Use of some carbon sources by *Pseudomonas* strains for synthesizing polyhydroxyalkanoates and/or rhamnolipids

Received for publication, January 20, 2014
Accepted, May 20, 2014

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Abstract

The study of several bacterial strains belonging to the *Pseudomonas* genus, isolated from environments contaminated with petroleum hydrocarbons, has demonstrated their ability to synthesize metabolites such as polyhydroxyalkanoates (PHA), rhamnolipids, and also hydrocarbon-degrading enzymes, when cultivated in the presence of unconventional carbon sources.

For the synthesis of rhamnolipids and polyhydroxyalkanoates mineral medium supplemented with different carbon sources (vegetable oil, sucrose and octanoate) was used. Thus, a simultaneous synthesis of both metabolites was achieved. The results were encouraging, the synthesized rhamnolipid amounts being predominant in all three strains under study, on all three carbon sources. The largest amount of rhamnolipids was synthesized by the CMM2 strain on medium with vegetable oil (174 mg/L), while the highest amount of PHAs was 32.11 mg/L in octanoate supplemented medium. For the synthesis of polyhydroxyalkanoates the influence of new carbon sources (xylene, n-hexadecane, and kerosene) was also tested.

Through synthesis of such metabolites, these bacterial strains demonstrate both their high resistance to the stress of contaminants and their biodegradative activity, which increases the interest for their characterization.

Keywords: rhamnolipids, polyhydroxyalkanoates, ALK-3f/ALK-3r primers

1. Introduction

Various studies on microorganisms isolated from hydrocarbon contaminated areas revealed the abilities of several bacterial species to produce surface-active compounds. Among the bacterial biosurfactants, rhamnolipids (RHL) produced by *Pseudomonas* strains are highly efficient in reducing surface tensions of aqueous media. Such biosurfactants are used for a wide range of industrial applications especially in cosmetics, food, pharmaceutical and as well as in bioremediation of pollutants (1,2).

Other important bacterial metabolites are represented by biopolymers like polyhydroxyalkanoates (PHAs): they are biodegradable and biocompatible compounds, with physical characteristics similar to those of petrochemical polyesters, reason for which they are considered an environmentally sustainable alternative to raw materials of plastic (3).

Previous studies noticed the simultaneously production of RHL and PHAs in some bacterial species like *Pseudomonas aeruginosa* (4, 5, 6), Thermus thermophilus (7), and *P. fluorescens* (2). Recently, an interrelationship between PHAs and RHL biosynthesis pathway was found in *P. aeruginosa*: when octanoic-1-13C acid was used it was shown that the fatty acid substrate was converted to PHAs and the acyl groups in the RHL with a minimum number of rearrangements (8). Moreover, the production of rhamnolipid in *P. aeruginosa* seemed to be very tightly regulated at the transcriptional level by the quorum-sensing response and by environmental conditions (8).
Due to the biotechnological importance of PHAs and RHL, attempts were made to use various carbon sources, preferably as inexpensive substrates, which could serve as growth support of bacterial cultures, with increased output in the synthesis of the mentioned metabolites.

Current efforts are focused on the selection and testing of bacterial strains that can use various renewable resources such as vegetable oils or waste materials (9). Using low-cost carbon sources, including agro-industrial wastes to produce PHAs and/or rhamnolipids can lead to significant economic benefits.

It was shown that *Pseudomonas aeruginosa* and other PHA producing bacteria were able to synthesize mcl-PHA (medium-chain-length) in media containing glucose, technical oleic acid, and wastes containing fatty acids or used cooking oil as carbon sources (10). Palm oil is indicated as a source of synthesis for simultaneous production of PHA and rhamnolipid by *Pseudomonas aeruginosa* (5). Moreover, the ability of *Pseudomonas stutzeri* to grow on o-xylene and the degradation of 2-metilbenzoic acid by *Burkholderia cepacia* MB2 was reported (11, 12).

Moreover, the isolation of indigenous microorganisms from polluted sites and augmentation of microbiota from accidentally or chronically oil polluted areas, in order to use them in bioremediation, requires the understanding of the action mode of hydrocarbon-oxidizing microorganisms, their selection depending on their tolerance degree towards the contaminant concentration and avoidance to generate hardly biodegradable intermediate byproducts.

