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Original paper

Screening and molecular analyses for biosurfactant production by *Pseudomonas aeruginosa* strains from oil polluted soil

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Abstract

In the last years the biosurfactants drawn considerable attention especially for their convenient properties. The main goal of the present study was to analyze Pseudomonas strains with high ability to produce biosurfactants during growth on n-hexadecane and Fluka-oil, and to evaluate the cell surface hydrophobicity. Biosurfactant production was screened by determining hemolysis on blood agar, blue halo on CTAB agar and emulsification activity. For tested Pseudomonas strains the emulsification index E24 values ranged between 18-58% (n-hexadecane) and respectively 15-40% for Fluka-oil. In the same time, specific genes responsible for biosynthesis of rhamnolipids were detected for bacterial strains. The obtained results showed that *Pseudomonas aeruginosa* K7 strain presented the lowest hydrophobic properties of the cell surface also for n-hexadecane (19.28%) and Fluka-oil (12.05%). The highest hydrophobicity was obtained for *Pseudomonas aeruginosa* K2 strain: 42.11% for n-hexadecane and respectively 72.73% for Fluka-oil. However, these results demonstrate that tested bacterial strains are the valuable for the future biotechnological applications.

Keywords *Pseudomonas aeruginosa*, biosurfactant, emulsification index, *rhl* gene.

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Introduction

Most of the bacterial strains isolated from contaminated sites with petroleum hydrocarbons are capable to produce biosurfactants. Compared to the synthetic surfactants, biosurfactants have many advantages, like: enhance the solubilization of hydrocarbons, have *less toxic effect and high stability* at *different* conditions (salinity, *pH, temperature),* good biodegradability and they are considered ecofriendly for the environment. Due to these advantages, lately biosurfactants have been widely studied (H. AMANI & al [1]; D.G. NOUDEH & al [2]; R. THAVASI & al [3]; O. CSUTAK [4]). Previous scientific reports describe several methods for the characterization of biosurfactants and different classification criteria, most of them based on chemical composition (P. DAS & al [5]; P.K.S.M. RAHMAN & al [6]; M.M. ASSADI & al [7]).

Based on their chemical structure, biosurfactants produced by *bacterial strains* can be classified into six main categories: glycolipids, lipopeptides, phospholipids, fatty acids/natural lipids, polymeric surfactants and particulate biosurfactants.

One of the most studied classes of biosurfactants is represented by the glycolipids class, especially rhamnolipids, which are produced by *Pseudomonas (P.) spp.* strains. Rhamnolipids containing one or two 3-hydroxy fatty acids of various lengths, linked to a mono- or di- rhamnose moieties can reduce surface tension between water and oil. The *Pseudomonas* species ar capable of producing rhamnolipids when using glucose, glycerol, *n*-alkanes as the sole carbon source (M. ABOUSEOUD & al [8]).

The aim of our study was focused on the screening and the investigation of biosurfactants produced by three *P. aeruginosa* strains isolated from oil polluted soil, in order to select the strain with the highest biosurfactant production, for further biotechnological applications/ environmental remediation.

Materials and Methods

Microbial strains

In our study we used three bacterial strains named K1B, K2, K7 that were previously isolated from contaminated soil. The strains were taxonomically identified using phenotypic and genetic analyses (R. Ionescu & al [9]) as *P. aeruginosa*. All microorganisms were maintained at -70ºC in Luria-Bertani broth (LB – peptone 10g/l, yeast extract 5g/l, sodium chloride 10g/l, agar 20g/l, pH 7-7,5) suplemented with *20% glycerol. Microbial strains were included* in the Microbial Collection of Center for Research, Consulting and Training in Microbiology, Genetics and Biotehnology (MICROGEN), Department of Genetics, Faculty of Biology, University of Bucharest.

