Identification and characterization of biosurfactants produced by the Arctic bacterium *Pseudomonas putida* BD2

Tomasz Janek\textsuperscript{a,*}, Marcin Łukaszewicz\textsuperscript{a,b}, Anna Krasowska\textsuperscript{a}

\textsuperscript{a) Department of Biotransformation, Faculty of Biotechnology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland  
\textsuperscript{b) Faculty of Chemistry, Wroclaw University of Technology, Gdańska 7/9, 50-344 Wroclaw, Poland}

Abstract

One hundred and thirty bacterial strains, isolated from Arctic soil on the Svalbard Archipelago, were screened for biosurfactant production. Among them, an isolate identified as *Pseudomonas putida* BD2 was selected as a potential biosurfactant-producer based on the surface/interfacial activity of the culture supernatant. The ability of the strain to produce simultaneously phosphatidylethanolamines and rhamnolipid, using glucose as a sole carbon source, was demonstrated. The rhamnolipid Rha-Rha-C10-C10 and two homologs of phosphatidylethanolamine were extracted from cell-free supernatant of *P. putida* BD2 culture with ethyl acetate and identified by UPLC-MS analysis. For Rha-Rha-C10-C10 the surface tension decreased from 72 to 31 mN/m and the critical micelle concentration was 0.130 mg/mL. The Rha-Rha-C10-C10 was able to form stable aggregates (80-121 nm). Pretreatment of a polystyrene surface with 0.5 mg/mL rhamnolipid inhibited bacterial adhesion by 43-79% and that of the pathogenic fungal species *C. albicans* by 89-90%. The same concentration of phosphatidylethanolamines inhibited bacterial adhesion by 23-72% and that of *C. albicans* by 96%- 98%. To our knowledge, this is the first report where one type rhamnolipid and two homologs of phospholipid biosurfactants were produced by *Pseudomonas putida* isolated from Arctic soil.

**Keywords**: *Pseudomonas putida* BD2; glycolipid; phosphatidylethanolamine; biosurfactant

* Corresponding author. Tel.: +48 713756210; fax: +48 713756234  
E-mail address: tomasz.janek@ibmb.uni.wroc.pl (Tomasz Janek).
1. Introduction

Biosurfactants are a structurally diverse group of surface-active compounds synthesized by microorganisms [1, 2]. The structure of these secondary metabolites is strictly dependent on the medium composition, e.g. carbon and nitrogen sources. Microbial surfactants encompass a wide spectrum of molecules, such as lipopeptides, glycolipids, phospholipids, fatty acids, neutral lipids and polymeric biosurfactants [3]. Microorganisms such as bacteria, filamentous fungi and yeast produce biosurfactants extracellularly or as compounds associated with cell membrane [4].

These natural compounds have many advantages over synthetic surfactants, such as non-toxic character or high biodegradability, and have extensive applications in many industrial fields such as production of food, cosmetics, pesticides, detergents, pharmaceuticals, oil recovery and bioremediation [5, 6]. Besides their potential application in industrial and environmental remediation, these surface-active compounds have been reported to possess several properties of therapeutic and biomedical importance. The features of biosurfactants allow for, e.g. antimicrobial and anti-adhesive action against several pathogenic microorganisms on medical implants [7, 8] restoration and maintenance of bacterial homeostasis in human body [9] or antibiotic activity against bacteria and fungi [10].

Glycolipids are one of the most promising biosurfactants. They consist of carbohydrates and long-chain aliphatic or hydroxyaliphatic acids. Rhamnolipids (RLs) produced by Pseudomonas strains have a glycosyl head group (a rhamnose moiety) and a 3-(hydroxyalkanoyloxy)alkanoic acid (HAA) [11]. The two most abundant RLs congeners are mono-rhamnolipids and di-rhamnolipids often referred to as Rha-C10-C10 and Rha-Rha-C10-C10, respectively [12]. Factors influencing RLs production in the genus Pseudomonas were reviewed by Muller et al. [13]. Pseudomonas was shown to be capable of using both water soluble carbon substrates, such as glycerol, mannitol and fructose [14] as well as water immiscible n-alkanes to produce rhamnolipid-type biosurfactants [15]. Not only the type of carbon and nitrogen source but also the respective C/N ratio strongly influence total RL production [16, 17].