The aim of this paper was to evaluate the abilities of some bacterial strains isolated from contaminated sites to produce simultaneously rhamnolipid and polyhydroxyalkanoates on various culture conditions. This could have potential implications in the selection of the most promising substrates for low-cost biotechnological important metabolites production, useful for decontamination and bioremediation processes.

### 2. Material and methods

Strains selected for this study belong to the genus *Pseudomonas* (*P.aeruginosa* C16, *P.aeruginosa* Ps3d and *P.fluorescens* CMM2). These were able to synthesize both rhamnolipids and PHA (2).

Bacterial strains were grown at 28 °C, at 150 rpm for 72 h on mineral medium (in g/L of distilled water: K2HPO4 • 3H2O 4.25, NaH2PO4 • H2O 1.00, NH4Cl 2.00, MgSO4 • 7H2O 0.2, FeSO4 • 7H2O 0.012, MnSO4 • H2O 0.0003, ZnSO4 • 7H2O 0.0003, CoSO4 • 7H2O 0.001, nitrilotriacetic acid 0.1), (13) supplemented with different carbon sources (waste vegetable oil, octanoate, sucrose) to a final concentration of 10%. For PHAs biosynthesis, bacteria were cultivated on plates with mineral medium supplemented with agar 2%, Nile Red 0.5 mg/mL (14) and xylene (mixture of ortho-, meta-, para-), kerosene or n-hexadecane to a final concentration of 10% as carbon sources. Controls were represented by mineral medium without carbon source (M1), and mineral medium supplemented with glucose 5% (M2), both containing Nile Red. The plates were incubated at 28°C for three days, and PHAs accumulation was detected under UV light (312 nm). UVP BioImaging Systems DIGI Doc – IT System and G.Box SYNGENE image capture system were used for PHA production examination.

Extraction of metabolites (PHAs and rhamnolipids) extraction was performed according to the methods described by Jiang (15) and Wang (16). Oil spread technique (17) was used for semi quantitative evaluation of RHL production.

Thin Layer Chromatography (TLC) was used for detection of rhamnolipids in extracts. Samples were spotted on silica gel 60 aluminum plates (Merck). Chromatograms were...
developed with a mixture of chloroform/methanol/acetic acid/water (85/22/5/10/4, v/v/v/v) and visualized with 10% molybdate phosphoric acid in ethanol. After drying the plates at 150°C the separated rhamnolipids appear as whitish spots. The rhamnolipids identification was based on their mobility (Rf), comparing with data from the literature (18).

The detection of some genes involved in the \( n \)-alkan or aromatic hydrocarbons catabolism was performed by colony PCR using two primer pairs: ALK-3f 5′-TCGAGCACATCCGGGCAGCCA-3′/ ALK-3r 5′-CGGTAGTGCTCGACGTAGTT (19, 20), and 2.3f 5′CGACCTGATCTCCATGACCAGCAT-3′/2.3r 5′TCAGGTCAGCAGCGTCA CAT-3′ (21, 20).

The amplification mixture for both PCR reactions consisted of: MangoMix (BIOLINE) 12 µl, 20 µM forward primer - 1µl, 20 µM reverse primer - 1µl, bacterial culture (transferred from a single colony), PCR water up to 25 µl. Amplification was performed in a thermal cycler (Biorad 1000) and the PCR program consisted of: initial denaturation 10 min at 96°C followed by 30 cycles (denaturation at 94°C for 1 min, annealing for 30 sec at 43°C and 56°C respectively, depending on the primer pair, extension at 72°C for 1 min) and final extension, 7 min at 72°C. The amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide and detected in UV light.

3. Results and discussions

*Evaluation of PHAs and rhamnolipids biosynthesis in the presence of different carbon sources*

The bacterial strains isolated from polluted areas were able to produce and accumulate important metabolites as rhamnolipids (RHL) and PHAs, as it was previously shown (2). In order to optimize the metabolites biosynthesis, the influence of three different carbon sources (vegetable oil, octanoate and sucrose) on PHAs and RHL was examined (fig.1).