Hydrocarbon

In order to determine biosurfactants production *n*-hexadecane with the highest purity grade (produced by SIGMA-ALDRICH, SUA) and Fluka-oil – mixture hydrocarbons with aromatic content 18% (FLUKA Germany) were used as carbon sources.

1. Cellular hydrophobicity assay

Cellular hidrophobicity of the bacterial strains was tested by the Microbial Adhesion to Hidrocarbons (MATH) method, described by M. ROSENBERG & al [10] with few modifications. The cells were grown to mid-logarithmic phase in LB broth, centrifuged 10 min at 10000 rpm, washed twice and resuspended in phosphate urea magnesium sulfate buffer (PUM – $K_2HPO_4X3H_2O$ 22.2 g/L, KH_2PO_4 7.26 g/L, urea 1.8 g/L, $MgSO_4X7H_2O$ 0.2 g/L, pH 7) and the initial absorbance at 600 nm was determined using a spectrophotometer ULTROSPEC 3000 (Pharmacia LKB). A mixture of 5 ml cell suspension and 500 μL hexadecane/petroleum was vortexed at high speed for 2 min. After 15 min 1 ml aqueous phase was used to determine optical density at 600nm. Each experiment was made in triplicate. Hydrophobicity was calculated using the formula: 100 x (1-OD_{600nm} of the aqueous phase/OD_{600nm} of the initial cell suspension).

2. Screening for biosurfactant production

An overnight culture of each of the three bacterial strains on LB broth was centrifuged and the cell pellet was washed and resuspended in Bushnell-Haas broth (BH - $MgSO_4$ 0.2g/L, $CaCl_2$ 0.02 g/L, KH_2PO_4 1.0g/L, K_2HPO_4 1.0 g/L, NH_4NO_3 1.0 g/l, $FeCl_3$ 0.05 g/L, pH 7.0). The 1% bacteria inoculum with $OD_{600\ nm}$ adjusted at 1.0 was added to ermetic flasks containg 15 mL liquid BH broth supplemented with *n*-hexadecane and, respectively, FLUKA-oil as sole carbon sources in final concentration 1%. No vitamins or oligoelements were added. After incubation at 28°C for 21 days in an orbital shaker at 150 rpm, cultures were centrifuged for 10 min at 10.000 rpm. The cell free supernatant was used for the hemolytic activity assay, CTAB (cetyltrimethylammonium bromide) Agar Plate assay and determination of the emulsification index (E24). All experiments were done in triplicate.

Hemolytic activity

Ten microliters of supernatant were spread on blood defibrillated calf agar Petri plates. Hemolytic activity was represented by the formation of a clear zone around the colonies after incubation for 48-72 h at 28°C.

CTAB agar plate method

Bacterial strains were cultivated on solid broth containing: KH_2PO_4 0.7 g/L, Na_2HPO_4 0.9 g/L, $NaNO_3$ 2.0 g/l, $MgSO_4$ 0.4 g/L, $CaCl_2$ 0.1g/L, glycerol 20 mL, CTAB 0.2 g/L, methylene blue 0.005g/L and agar 20 g/L, was used to detect glycolipid production according with the method proposed by I. SIEGMUND & al [11]. After 48-72 h incubation at 28°C was monitored appearance of blue halo around the colonies, showing a positive reaction for the rhamnolipids production. The results were noted "–" or "+", corresponding to negative and complete production of glycolipids.

Emulsification index (E24) was estimated using 1 mL of bacterial culture over which was added 1 ml of *n*-hexadecane/oil. The samples thus prepared were vortex vigorously for 2 minutes. After 24 h incubation at room temperature E24 was calculated using the following formula:

E24 = (height of emulsified layer divided by total height of the liquid column) x 100

3. Rhamnolipid quantification

In order to determined rhamnolipids concentration phenol-sulphuric method, described by DuBois (1956) was used. Development of orange color indicated the presence of rhamnolipids. We used 96-well plates in which 25 µL cell supernatant was added to 25 µL phenol solution 5% and 125 µL concentrated sulfuric acid. Plates were incubated for 15 minutes at room temperature and absorbance at 490 nm was determined using spectrophotometer Apollo1LB911 (Berthold Technologies). Standard curve was obtained using different concentration of rhamnose.