Desai and Banat [1] have reviewed a wide range of techniques to determine the presence of biosurfactants in culture media. The chemical and physico-chemical methods used for this purpose include determination of surface tension (ST), emulsification index (E24) determined over a 24-h period [18], drop-collapsing test [19], thin layer chromatography...
(TLC), high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR) and mass spectrometry (MS).

The aims of this work included identification of a new Arctic strain, purification of biosurfactants secreted to minimal medium with glucose as a sole carbon source, characterization of chemical structures of isolated biosurfactants and their physico-chemical properties (minimum surface tension, critical micelle concentration, emulsification activity) and the anti-adhesive activities against several microorganisms. Furthermore, the size of micelles of Rha-Rha-C10-C10 was also studied.

2. Experimental

2.1. Microorganism and growth culture

*Pseudomonas putida* BD2 was isolated from a soil sample from the Arctic Archipelago of Svalbard (latitude 77° 04' N, longitude 15° 14' E). After being grown on LB medium containing 5 g/L yeast extract, 10 g/L bacto-tryptone and 10 g/L NaCl, the bacterial strain was maintained at −70 °C in 15% glycerol. Experiments were carried out in 1000 mL baffled Erlenmeyer flasks containing 500 mL of medium with the following composition: 7 g/L K$_2$HPO$_4$, 2 g/L KH$_2$PO$_4$, 1 g/L (NH$_4$)$_2$SO$_4$, 0.5 g/L sodium citrate x 2H$_2$O, and 0.1 g/L MgSO$_4$ x 7H$_2$O (pH 7.0) [20]. Finally, 20 g/L of glucose was used as the carbon source. Medium components were sterilized separately at 120 °C, 1 atm for 20 min. The flasks were inoculated with 5 ml overnight pre-culture of the strain and incubated at 28 °C on a reciprocal rotary shaker at 170 rpm.

2.2. Identification of bacterial strain

Strain BD2 was identified by the API 20E test for Enterobacteriaceae (BioMerieux, Marcy l’Etoile, France) and genomic DNA was obtained by the method of Janek et al. [20]. Genomic DNA was isolated from a one-day liquid culture using a GeneMATRIX Bacterial and Yeast Genomic DNA Purification kit 50 (EURx, Gdansk, Poland) following the manufacturer’s standard protocol, and used as a target for polymerase chain reaction (PCR) amplification using primers 27F (5’-AGR GTT YGA TYM TGG CTC AG-3’) and 1492R (5’-GGT TAC CTT GTT ACG ACT T-3’). The PCR products were subjected to agarose gel electrophoresis, purified using a GeneMATRIX PCR/DNA Clean-up Purification kits (EURx, Gdansk, Poland), and their nucleotide sequence was determined at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland) using the
standard shotgun sequencing reagents and a 454 GS FLX Titanium Sequencing System (Roche, Basel, Switzerland), according to the manufacturer's instructions. The rDNA gene sequence obtained from the BD2 isolate was compared with other bacterial sequences by using GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Biosurfactant production and purification

Microbial growth was evaluated by measuring the absorbance of the culture at 600 nm. Glucose released was measured enzymatically with the Glucose kit from Biosystems (Barcelona, Spain). Emulsification assays of the biosurfactants were performed using a previously described method [18]. The emulsification activity of the supernatant was measured by adding 6 mL petroleum ether to 4 ml of aqueous sample in a test tube, vortexing for 2 min, and then leaving it to settle for 24 h. The emulsification index (E24) was estimated as the height of the emulsion layer, divided by the total height, multiplied by 100. The surface tension of the free culture supernatant was measured with a tensiometer using the du Nouy procedure with a platinum ring at 25 °C.

Cell-free supernatant was obtained from the culture by centrifugation (8000 g) at 4 °C for 30 min. It was extracted three times with ethyl acetate and evaporated under vacuum. The crude biosurfactants were dissolved in 5 mL of methanol, filtered through a 0.2 µm syringe filter and 40 mg crude biosurfactants were separated by preparative layer chromatography (PLC) (silica gel 60 F254 20 cm x 20 cm with concentrating zone, 1 mm x 20 cm x 4 cm, Merck; chloroform/methanol/water 65:15:2). Visualization was carried out by UV transilluminator. In preparative mode, visualized spots were scraped off and the biosurfactants were extracted with 15 mL of chloroform/methanol (2:1). The surface-active fractions were evaporated under vacuum and stored at -20 °C for further studies.