![Graph showing the influence of carbon source on PHAs and RHL biosynthesis by various strains.](image)

The best results regarding the rhamnolipids production (174 mg/L) were obtained with the strain *P. fluorescens* CMM2 cultivated in the presence of sunflower oil as carbon sources. It was shown that all the strains produced largest amounts of biosurfactants in sunflower oil...
Use of some carbon sources by *Pseudomonas* strains for synthesizing polyhydroxyalkanoates and/or rhamnolipids

containing medium, comparing with the other carbon sources. These results are in concordance with data from literature (22) and could be explained by glycolipidic nature of the biosurfactant. Concerning the PHAs production, the highest level was obtained in octanoate containing medium, with CMM2 strain (32.11 mg/L). Our data suggest that the selected strains are able to produce simultaneously both metabolites but their level is dependent on carbon source used. It is possible that in the presence of specific carbon sources (like octanoate and sucrose) certain precursors in the metabolic pathway for PHAs and RHL synthesis are directed toward PHAs synthesis, rather than RHL.

When sucrose was added in culture medium, the amount of rhamnolipids prevailed in all three strains of interest, as with the other two carbon sources, but the level was generally higher.

In the case of the *P. aeruginosa C*<sub>16</sub> train, both the rhamnolipid and the PHA quantities obtained with all three carbon sources had close values, as shown in the graphs above.

Rhamnolipid extracts were checked by the oil spreading test, as well as TLC (thin layer chromatography).

The extracts of the three strains of interest tested positively by the oil spreading assay, confirming thus the presence of rhamnolipids (Table 1), according to Nasr (17).

Table 1. Oil spreading test in rhamnolipid extracts.

<table>
<thead>
<tr>
<th>Rhamnolipid extract / strain</th>
<th>Medium with sucrose</th>
<th>Medium with octanoate</th>
<th>Medium with waste vegetable oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps3d</td>
<td>6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>CMM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
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When strains were cultivated on medium with sucrose, an improvement of the oil spreading assay for rhamnolipid extracts was noticed as compared to the oil spreading test performed on the supernatant (in this case the results were: Ps3d = 3 mm, C<sub>16</sub> = 3 mm, CMM<sub>2</sub> = 10 mm). In case of the Ps3d strain, the results obtained with the oil spreading test for rhamnolipid extract was 6 mm as compared to 3 mm obtained when testing the supernatant by the same assay (Figure 2), probably due to the higher concentrations of rhamnolipids in the extract than in the supernatant.

Figure 2. Comparative oil spreading test between the supernatant and rhamnolipid extract.
Upon verification of rhamnolipid extracts by TLC, the Rf values obtained by us ranged between 0.68 and 0.75; according to Nereus, 2005, Rf factor values between 0.62 și 0.84 are considered various forms of mono-rhamnolipids (Fig. 3).

Fig.3. Verification of rhamnolipid extracts by TLC: A – rhamnolipid extracts from sucrose containing medium line 1 - C16, line 2 – CMM2, line 3 - Ps3d; B - rhamnolipid extracts from octanoate containing medium: line 1- C16, line 2 - CMM2, line 3 - Ps3d; C – rhamnolipid extracts from used sunflower containing medium: line 1 - C16, line 2 – CMM2, line 3- Ps3d.

In order to test the metabolic capacity and to render more effective the growth media for the synthesis of polyhydroxyalkanoates, some compounds of great industrial importance, such as xylene, kerosene, n-hexadecane were selected.

So far, we did not find in the available literature data concerning the assessment of the ability to produce polyhydroxyalkanoates in the presence of xylene, kerosene, n-hexadecane, as well as to synthesize of PHA using these substances as carbon source, in mineral medium.

The controls, as shown in Figure 4, show no development of bacterial cultures on M1 (this was expected because the used mineral medium had no carbon source), while on M2, not only growing of the three strains was recorded, but also accumulation of PHA granules, thus indicating that the three bacterial strains were able to synthesize polyhydroxyalkanoates.
Use of some carbon sources by *Pseudomonas* strains for synthesizing polyhydroxyalkanoates and/or rhamnolipids

Figure 4. Used controls: $M_1$ (left) – mineral medium without carbon source, $M_2$ (right) - mineral medium glucose inoculated with $C_{16} = P. aeruginosa$ C16, CMM2 = *P. fluorescens* CMM2 and Ps3d = *P. aeruginosa* Ps3d

Figure 5 shows the Petri dishes cultivated in the presence of n-hexadecane (A), xylene (B), kerosene (C). In the presence of xylene, the three bacterial strains did not grow.

