 10x PCR buffer (10 mM of Tris-HCl, 50 mM of KCl, pH 9.0 at 25 °C), 1.5 mM of $MgCl_2$, 200 mM of each dNTP, 100 pM of each primer, 2µl of DNA solution and 1.25 U of polymerase enzyme. To evaluate the reaction, an horizontal agarose gel (2.4%) electrophoresis of the PCR product was performed. 16S rDNA amplification was carried out on a Stratagene Robo Cycler Gradient.

2.5.2.2. 16S rDNA sequencing

DNA sequencing was done at Beckman Coulter Genomics (United Kingdom).

The applied primers were the same pair of primers used to amplify 16S rDNA, 10-30F (5'-GAG TTT GAT CCT GGC TCA-3 ') and 1500R (5'-AGA AAG GAG GTG CAG ATC CC-3 ').

Phylogenetic identification of neighboring was first carried out by the program BLASTN [18]. The best sequences with the highest scores are selected for the calculation of "pairwise sequence similarity" using the global alignment algorithm located at the EzTaxon-e server [19] (http://eztaxon-e.ezbiocloud /; [19]). Phylogenetic analysis and molecular evolution were performed using software included in the package MEGA version 6.0 [20].

16S rDNA sequence of the most powerful strain was aligned to the "ClustalW" program [21], 15 corresponding to the closest taxa nucleotide sequences were retrieved from the results in Blast EZ Taxon-e (Files in FASTA format). Distances calculation of the evolution matrix and the algorithm of the "Neighbor-Joining" were generated [22] and a phylogenetic tree was prepared which enabled the construction of the topology of the tree evaluated by the bootstrap test [23].

2.6. Statistical analysis

Presented results were the average values of three determinations (All experiments data are expressed in terms of arithmetic averages obtained from at least three replicates) and the standard deviations for all measurements were less than 5%, the analysis were done using XLSTAT software, version 2014.3.01 (Adinosoft).

RESULTS AND DISCUSSION

3.1. Isolation of *Pseudomonas aeruginosa* strains

Cetrimide agar is a medium for promoting pigmentation, it is King A supplemented with nalidixic acid and tétradonium bromide (Cetrimide) that inhibit the Gram positive growth, leaving advantage to *Pseudomonas sp*., *Pseudomonas aeruginosa* is characterized by two pigments production: blue green pigment (pyocyanin) and yellow green pigment (pyoverdin) [7].

30 environmental strains with fluorescent pigments were isolated, identified, purified and conserved on glycerol broth at –80 °C.

Cetrimide agar is a medium for promoting pigmentation, it is King A supplemented with nalidixic acid and tétradonium bromide (Cetrimide) that inhibit the Gram positive growth, leaving advantage to *Pseudomonas sp*., *Pseudomonas aeruginosa* is characterized by two pigments production: blue green pigment (pyocyanin) and yellow green pigment (pyoverdin) [7].

30 environmental strains with fluorescent pigments were isolated, identified, purified and conserved on glycerol broth at –80 °C.

Cetrimide agar is a selective medium for *Pseudomonas* isolation, the use of Cetrimide was advocated by Lowbury in 1955 [24]. Nalidixic acid blocks DNA replication of sensitive bacteria [25]. Magnesium chloride and potassium sulfate promotes pyocyanin production [26] and grown in iron limiting conditions, *Pseudomonas* produces yellow green fluorescent siderophores called pyoverdins [27].

3.2. Biosurfactants producing tests

3.2.1. Drop collapsing

In absence of biosurfactants, a drop of water applied to a hydrophobic surface, form a pearl because polar molecules of water are repelled from hydrophobic surface, but if the water drop contains biosurfactants, the latter falls and spread over the blade [28]. Interfacial tension between water droplet and hydrophobic surface is reduced in biosurfactants presence, resulting in the spread of the water drop on hydrophobic surface [29]. Presented results, show biosurfactants presence in all tested supernatants.

3.2.2. Oil displacement

The oil displacement is closely related to biosurfactants presence in the supernatant of the tested bacteria [30], clear halos diameters are function to the biosurfactants amount present in supernatant of each sample. All tested strains showed a positive result for oil displacement with different diameters (Fig. 2).