2.4. Thin-layer chromatography

The purified biosurfactant fractions obtained after preparative TLC purification were separated by thin layer chromatography (Silica gel 60; Merck, Darmstadt, Germany). The compounds were separated using a mobile phase of chloroform/methanol/water in a 65:15:2 ratio (vol/vol/vol). The resulting spots on the TLC were visualized by spraying with a solution of 0.25% ninhydrin in acetone [20] for amine groups and orcinol solutions [8] for sugar detection. For the detection of lipids, the plates were treated with 0.1% bromothymol blue in 10% aqueous ethanol [21] and iodine.
2.5. Liquid chromatography

Aliquots of the biosurfactant extracts or of the chromatographed samples were dissolved in methanol to obtain 1 mg/mL solutions.

Chromatographic equipment consisted of an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). Separation was performed on a BEH C18 column (1.7 µm, 2.1 x 100 mm, Waters) held at 40 °C. A multistep linear gradient composed of eluent A (water + 0.1% trifluoroacetic acid) and eluent B (acetonitrile + 0.1% trifluoroacetic acid) was applied. The autosampler temperature was maintained at 10 °C and 10 µL of sample solution was injected. From 0.00 min to 13.00 min a linear gradient was applied from the mixture A:B (70:30, vol/vol) to A:B (0:100, vol/vol). A plateau of 100% eluent B from 13.00 min to 15.00 min was set before going back to 70% eluent A from 15.00 min to 16.00 min. Flow rate was 0.3 mL/min.

2.5. Mass spectrometry analysis

The LC system was coupled to a Waters Xevo QTOF MS mass spectrometer with an atmospheric pressure electrospray interface. The ESI source was set in positive and negative ionization mode. The parameters used for the mass spectrometer under the ESI mode were as follows: ESI+ (capillary voltage 4.50 kV, source temperature 80 °C, desolvation temperature 250 °C, desolvation gas flow 650 L/h, cone gas flow 18 L/h) and ESI- (capillary voltage 3.50 kV, source temperature 120 °C, desolvation temperature 250 °C, desolvation gas flow 700 L/h, cone gas flow 20 L/h). Different cone voltages were tested to study the fragmentation of biosurfactants. LC/MS full scan positive and negative modes were performed from m/z 200 to 800; alternatively, LC/ESI-MS/MS modalities were applied to the selected precursor ions, following the conditions set during the infusion analysis. Waters MassLynx version NT 4.1 was used for LC/MS system control and data analysis.

2.6. Physico-chemical characterization

The surface tension was measured at 25 °C with a Kruss K100 (Kruss GmbH, Hamburg, Germany), tensiometer by the du Nouy’s ring method. The instrument was calibrated against Mili-Q ultrapure distilled water. Aqueous solutions of purified rhamnolipids in the concentration range of 180–5 mg/L were obtained by successive dilutions of a concentrated sample prepared by weight in ultrapure water.
The mean particle size and polydispersity index (PDI) of rhamnolipids diluted in Milli-Q water were determined using a Zetasizer Nano-ZS (Malvern Instruments Ltd., Malvern, UK) and PCS software. The analysis of particle size and PDI, determined by photon correlation spectroscopy, was done using the volume distribution algorithm. The polydispersity index qualifies the particle size distribution, which here ranged from 0 for monodisperse to 1.0 for entirely heterodisperse emulsions. In order to obtain the optimum light scattering intensity and for the size analysis, approximately 100 μL of the rhamnolipid was added to 900 μL of Milli-Q water. All the measurements were carried out at 25 ºC.

Emulsification activity of phospholipids and rhamnolipids was tested on different organic compounds: petroleum ether, benzene, n-hexane, cyclohexane, n-hexadecane, xylene, toluene, sunflower, olive and rapeseed oil. The purified biosurfactants dissolved in 5.0 mL distilled water (0.2% w/v) were mixed with 5 mL of each hydrophobic compound, and then vortexed at high speed for 2 min, after which the mixture was kept at 25 ºC for 24 h.

