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Isolation of rhamnolipids-producing cultures from faeces: Influence of interspecies communication on the yield of rhamnolipid congeners

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ABSTRACT

The aim of this study was to evaluate the ability of bacterial cultures isolated from cattle, poultry or pig faeces and manure to produce rhamnolipids, as well as to investigate the influence of interspecies communication on possible quantitative differences in the production of rhamnolipid congeners. Initial screening methods (oil spreading, drop collapse, haemolytic activity and emulsification activity) showed that approximately 36% of the 51 isolated cultures exhibited the ability to produce biosurfactants. Subsequent studies using a selected culturable mixed culture (which included *Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli*) revealed that only *P. aeruginosa* was able to produce this biosurfactant. HPLC–MS analysis showed that the surface active compounds were rhamnolipids. Further comparative studies confirmed that the total yield of rhamnolipids was notably higher in the bioreactor inoculated with the selected mixed culture (940.58 ± 1.10 mg/L) compared to the bioreactor inoculated with the axenic strain of *P. aeruginosa* (108.47 ± 0.41 mg/L). Twelve rhamnolipid congeners were identified during cultivation of the selected mixed culture, whereas six congeners were detected during cultivation of the sole axenic strain of *P. aeruginosa*. Furthermore, increased production of rhamnolipids was observed when the concentration of autoinducer molecules (AI-2) responsible for interspecies signaling increased, suggesting the influence of quorum-sensing communication on biosynthesis efficiency. This observation may be of importance for large-scale production of this biosurfactant, as it opens new possible solutions based on the use of mixed cultures or external addition of stimulating autoinducers.

Introduction

Rhamnolipids are among the best studied surfactants of biological origin. They belong to the group of glycolipids and are composed of hydrophilic rhamnose sugar and hydrophobic 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) units [1]. Their basic classification is determined by the number of rhamnose molecules, which results in either mono-rhamnolipids (single rhamnose unit) or di-rhamnolipids (two rhamnose units) [2]. Furthermore, the factors associated with HAA units (carbon chain length, saturation and possible esterification) may also differ, which contributes to approximately 60 different known congeners [3]. Due to their amphiphilic structure, low toxicity and high biodegradability, rhamnolipids have attracted attention as possible alternatives to synthetic surfactants [4]. Their great structural variety is associated with numerous interesting properties, such as solubilisation of hydrophobic compounds, stabilisation of emulsions, antimicrobial activity and complexation of heavy metal ions [5]. As a result, rhamnolipids have found applications in numerous industrial fields, such as cosmetics (stabilisation of oil-in-water formulations) [6], petroleum extraction (microbially enhanced oil recovery) [7] and environmental protection (bioremediation of petroleum-contaminated soil, phytoextraction of heavy metals) [8,9].

Adversely, clinical studies have provided evidence that rhamnolipids also exhibit the ability to cause necrosis of epithelial cells, polymorphonuclear leukocytes or macrophages, and have been considered as a major virulence determinant [10]. This is

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directly associated with the fact that predominant rhamnolipid-secreting bacteria are opportunistic pathogens. In general, *Pseudomonas aeruginosa* is the most frequently reported producer [11]. Other notable bacteria capable of rhamnolipid production include *Burkholderia*, *Enterobacter*, *Staphylococcus* and *Bacillus* species [12]. Such microorganisms were often isolated from the lungs, sputum and respiratory tract of infected patients, oily skin areas of small animals [13] and breast skin of female and male poultry [14]. Non-pathogenic producers are uncommon and often originate from specific environmental niches (i.e. oil-contaminated soil, plant rhizosphere or thermophilic reservoirs) [15,16].

It has been established that the mechanism of rhamnolipid production is associated with bacterial quorum-sensing systems as well as virulence factors. In brief, the biosynthesis of rhamnolipids in *P. aeruginosa* is based on three sequential reactions catalysed by the corresponding proteins, RhlA, RhlB and RhlC. RhlA is associated with the synthesis of HAA during the first step, RhlB enables the coupling of rhamnose and HAA in order to form mono-rhamnolipids, whereas RhlC governs the addition of another rhamnose unit, resulting in the production of di-rhamnolipids [3]. The activity of these proteins is controlled by *las* and *rhl* quorum-sensing systems, which regulate the concentration of two *Pseudomonas* autoinducer (PAI) compounds belonging to N-Acyl homoserine lactones (AHLs): *N*-(3-oxododecanoyl) homoserine lactone (PAI-1, part of the *las* system) and *N*-butyrylhomoserine lactone (PAI-2, part of the *rhl* system) [17]. The *las* system also governs the induction of several virulence-related genes, such as *lasB* which encodes elastase. Moreover, reports confirm that the concentration of AHLs acts as a controller of multicellular processes, namely formation of biofilms or detachment and subsequent activation of cellular motility and virulence [5].