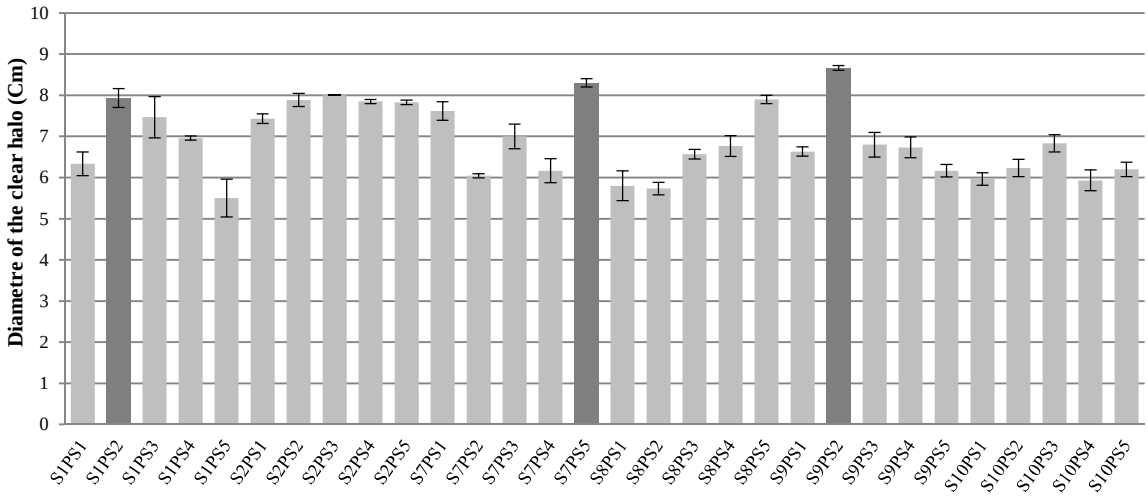


Fig. 2 Comparison of clear halos diameters due to biosurfactants of the different strains

Biosurfactants have two different polarities, and there density is less than water, so they float to the surface for the oil displacement test, micelles have a greater affinity for water then oil, therefore, they will return in competition with the latter to the surface occupation. If the oil volume was higher than that of biosurafactant, this latter is adsorbed at the water / oil interface [31], but because of the very small volume of oil, its hydrophobic molecules will move leaving the surface to biosurfactant molecules.

3.2.3. Emulsification activity

Biosurfactants increase apparent solubility of hydrophobic molecules by trapping them in a pseudohydrophobic phase formed by micelles [32]. All tested supernatants solubilize the oil and form pseudohydrophobic phase, emulsion indexes (E_{24}) varies according to the biosurfactants concentration (Fig. 3).

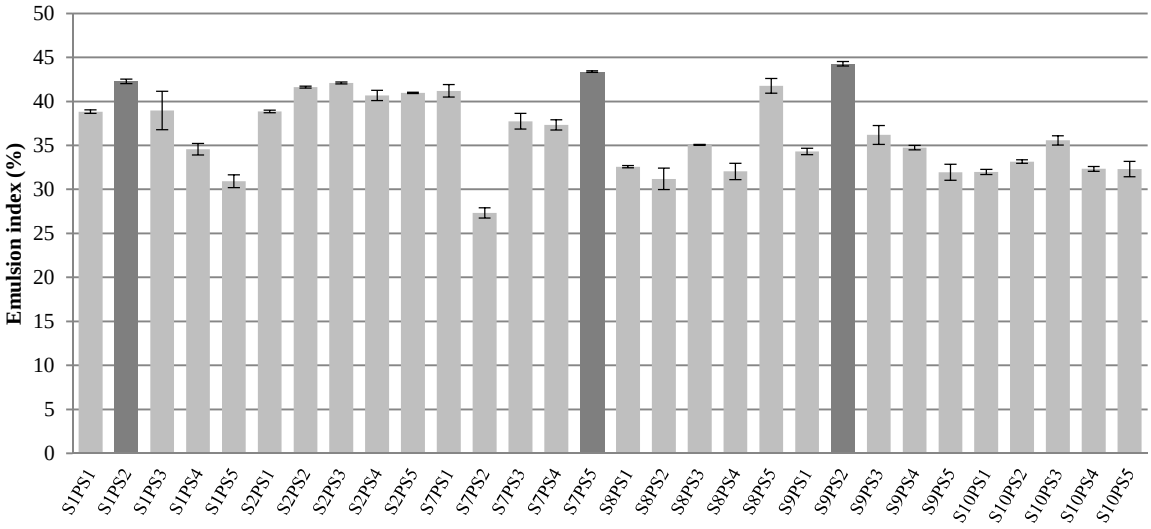


Fig. 3 Comparison of emulsion indexes of the different strains

Micelles are formed when hydrophobic portions unable to form hydrogen bonds in aqueous phase, unite and move towards the center leaving the hydrophilic portions outward; agitation provided by vortex was made to isolate hydrophobic molecules of oil, and trapping them inside micelles [33].