2.7. Biological assays

The anti-adhesive properties of phospholipids and rhamnolipids were tested on several pathogenic strains that colonize medical devices or human body. A wide range of Gram-positive and Gram-negative bacteria were tested: Escherichia coli ATCC 25922, Escherichia coli ATCC 10536, Escherichia coli 17-2 (clinical isolate, Wroclaw Medical University), Enterococcus faecalis ATCC 29212, Enterococcus faecalis JA/3 (clinical isolate, Wroclaw Medical University), Enterococcus hirae ATCC 10541, Staphylococcus epidermidis KCTC 1917 and Proteus mirabilis ATCC 21100. Yeast strains: Candida albicans ATCC 20231, Candida albicans SC5314.

Inhibition of microbial adhesion by the biosurfactants was tested in 96-well plates (Sarstedt, Nümbrecht, Germany) by the method of Janek et al. [7]. The wells of a sterile 96-well flat-bottom plate were filled with biosurfactants dissolved in PBS. Next, the plates were incubated for 2 h at 37 ºC on a rotary shaker (MixMate, Eppendorf, Hamburg, Germany) at 300 rpm and subsequently washed twice with PBS. The overnight cultures of microbial strains were centrifuged, washed twice with PBS (pH 7.4) and re-suspended in PBS. The microbial suspension was added to each well of the microtiter plate. After a 2-h incubation at 37 ºC in a rotary shaker at 300 rpm, nonadherent cells were removed by three washes with PBS. Then the plates were stained with 0.1% crystal violet for 5 min and again washed three times with PBS. The adherent microorganisms were permeabilized and the dye was resolubilized with 150 μL of isopropanol-0.04 N HCl and 50 μL of 0.25% SDS per well.
Crystal violet optical density readings of each well were taken at 590 nm on the Asys UVM 340 (Biogenet) microplate. All the experiments were carried out in triplicate with suitable controls (uninoculated microtiter plates).

3. Results and discussion

3.1. Characterization of Pseudomonas putida BD2 and purification of secreted biosurfactants

The strain BD2 isolated from Arctic soil was Gram-negative, aerobic, oxidase-positive and these results classified it as belonging to the *Pseudomonas* genus. The optimal temperature for growth of strain BD2 in MSM with 2% glucose was 28 °C. Comparison of 16S rRNA nucleotide sequence from strain BD2 with sequences in the GenBank database revealed 100% identity with the corresponding sequences from *Pseudomonas putida* [22]. When *P. putida* BD2 was grown at 28 °C in submerged culture in a sterile mineral salts medium with 2% (w/v) glucose, 0.15 g/L of the crude biosurfactant was produced after 7 days of culture (Fig. 1). The maximum decrease in the surface tension of the culture medium (45.8 mN/m) occurred after 48 h. The emulsification index (E24) of the culture filtrate with petroleum ether increased from 2% to 62.9% and stabilized after 144 h. The extracted biosurfactants were then subjected to PLC. A series of bands were observed after PLC (Fig. 2). These fractions were divided into 4 groups and scraped off from the preparative plates, and then eluted by a chloroform–methanol mixture (2:1) and evaporated. These 4 samples, designated A to D, were re-dissolved in 200 μL of methanol and stored for future studies. Further assays were carried out with these 4 samples in order to determine their surface activity by the drop-collapsing assay. Due to their strong surface tension reducing activity, samples A (5 mg) and B (20.5 mg) from 40 mg crude extract were selected and subjected to further analytical UPLC. Four more cycles of the biosurfactant separation and extraction experiments had to be repeated in order to obtain sufficient quantity of samples for the analytical UPLC, physico-chemical and biological assays.