Based on the link between rhamnolipids, potential pathogenicity and bacterial biofilms, it can be hypothesised that bacteria originating from gastro-intestinal tracts and manures may exhibit promising abilities to produce these biosurfactants. Microorganisms which proliferate in the gastro-intestinal tract of mammals exist in the form of biofilms and are among the most abundant and diverse populations [18,19]. Moreover, it should be emphasised that most of the previous studies regarding the production of rhamnolipids was carried out with the use of axenic strains, but results of previous reports indicate that the production of rhamnolipids may be enhanced in the presence of other auto-inducer (AI) molecules [20]. Therefore, it is of interest to investigate whether different species of bacteria may influence the production of rhamnolipids by interspecies communication *via* quorum-sensing systems.

Having regard to the above hypothesis, this study was focused on the following aims: (1) to evaluate the ability of bacterial cultures isolated from cattle, poultry and pig faeces, as well as from manure, to produce biosurfactants; (2) to compare the production of rhamnolipids conducted by an axenic strain and a mixed culture; and (3) to assess the potential influence of autoinducer molecules on the production of rhamnolipids by the mixed culture.

Materials and methods

Materials

Commercial diesel fuel (EN 590:2004) was purchased from a petrol station (PKN Orlen, Poland). It was sterilized by filtration prior to use (Millex, pore size 0.2 μm; Millipore). Rhamnolipids (90% purity) used as standards for HPLC–MS analysis were purchased from AGAE Technologies LLC (Corvallis, Oregon, USA) as a 5% water solution. MS-grade acetonitrile, ammonium acetate, ethyl acetate and methanol, used as mobile phase additives, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Double distilled water was produced by reverse osmosis in a Demiwa system from Watek (Ledec nad Sazavou, Czech Republic), followed by double distillation in a quartz apparatus.

First experimental stage

Evaluation of the ability of bacterial cultures isolated from faeces and manure to produce biosurfactants.

Collection of samples

Samples originating from cow, chicken and pig faeces as well as from manures were collected between August and December 2012 from the western part of Poland (70 km southeast of Poznan). A total of 24 samples (approx. 5 g each) were collected separately in sterile plastic jars and were stored at 4 °C until the start of experiments.

Isolation of microorganisms

Isolation of biosurfactant-producing cultures was carried out according to the modified procedure described by Bushnell and Haas [21]. The samples were pre-cultured on a sterile modified BH mineral medium consisting of (g/L): $MgSO_4$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1, $FeCl_3$ 0.05. 1 g of each sample was transferred to flasks containing 50 mL of the mineral medium and supplemented with 2% diesel oil (v/v). The hydrophobic substrates were applied in order to stimulate the production of rhamnolipids [5,22]. The cultures were cultivated at 30 °C for 7 days. The enrichment cultures were obtained after five transfers of original microbiocoenoses (1 mL) to fresh mineral medium (50 mL) supplemented with 2% diesel oil (v/v). Subsequently, the cultures were diluted with sterile 0.85% (v/v) solution of NaCl and then spread onto TSA agar plates (Oxoid, UK). The plates were incubated at 37 °C for 24 h. Selected cultures were transferred to a TSB broth (Oxoid, UK) and incubated at 37 °C for 24 h. Overall, 51 cultures were isolated from different samples: 16 isolates were obtained from cow faeces, 9 isolates from chicken faeces, 8 from pig faeces and 18 from manures.

Screening of isolated cultures for production of biosurfactants

Four simple and rapid screening techniques were applied in order to determine the ability of the isolated cultures to produce biosurfactants: oil spreading (i), drop collapse (ii), haemolytic activity (iii), and emulsification activity (iv) [23]. The cultures were grown at 30 °C for 7 days (without shaking) in the modified BH mineral medium (10 mL) with 2% diesel fuel (v/v) as a sole carbon source. Before implementation of screening techniques, the biomass was washed three times with NaCl (0.85% w/v) and resuspended in a TSB broth. During the initial studies, two strains were selected to serve as positive and negative control, respectively: *P. aeruginosa* ATCC 10145 (with confirmed ability to produce biosurfactant) and *E. coli* ATCC 4157 (which was unable to produce biosurfactant).

For the oil spreading technique (i), a Petri dish (100 × 20 mm) was filled with 50 mL of double distilled water (1/3rd of its volume) and crude oil (20 μL) was added to the surface. After 3 days incubation, 1 mL of each microbial culture was centrifuged at 18,000 × *g* for 10 min and the cell-free supernatant (10 μL) was injected onto the surface of crude oil. The diameters of clean zone on oil surface, which reflected biosurfactant production, were measured [24].

The drop collapse method (ii) was performed in a 96-microtiter plate with flat wells filled with 2 μL of diesel oil. The plate was closed and equilibrated at RT for 60 min and afterwards 5 μL of cell-free supernatant was added to the surface of diesel oil. The shape of the drop was observed after 1 min [25].