3.2.4. Surface activity measurements

Surface tension of the different cultures supernatants was measured function to biosurfactants concentration excreted in order to compare the surface activities of all isolates (Fig. 4). Surface tension of the nutrient broth (5.66 mN / m) is rapidly decreased as the biosurfactant concentration increased.

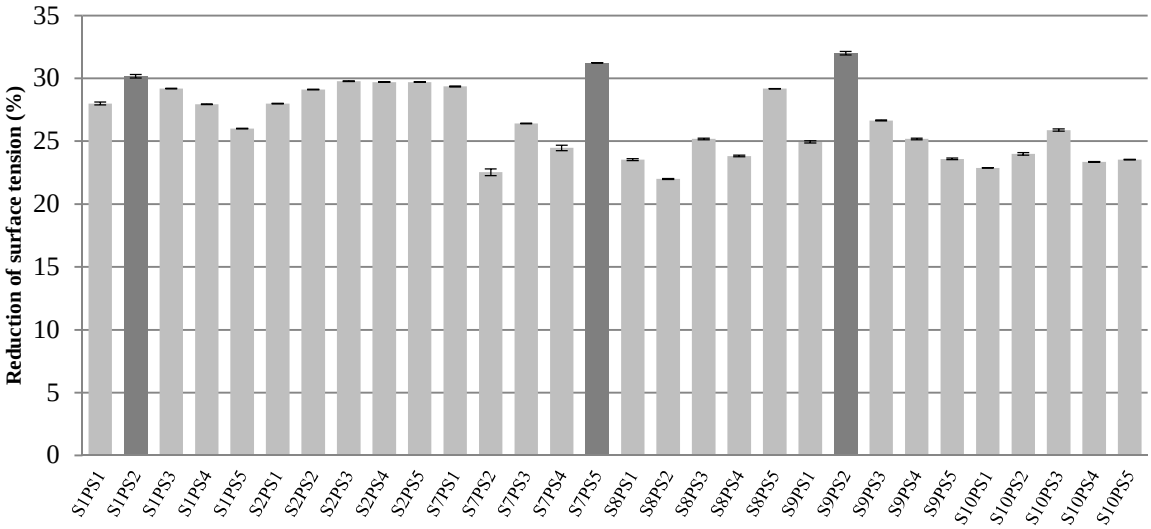


Fig. 4 Comparison of percentages reduction of surface tension of the different strains

Biological surfactants produced by *Pseudomonas aeruginosa* strains showed a reduction of the surface tension of pure water from 72 to 30% with a CMC ranging from 5 to 200 mg / l, depending on their components [34]. It is clear that the highest surface activity (32.00%) is proportional with the lowest surface tension (3.85 mN / m).

3.3. Selection of the most efficient strains

Table 1 Values of emulsion indexes (%), clear halos diameters (cm) and reduction of surface tension (%) for S1PS2, S7PS5 and S9PS2 strains

Strain	Emulsion index (%)	Clear halos diameters (cm)	Reduction of the surface tension (%)
S1PS2	42,26 ± 0,25	7,93 ± 0,23	30,17 ± 0,12
S7PS5	43,38 ± 0,07	8,3 ± 0,10	31,23 ± 0,01
S9PS2	44,27 ± 0,26	8,66 ± 0,05	32,00 ± 0,13

According to the obtained results (Table 1), bacteria that have shown significant potential for biosurfactants production are bacteria with maximum values of percentage reduction of the surface tension, of oil displacement and emulsion index (E_{24}). Strains S1PS2, S7PS5 and S9PS2 are the best biosurfactants producers.

3.4. Culture medium and growth conditions for the production of biosurfactants

3.4.1. Optimizing the olive oil amount

Olive oil concentration in the nutrient broth was varied from 1 to 5%. As shown in Figure 5, the best biosurfactants performance is obtained with 1% olive oil in the different cultures and represented by percentage reducing of surface tension which is 29.41 ± 00% for S1PS2, 38.35 ± 0.13% for S7PS5 and 35.23 ± 0.03% for S9PS2.