Until recently, *Pseudomonas aeruginosa* was considered as potentially the main producer of rhamnolipids [23, 24]. However, *P. aeruginosa* species is pathogenic. Thus, our research was focused on characterizing other RLs producer species. This paper describes a new strain of *P. putida* BD2 which is generally considered non-pathogenic [25]. Besides production of the rhamnolipid, another important tenet is the finding that *P. putida* BD2 also produces phospholipids.
Strain BD2 grew in culture media containing different types of carbon source (data not shown) but maximum cell growth and biosurfactant production occurred with glucose used as a sole carbon source. While this finding was in agreement with that of Rendell et al. [26], some other strains have shown the highest biosurfactant production on hydrophobic substrates such as soybean oil [27, 28]. To our knowledge, non-pathogenic \textit{P. putida} strains produce less rhamnolipids than the pathogenic \textit{P. aeruginosa}. The production of rhamnolipids was observed when a strain of \textit{P. putida} was grown on soluble substrates such as glucose, molasses or on poorly soluble substrates such as hexadecane, reaching values from 0.52 to 1.2 g/L; however the exact structures of the produced rhamnolipids were not determined [29, 30].

3.2. UPLC-MS analysis

The molecular mass of the purified active compounds, i.e. fractions A and B, was measured using UPLC/ESI-MS/MS. Previous studies revealed that the active ingredient of biosurfactants produced by \textit{Pseudomonas} was rhamnolipids [31, 32]. UPLC/MS analysis revealed the presence of one major component with retention time 5.23 min. The mass spectrum and chemical structure of the component is illustrated in Fig. 3A. One [M-H]⁺ pseudomolecular ion with m/z 649 was observed. ESI-MS showed a molecular ion of Rha-Rha-C10-C10, which gave rise to fragments with m/z 479 and 310. The possible di-rhamnolipid structure and fragmentation is also shown in Fig. 3A.

In the generally accepted pathway for the rhamnolipid synthesis, Rha-C10-C10 is the precursor for Rha-Rha-C10-C10 [33]. This would suggest that for each di-rhamnolipid detected, the mono-rhamnolipid congener should also be detected, although this is not always the case [34]. In our work the di-rhamnolipid did not have a mono-rhamnolipid congener. Rhamnolipids containing only one hydroxyaliphatic acid were also found by other authors [35, 36]. In our study we did not find glycolipids with one \(\alpha\)-hydroxy fatty acid connected, which might be due to differences either in the culture conditions or in the strain used.

ESI-MS spectra of \textit{P. putida} BD2 phospholipids showed that the dominant species are protonated phosphatidylethanolamines (PE(32:1)) (PE(33:1)) at m/z 690.5 and 704.5, respectively. Dimers of phospholipids were observed in the range of m/z 1300–1500 and verified by tandem mass spectrometry (MS/MS). Tandem mass spectra display the fragmentation pattern of [PE(16:1/16:0) + H]⁺ and [PE(16:1/17:0) + H]⁺, which mainly lose neutral phosphoethanolamine (141 mass units) to yield the fragment ion at m/z 549.5 and 563.5 respectively. The peak of m/z 549.5 was dissociated to form two major fragment ions to
form $[C_{16}H_{31}O]^+$ of m/z 239.2, and $[C_{16}H_{29}O]^+$ of m/z 237.2 (Fig. 3B). The m/z 563.5 was used as precursor ions for further ESI-MS/MS analyses (Fig. 3C).

3.3. TLC analysis

Thin-layer chromatography showed that the crude extract was a mixture of compounds that could be classified as glycolipid and phospholipid. Visualization was carried out with orcinol and ninhydrin followed by heating. The lower spot ($R_f = 0.26$) is from the di-rhamnolipid, while the higher spot ($R_f = 0.47$) comes from phosphatidylethanolamines (data not shown). Our results are similar to previously reported results of TLC of rhamnolipids from different strains of *Pseudomonas* [23]. The $R_f$ values obtained by these authors are 0.27 and 0.57 for di- and mono-rhamnolipid, respectively.