4. Detection of rhamnosyl transferase gene by PCR

Rhamnosyl transferase gene encoding for the enzymes responsible for the final steps of rhamnolipid synthesis (A.M. ABDEL-MAWGOUD & al [12]), was detected using the primers presented in Table 1. The amplification reaction was performed in a total volume of 25 µL consisting from: PCR master mix (2X-Promega), 0,5 µM of each forward and reverse primer and 50 ng DNA. The total DNA samples were obtained with the CTAB method, adapted from T. VASSU, & al [13]).

For the PCR reaction the following program was used: 2 min at 95°C, 30 cycles (15 s at 94°C, 15 s at 54°C, 15 s at 72°C), and final extension at 72°C for 2 min. The amplicons were separated by electrophoresis using 2% agarose gel in 1X TBE buffer (Tris base 54 g/L, boric acid 27.5 g/L, EDTA 3.7 g/L).

Table 1. Primers for rhamnosyl transferase gene detection (from A. BODOUR & al [14]; J.W. NEILSON & al [15]; A. SCHMIDBERGER & al [16])

Notation	Sequence 5'→3'	Expected length of the amplified fragment (bp)
*rhl*A **F**	GATCGAGCTGGACGACAAGTC	95
*rhl*A **R**	GCTGATGGTTGCTGGCTTTC	
*rhl*B **F**	GCCCACGACCAGTTCGAC	226
*rhl*B **R**	CATCCCCCTCCCTATGAC	
*rhl*C **F**	ATCCATCTCGACGGACTGAC	159
*rhl*C **R**	GTCCACGTGGTCGATGAAC	

Results and Conclusions

MATH assay are a simple method based on the properties of bacterial cells to adhere at various hydrocarbons. According to studies made by A. MELIANI & al [17]), for bacteria with hydrophilic properties values are less 30%, values between 30-40% characterize strains with moderate hydrophobic character and values above 40% are typical for hydrophobic bacteria.

Our results indicated that strains *P. aeruginosa* K1B and *P. aeruginosa* K7 presented hydrophilic properties for *n*-hexadecane and FLUKA-oil, while *P. aeruginosa* K2 strain presented hydrophobic properties (Table 2). In the present study values obtained for *P. aeruginosa* PAO1 strain used as control were similar to those obtained by P.C.Y. LAU & al [18]), which reported 33.5% ± 9.1 affinity to *n*-hexadecane.

Table 2. Values of MATH test

Bacterial strains	*n*-hexadecane	FLUKA-oil
P. aeruginosa PAO1	29.25	28.75
P. aeruginosa K1B	28.36	28.95
P. aeruginosa K2	42.11	72.73
P.aeruginosa K7	19.28	12.05

Screenig for biosurfacant activity

All *P. aeruginosa* strains were tested for biosurfactant production. The first screening test was **blood agar plates** based on the capacity biosurfactants produced by bacteria to lyse erythrocytes. Because appearance of the clear zone may be due the other hemolytic factors, this method is considered not accurate and is therefore required to use simultaneous other screening methods like CTAB agar plate method (V. WALTER & al [19]; A.M. ABDEL & al [20].

CTAB agar plate method is based on the fact that *anionic rhamnolipid* could react with cationic bromide salt and to *form* an insoluble *complex* revealed by **methylene *blue* staining** (S.B. Hamed & al [21]). For all tested bacterial strains blue halo around the colonies was present. Emulsification index assay revealed that all bacteria strains presented the **emulsification capability** for *n*-hexadecane and FLUKA-oil. The highest value of emulsification index were obtained for *n*-hexadecan compared with FLUKA-oil (Fig. 1).