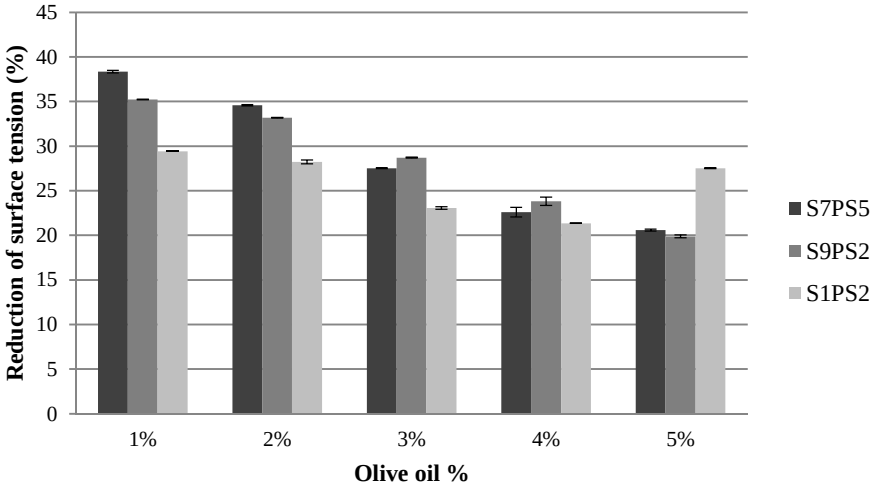


Fig. 5 Effect of the olive oil amount on percentages reduction of surface tension of S7PS5, S9PS2 and S1PS2 strains

Therefore, in a nutrient broth with 1% olive oil is selected as a suitable culture medium for the next experiments of biosurfactants production for the three selected bacteria with S7PS5 strain that gave the best rate of biosurfactants production for this olive oil concentration.

3.4.2. Inoculums optimization

3.4.2.1. Growth Kinetics

Growth kinetics of S1PS2, S7PS5 and S9PS2 strains were studied to find the best time for an inoculum culture preparation, it is time taken for each bacterium to enter in exponential phase; the three strains were monitored for 52 h by measuring their absorbance at 600 nm every 2 hours at room temperature. From Figure 6, S1PS2 reached the exponential phase after 24 h of culture, S7PS5 reached exponential phase after 22 h and finally S9PS2 reaches that after 26 h. S7PS5 strain has the shorter lag and acceleration phases and is therefore best suited to this medium.

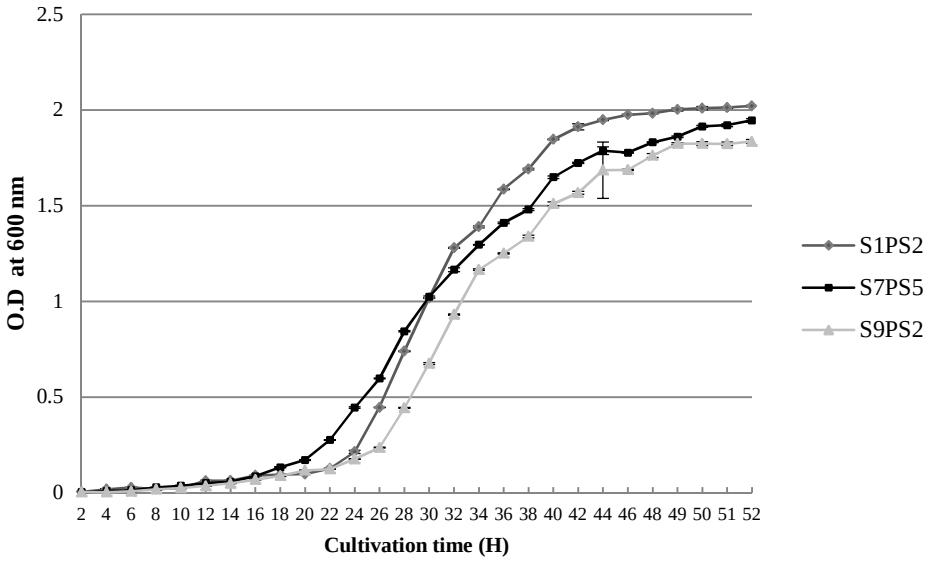


Fig. 6 Growth kinetics of S1PS2, S6PS5 and S9PS2 strains in nutrient broth at room temperature with shaking at 75 rpm / min.