3.4. Physico-chemical properties of biosurfactants

Biosurfactants tend to form aggregates with their hydrophilic head groups in contact with water and their hydrophobic tails directed toward the interior of the micelles and away from water. These aggregates are generally called micelles. Depending on their shape and structure, micelles can be generally divided into four classes: spheres, rods, lamellar structures, and vesicles. The aggregation behavior of Rha-Rha-C10-C10 was characterized by measuring the change in the size of aggregates with changing compound concentration. Fig. 4 shows the aggregation behavior of Rha-Rha-C10-C10. The size of the aggregates increased with concentration in the range of 0.15–0.5 mg/mL and reached 80-121 nm. In previous studies, we have demonstrated that aggregation above the CMC was also observed for pseudofactin II, a surface active lipopeptide produced by *Pseudomonas fluorescens* [37]. The mean hydrodynamic diameter of pseudofactin-biosurfactant micelles in water ranged from 40.2 nm to 60.3 nm. Xu et al. [38] demonstrated that the type and concentration of surfactant significantly influence the properties of aggregates. The aggregation behavior of the surface active compounds affects the size distribution and stability of aggregates. There is still little information available about aggregation and polydispersity index (PDI) of various rhamnolipids. In this work, we show the size of aggregates for one type of rhamnolipid (Rha-Rha-C10-C10), while other reports focused on mixtures of rhamnolipid homologs [39, 40]. The new data about each described biosurfactant will contribute to understanding the aggregation process and compounds the final activity of the product.
The emulsifying properties of 0.2% aqueous solution of biosurfactants produced by BD2 strain were examined with different vegetable oils and hydrocarbons. The purified phospholipids and the rhamnolipid emulsified vegetable oils more efficiently than hydrocarbons (Fig. 5). Biosurfactants could emulsify 70% of olive oil, while emulsification of n-hexane, xylene, hexadecane, and petroleum ether ranged from 51 to 65%.

The rhamnolipid (Rha-Rha C10 C10) reduced surface tension of the water from 72 to 31 mN/m. Critical micelle concentration (CMC) determined with the aid of a series of concentrations was around 0.130 mg/mL (Fig. 6). The surface tension properties of rhamnolipids are dependent on bacterial strain, medium composition, and culture conditions that determine the composition and distribution of homolog molecules present in the final product [41]. CMC values in a wide range from 5 to 386 mg/L and surface tension from 25 to 31 mN/m have been reported for different rhamnolipid mixtures [42]. Abalos et al. [43] reported CMC values of 106, 150 and 234 mg/L for different mixtures of mono- and di-rhamnolipids. More hydrophilic rhamnolipids like Rha-C10 or Rha-Rha-C10 yielded CMC as high as 200 mg/L whereas lower values of 5-60 mg/L have been reported for mixtures containing mainly mono-rhamnolipid Rha-C10-C10 [44].

3.5. Biological activity of biosurfactants

The microtiter-plate based anti-adhesion assay estimated the rhamnolipid and the phospholipids concentrations that were effective in inhibiting adhesion of the pathogenic microorganisms. The highest percentage of microbial adhesion inhibition was obtained against *C. albicans* SC5314, 90% for the rhamnolipid (Table 1) and 98% for the phospholipids (Table 2) at 0.5 mg/mL concentration of the biosurfactants. The order of anti-adhesive action against microorganisms studied was *C. albicans>*E. faecalis>*P. mirabilis>*E. coli>*S. epidermidis>*E. hirae*.

Attempts to reduce or inhibit microbial adherence is a viable means to control infection, since such adherence is one of the initial stages of the infectious process. Thus, pre-coating of solid surfaces by biosurfactants might constitute a new and effective means of combating colonization by pathogens and an effective strategy to reduce microbial adhesion [10]. Thanks to their amphiphilic structure, surface active compounds reduce the surface tension at interfaces, and thus affect the adhesion and detachment of microorganisms [10]. Gudina et al. [45] characterized the anti-adhesive activity of biosurfactants against several microorganisms including Gram-positive and Gram-negative bacteria. Several investigations have pointed that biosurfactants produced by probiotic microorganisms inhibited adhesion of

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bacteria or fungi to artificial surfaces [46-48]. For example, biosurfactants from *Streptococcus thermophilus* and *Lactobacillus acidophilus* suppressed *C. albicans* adhesion to silicone rubber [8, 49].

In conclusion, we suggested that the phospholipids and rhamnolipid produced by nonpathogenic *P. putida* BD2 could be used as alternative anti-adhesive agents against pathogenic microorganisms responsible for diseases and infections in the urinary, vaginal and gastrointestinal tracts. This points to their potential utility as coating agents for medical insertional materials.