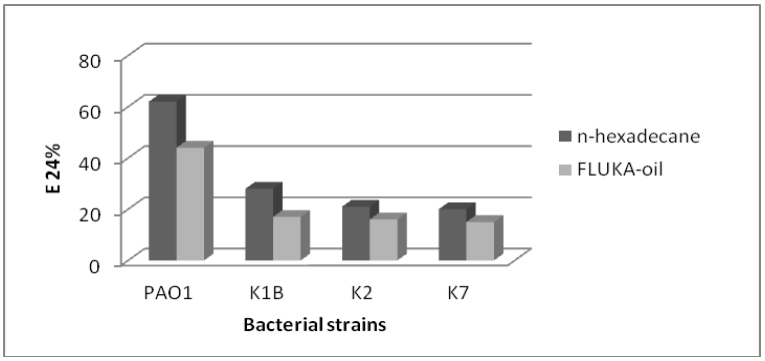

Figure 1. Emulsifying activity

The rhamnolipid production was confirmed by investigating the presence of rhamnose fraction using **DuBois method.** The highest rhamnose concentration was obtained for *P. aeruginosa* K1B, respectively more than 2.2 µg/mL for both *n*-hexadecane and Fluka-oil.

The results obtained using the mentioned methods showed positive results for all tested *Pseudomonas* strains.

Moreover molecular experiments revealed the presence of the specific genes responsible for the biosynthesis of mono- (*rhlA*, *rhlB*), and dirhamnolipids (*rhlC*). Because all rhamnosyl transferase genes were highlighted for bacterial strains, we might conclude that the newly isolated strains could be used in biotechnological applications to produce biosurfactants, especifically rhamnolipids (Fig. 2).

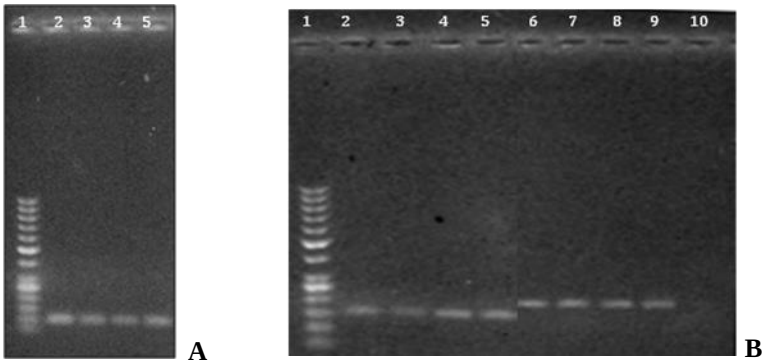

Figure 2. PCR amplification of the rhamnosyl transferase genes. A- *rhlA; B- rhlC* and *rhlB*
(**A**) Lanes: 1- molecular markers 50pb DNA Step Ladder (Promega) 2- *P. aeruginosa* PAO1, 3- *P. aeruginosa* K1B, 4- *P. aeruginosa* K2, 5- *P. aeruginosa* K7; (**B**) Lanes: 1- molecular markers 50 pb DNA Step Ladder (Promega), 2- *P. aeruginosa* PAO1, 3- *P. aeruginosa* K1B, 4- *P. aeruginosa* K2, 5- *P. aeruginosa* K7, 6- *P. aeruginosa* PAO1, 7- *P. aeruginosa* K1B, 8- *P. aeruginosa* K2, 9- *P. aeruginosa* K7, 10- negative control.

In conclusion, our results showed that screening methods are efficient for the detection of biosurfactant production in *P. aeruginosa* strains. Molecular analyses confirmed the presence of the specific genes involved in rhamnolipid synthesis. Results obtained for biosurfactant production in presence of different pollutants such *n*-hexadecane and Fluka-oil indicated the potential of strains to be used in environmental biotechnology. Future studies will focus on biosurfactant purification and on the expression of *rhl* genes in different growth condition.

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