Consequently, selected time to prepare inoculum culture is 22 h for S7PS5, 24 h for S1PS2 and 26 h for S9PS2 ; this is time to reach the exponential stage and where the growth rate is maximum and stable.

3.4.2.2. Optimizing the inoculums amount

Inoculum amount in the nutrient broth was varied from 1 to 5% (v / v). The best performance biosurfactants production is obtained with 1% inoculum used in the different cultures (Fig. 7) and represented by the percentage reducing of surface tension which is 37.17 ± 0.14% for S1PS2, 42.17 ± 0.07% for S7PS5 and 36.88 ± 0.06% for S9PS2.

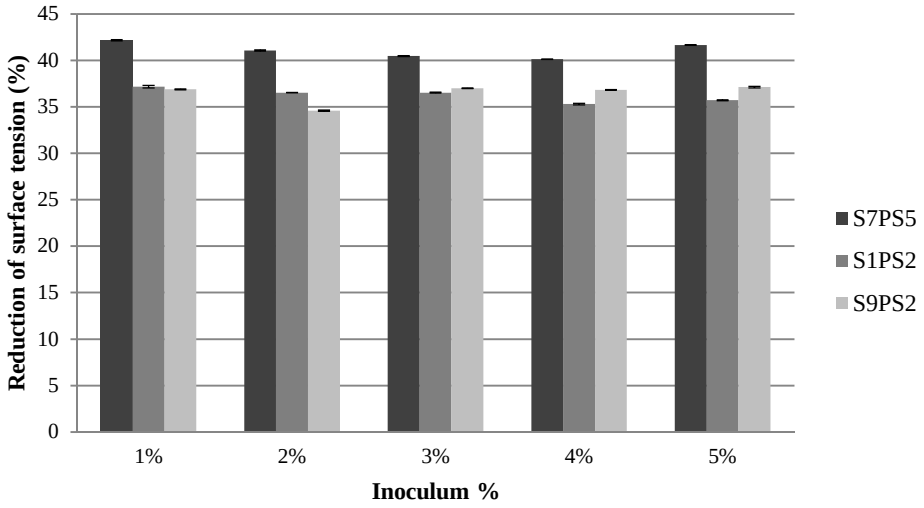


Fig. 7 Effect of the inoculum amount on percentages reduction of surface tension of S7PS5, S9PS2 and S1PS2 strains

Therefore, nutrient broth inoculated with 1% microbial culture was selected as the most appropriate concentration for biosurfactants production by the three selected strains.

Cell number of bacteria less than the best will probably not lead to better production, but when cells number is high, they use all the culture medium compounds for their growth, which leads to culture medium depletion without achieving the desired metabolite production. The most powerful strain is S7PS5 and gave the best rate of poucentage reducing of surface tension which is 42.17 ± 0.07%

3.5. Identification of the most powerful strain

3.5.1. API 20NE

S7PS5 macroscopic and microscopic study are identical to those obtained by Palleroni in 1984 on *Pseudomonas aeruginosa* [35]. The Bacterial Identification Program suggests that S7PS5 strain is a *Pseudomonas aeruginosa* with an identification score of 0,99.

3.5.2. 16S rDNA sequencing

16S rDNA sequence has long been used as a taxonomic gold standard in determining the phylogenies of bacterial species [36], it has been used to detect and differentiate *Pseudomonas* species.

The 16S rDNA sequence (810 bp) of strain S7PS5 has been deposited in the GenBank data library and has been assigned the accession number KR349493 and allowed the link S7PS5 to the genus *Pseudomonas*. Alignment of this sequence by the blast showed 98.84% of similarity with *Pseudomonas aeruginosa LMG 1242^T/Z76651* as the closest specie. Other *Pseudomonas* species have lower percentages of similarity, situated between 97.54 and 95.86%, Figure 8 shows on the dendrogram, phylogenetic position of the S7PS5 strain.