4. Conclusions

Our data allow several conclusions:
- The new bacterial isolate BD2 was identified as *Pseudomonas putida*.
- Two distinct fractions of the crude biosurfactants correspond to a mixture of phosphatidylethanolamines PE(32:1), PE(33:1) and di-rhamnolipid (Rha-Rha–C10–C10).
- *P. putida* species has not been previously described as rhamnolipid and phospholipid producer.
- The physico-chemical properties such as CMC (0.130 mg/mL), surface tension (31 mN/m), emulsification (51- 70%), and size of aggregates above CMC 80-121 nm were evaluate.
- Described biosurfactants have strong anti-adhesive activities against bacterial and yeast strains on a polystyrene surface.

Acknowledgments

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References


Pseudomonas aeruginosa strain 57RP grown on mannitol or naphthalene, Biochim Biophys Acta, 1440 (1999) 244-252.


[47] M.M. Velraeds, H.C. van der Mei, G. Reid and H.J. Busscher, Inhibition of initial adhesion of uropathogenic Enterococcus faecalis to solid substrata by an adsorbed biosurfactant layer from Lactobacillus acidophilus, Urology, 49 (1997) 790-794.


Tables

Table 1. Inhibition of microbial adhesion in the microtiter plate by purified di-rhamnolipid. PBS was used as control and set at 0% as no inhibition occurs. Values ± confidence interval, n=9, α=0.05.

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<th>Microorganism</th>
<th>Rhamnolipid concentration (mg/ml)</th>
<th>Control (PBS)</th>
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Table 2. Inhibition of microbial adhesion in the microtiter plate by purified phosphatidylethanolamines. PBS was used as control and set at 0% as no inhibition occurs. Values ± confidence interval, n=9, α=0.05.

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<th>Microorganism</th>
<th>Inhibition of microbial adhesion (%)</th>
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<td>Phosphatidylethanolamines concentration (mg/ml)</td>
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Figures

Fig. 1. Time course of biosurfactant production, cell growth, surface tension and emulsification activity of *P. fluorescens* BD5 grown on mineral salt medium with 2% glucose as carbon source at 28 °C.

Fig. 2. Preparative layer chromatography (PLC) of compounds obtained from *P. putida* BD2 by using a mobile phase of chloroform/methanol/water in the 65:15:2 ratio (vol/vol/vol). Fractions were visualized by UV transilluminator.

Fig. 3. UPLC-ESI–MS spectrum, negative and positive ion mode of rhamnolipid and phospholipids respectively. (a) Negative mass spectrometry showing the predominance (retention time of 5.23 min) of m/z 649 (Rha-Rha-C10-C10). Product ion spectra of the protonated molecules [M + H]+ of (b) PE(32:1) at retention time 10.13 min and (c) PE(33:1) at retention time 10.64 min.

Fig. 4. The effect of di-rhamnolipid concentration on aggregate size.

Fig. 5. Emulsifying index (E24) of biosurfactants with different hydrocarbons, mean ± SD (n=3).

Fig. 6. Effect of Rha-Rha-C10-C10 concentration on surface tension. The CMC was determined from the intersection of regression lines that describe the two parts of the curve, below and above CMC. Results represent the average of three independent measurements.
Graphical abstract

**Highlights**

1. *Pseudomonas putida* BD2 was isolated and identified from an Arctic soil.
2. The strain was found to release to growth medium biosurfactants.
3. Biosurfactants were purified and indentified using various physico-chemical methods.
4. Structures were identified using UPLC-MS method.
5. One glycolipid and two phospholipids were identified.
Graphical Abstract (for review)

Biosurfactants production by *Pseudomonas putida*

Biosurfactants separation

Physico-chemical properties

Biological activity

Growth (OD=600nm), Glucose (g/L)

Surface Tension (mN/m), Θ24 (%)
Fig. 1.
Fig. 2.
Fig. 3.

Fig. 4.
Fig. 5.
Fig. 6.

The graph shows the relationship between surface tension (mN/m) and rhamnolipid (mg/mL). The graph indicates a high correlation with an $r^2$ value of 0.9882. The CMC (Critical Micelle Concentration) is at 0.130 mg/mL. The linearity of the data is confirmed with an $r^2 = 1$.