![Figure 5](image)

When strain *P. aeruginosa* Ps3d was grown on n-hexadecane as carbon source, polyhydroxyalkanoates accumulation was demonstrated (Fig. 5A). The *Pseudomonas aeruginosa* C16 and *Pseudomonas fluorescens* CMM2 strains grew in the presence of n-hexadecane, which can be regarded, as well as xylene, as a stress factor.

Kerosene, a mixture of hydrocarbons containing 6 to 16 C atoms/molecule (23), obtained by fractional distillation of petroleum, was the sole carbon source (Figure 8 C) for C16 strain, strain showing slight accumulation of PHA.

The ability of microorganisms to metabolize different substances such as xylene, toluene, n-hexadecan, and other oil-derived hydrocarbons and even petroleum, is due to the presence of some genes such as: alkB, alkM, alkB/alkB1, catechol 2,3-dioxygenase, etc. (20).

To make the above experiments more reliable as well as those related to metabolism of crude oil that will be later presented, we tried to render evident some genes which encode enzymes of alkan hydroxylase group III type and catechol 2,3-dioxygenase respectively (Figure 6); these enzymes are involved in the metabolism of alkanes and aromatic hydrocarbon.
The ALK-3f 5'-TCGAGCACATCCGCGGCCACCA-3'/ ALK-3f 5'-CCGTAGTGCTCGACGTAGTT ALK-3r-3' primers (19, 20) were used to reveal the presence of the alkB/alkB1 genes, genes responsible for the synthesis of enzyme of alkan hydroxylase group III type that hydrolyze n-alkanes with chains containing more than 12 carbon atoms.

We also intended to show the genes encoding for catechol 2.3 dioxygenase by using the following primers: 2,3-f 5'CGACCTGATCTCCATGACCGA CAT -3'/ 2.3 r 5'TCAGGTCAGCAGGTCAT-3'. This pair of primers was recommended Mesarch and Stancu (21, 20).

According to the literature, using the ALK-3f/Alk-3r primer pair, we should obtain an amplification product of 330 bp (19). In our case, we obtained a similar product only for the Ps3d strain; for the other two strains, C16 and CMM2 respectively, no single amplification product could be obtained, but it can be noticed the presence of two well marked PCR products, one of 250 bp, size close to the amplicon of interest, and a second one with the size of about 700 bp.

Thus, we can conclude that the Ps3d strain possesses the enzymatic support required for the metabolization of n-alkans with chains containing more than 12 C atoms, fact confirmed by our former described experiment.

When using the 2.3CAT-f/2.3CAT-r primer pair, no amplification product close to that recommended by Mesarch et al. of 238 bp was obtained for any of the three bacterial strains. For the C16 (Pseudomonas aeruginosa) and CMM2 (Pseudomonas fluorescens) strains a unique amplicon was obtained, weakly represented of about 1000 bp, while for the Ps3d strain (Pseudomonas aeruginosa) no amplification product was obtained.
4. Conclusions

Areas contaminated by hydrocarbons or oil residues show a microbiota tolerant to environmental contaminants; hydrocarbon-oxidizing bacterial strains, or able to synthesize metabolites of biotechnological interest can be isolated and selected.

In order to stimulate the rhamnolipid synthesis, mineral culture media were used, supplemented with various carbon sources, such as edible vegetable oil, octanoate and sucrose.

Cultivation on all three carbon sources led to appreciable amounts of rhamnolipids synthesized by all the three bacterial strains analyzed.

In order to emphasize the ability to synthesize polyhydroxyalkanoates, it can be noticed that *Pseudomonas aeruginosa* Ps3d strain exhibits the capacity to use hexadecane for synthesizing polyhydroxyalkanoates.

None of the three bacterial strains were able to grow on xylene as sole carbon source.

The ability of some bacterial strains to metabolize oil compounds is due to the presence of alkB and alkM genes responsible for the production of degrading enzymes such as alkane hydroxylases and catechol 2, 3-dioxygenases.

In case of the *Pseudomonas aeruginosa* Ps3d strain an unique amplification product was obtained, similar to 330 bp value, which shows the ability of this bacterial strains to synthesize enzymes capable of metabolizing n-alkans with chains containing over 12 carbon atoms.

References