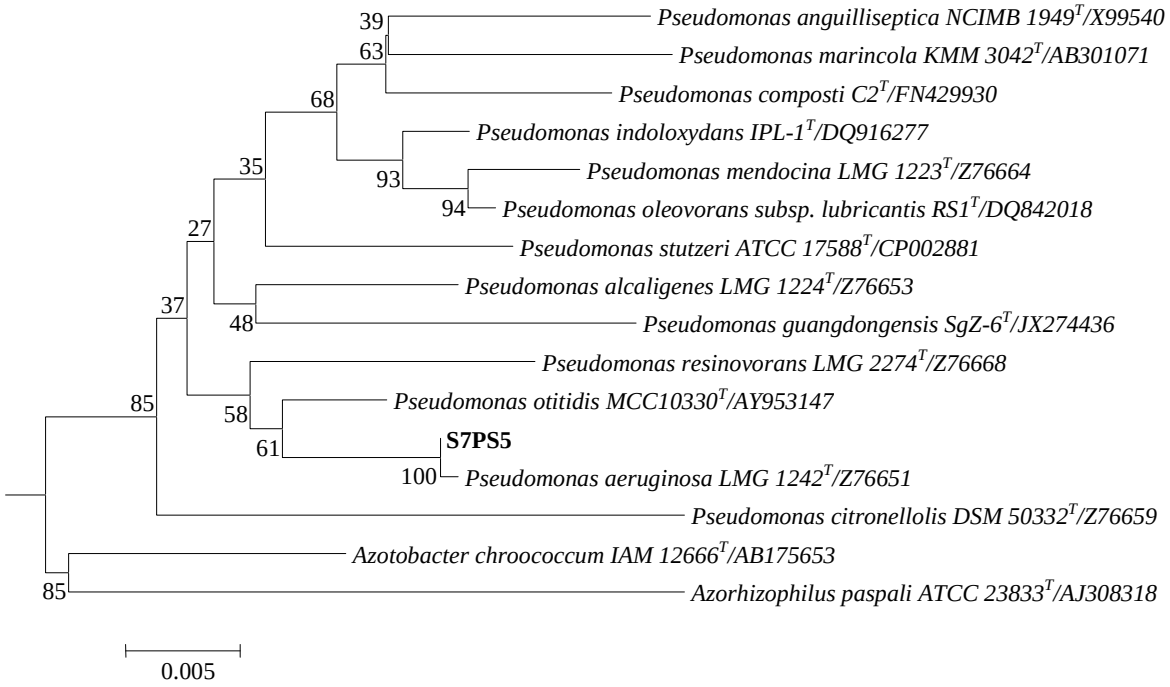


Fig. 8 Phylogenetic tree based on sequence analysis of 16S rDNA and showing the relationships between S7PS5 and and the other similar species. Note: The numbers at the nodes indicate "bootstrap" levels (As a percentage of 1000 re-sampling). The bar indicates 0.005 substitutions per nucleotide position

CONCLUSION

We have designed an isolation assays that provide rapid, simple, and reliable identification of *P. aeruginosa*. These assays should serve as a useful adjunct in the evaluation of rhamnolipids producing bacteria. Three types of microorganisms were isolated from soil contaminated by hydrocarbons in the north west of Algeria. Rhamnolipids production was made using a nutrient broth supplemented with olive oil as the best carbon source. To study microbial growth, drop collapsing, oil displacement, emulsification tests and surface activity measurements were performed. It was found that S7PS5 strain could grow in the culture medium better than the other strains resulting in a shorter culture time for biosurfactant production and a higher yield of rhamnolipids, 16S rDNA sequencing proved the *Pseudomonas aeruginosa* producing biosurfactants isolation strategy.

Acknowledgments
The authors of this paper thank Mr. SELOUANI M.M the responsible of the microbiology laboratory at the biology department, faculty of science, Sidi Bel Abbess University (Algeria) and Mr. REGUIG O. for their support and help.

REFERENCES

[1] K Urum; T Pekdemir. *Chemosphere*., **2004**, 57, 1139–1150.
[2] M Pacwa-Płociniczak; GA Płaza; Z Piotrowska-Seget; SS Cameotra. *Int. J. Mol. Sci*., **2011**, 12, 633–654.
[3] GM Garrity, JT Staley, DR Boone, DJ Brenner, P De Vos, M Goodfellow, NR Krieg, FA Rainey, KH Schleifer. Bergey's manual of systematic bacteriology, second edition ;volume two: the proteobacteria; part b: the gamma proteobacteria, USA (Michigan state), Library of congress cataloging-in-publication, **2000**, 323–351.
[4] G Hauser; ML Karnovsky. *J. Bacteriol*., **1954**, 68, 645–654.
[5] K Zhu; CO Rock. *J. bacterial.*, **2008**, 190(9), 3147–3154.
[6] JD Desai; IM Banat. *Microbiol. Mol. Biol. Rev*., **1997**, 60, 47–64.
[7] M Dworkin, S Falkow, E Rosenberg, KH Schleifer, E Stackebrandt. The prokaryotes: a handbook on the biology of bacteria, third edition, volume 6: proteobacteria: gamma subclass. Singapore: springer science + business media, **2006**, 654–655.
[8] B Atzél; S Szoboszlay; Z Mikuska; B Kriszt. *Int. J. Hyg. Environ. Health*., **2008**, 211, 143–155.
[9] O Pornsunthorntawee; N Arttaweeporn; S Paisanjit; P Somboonthanate; M Abe; R Rujiravanit; S Chavadej. *Biochem. Eng. J*., **2008**, 42, 172–179.
[10] M Abouseoud; R Maachi; A Amrane; S Boudergua; A Nabi. *Desalination*., **2008**, 223, 143–151.

[11] YH Wei; CL Chou; JS Chang. *Biochem. Eng.J*., **2005**, 27, 146–154.
[12] DK Jain; DLC Thompson; H Lee; JT Trevors. *J. Microbiol. Meth*., **1991**, 13, 271–279.
[13] M Morikawa; H Daido; T Takao; S Murata; T Imanaka. *J. Bacteriol*., **1993**, 175, 6459–6466.
[14] K Paraszkiewicz; A Kanwal; J Dlugonski. *J. Biotechnol*., **2002**, 92, 287–29.
[15] JE Zajic; W Seffens. Biosurfactants. *C.R.C. Crit. Rev. Biotechnol*., **1984**, 1(2), 87–107.
[16] TN Bryant. *J. Appl. Microbiol*., **2004**, 97, 1326–1327.
[17] D Liu; S Coloe; R Baird; J Pedersen. *J. Clin. Microbiol*., **2000**, 38, 471.
[18] SF Altschul; TL Madden; AA Schäffer ; J Zhang; Z Zhang; W Miller; DJ Lipman. *NucL. A. Res*., **1997**, 17, 3389–402.
[19] OS Kim; YJ Cho; k Lee; SH Yoon; M Kim; SC Park; YS Jeon; JH Lee; H Yi; S Won; J Chun. *Int. J. Sys. Evo. Microbiol*., **2012**, 62(3), 716–721.
[20] K Tamura; G Stecher; D Peterson; A Filipski; S Kumar. *B. Mol. Biol. Evo*., **2013**, 30, 2725–2729.
[21] JD Thompson; DG Higgins; TJ Gibson. *Nucl. A. Res*., **1994**, 22, 4673–4680.
[22] TH Jukes, CR Cantor. Evolution of protein molecules. In: HN Munro (Ed), Mammalian protein metabolism, New York, Academic press, **1969**, 3, 21–132.
[23] J Felsenstein. *Evolution*., **1985**, 39, 783–791.
[24] EJL Lowbury; AG Collins. *J. Clin. Pathol*., **1955**, 8, 47.
[25] E Jawetz, JL Melnick, EA Adelberg.
J. Lab. Clin. Med., **1954**, 44, 301–307.
[27] JM Meyer; MA Abdallah. *J. Gen. Microbiol*., **1978**, 107, 319–328.
[28] AA Bodour; RM Miller-Maier. *J. Microbiol. Met*., **1998**, 32, 273–280.
[29] EV Candrasekaran, JN Bemiller. Constituent analyses of glycosamino-glycans, In: methods in carbohydrate chemistry, RL Whisler (Ed), New York, Academic Press Inc, **1980**, 89–96.
[30] LR *Col. Surf. B. Biointerf*., **2006**, 49, 79–86.
[31] LL Schramm; EN Stasiuk; DG Marangon. *Annu. Rep. Prog. Chem. Sect. C*., **2003**, 99, 3–48.
[32] G Bai; ML Brusseau; RM Miller. *J. Contam. Hydrol*., **1998**, 30, 265–279.
[33] SD Haigh. *Sc.Tot. Env*., **1996**, 185, 161–170.
[34] MG Healy; CM Devine; R Murphy. *Resour. Conserv. Recycl*., **1996**,18, 41–57.
[35] NJ Palleroni. Genus I. *Pseudomonas* Migula 1894. In: NR Kreig, JG Holt (Eds.), Bergeys Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, MA, **1984**, 141–199.
[36] CR Woese. *Microbiol. Rev*., **1987**, 51, 221–271.