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The impact of long-term contact of *Achromobacter* sp. 4(2010) with diesel oil – Changes in biodegradation, surface properties and hexadecane monooxygenase activity

Ewa Kaczorek [a,*], Karina Sałek [a], Urszula Guzik [b], Beata Dudzińska-Bajorek [c], Andrzej Olszanowski [a]

[a] Institute of Chemical Technology and Engineering, Poznan University of Technology, M. Skłodowskiej-Curie 2, 60-965 Poznań, Poland
[b] University of Silesia, Faculty of Biology and Environment Protection, Department of Biochemistry, Jagiellonska 28, 40-032 Katowice, Poland
[c] State Higher Vocational School, Ks. Kard. Stefana Wyszyńskiego 38, 62-200 Gniezno, Poland

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ABSTRACT

A bacterial strain was isolated from soil that was contaminated with diesel oil and was used in our experiments. The strain was then phenotypically, biochemically and genetically tested and named as *Achromobacter* 4(2011). In order to examine the impact of long-term contact with diesel oil of bacterial cells, the strain was stored under different conditions – on standard nutrient agar plates and on agar plates with 50 μl diesel oil as a sole carbon and energy source. The results clearly indicated that longer contact with diesel oil led to changes in both the bacterial surface and biochemical properties, as well as the hexadecane monooxygenase activity. Moreover, the fatty acid profiles also changed, leading to an increased content of saturated fatty acids. In addition, the rates of biodegradation of diesel oil were higher even when supplemented with the surfactants – rhamnolipids and saponins. This work demonstrates that prolonged contact of microorganisms with diesel oil can lead to many changes, not only in biodegradation potential, but also in their surface and genetic properties.

1. Introduction

Over the last few years the microbiological degradation of hydrocarbons has been widely described in literature and interest in this method is still growing (Márquez-Rocha et al., 2001; Medina-Bellver et al., 2005). One of the reasons for this trend is that biodegradation is considered to be a very effective and relatively cheap method helpful in reducing water and soil pollution. Petroleum fuels, crude oil or polycyclic aromatic hydrocarbons in contaminated water and soil are important ecological problems due to their poor water solubility (Bouchez et al., 1995). Limited water solubility of these compounds significantly reduces their bioavailability which, as a consequence, negatively affects biodegradation (Megharaj et al., 2011). Thus, many new ideas are being applied to enhance this process and increase it in a significant way.

Surface active agents belong to compounds that have recently been discovered to increase biodegradation rates. Thus, the use of these compounds enhances the desorption and solubilization of hydrophobic substances (Kuyukina et al., 2005). Furthermore, surfactants can also improve hydrocarbon utilization due to their emulsifications, as well as through adhesion on microbial cell surfaces (Singh et al., 2007). Special attention is focused on biosurfactants, (produced by microorganisms), as they are less toxic and easily biodegradable (Costa et al., 2006). Moreover, biosurfactants can be synthesized from renewable resources, as well as from industrial and domestic wastes. Surfactant adsorption onto bacterial cell surfaces leads to various modifications. The type and the concentration of surface active compounds influences the nature of the cell surface, especially whether hydrophobic or hydrophilic. Zhong et al. (2007) observed that rhamnolipid adsorption plays a role in modifying cell surface hydrophobicity, and the effect of monomer adsorption at low levels of rhamnolipid concentration is more significant than that of micelle adsorption. Increased cell surface hydrophobicity would favor cell adhesion on both hydrophilic and hydrophobic support surfaces (Liu et al., 2004). Cell surface hydrophobicity (CSH) depends on the ratio of hydrophobic and hydrophilic regions on the microbial cell surface (Ron and Rosenberg, 2002). Addition of surfactants exhibits both positive and negative effects when used as additives in a bioremediation process. Different research groups reported an improved

* Corresponding author. Tel.: +48 61 665 3688; fax: +48 61 665 3649.
E-mail address: ewa.kaczorek@put.poznan.pl (E. Kaczorek).

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hydrocarbon biodegradation by the addition of rhamnolipids (Straube et al., 2003; An et al., 2011). The hydrophobic cell surface often accesses hydrophobic contaminants by direct adhesion to insoluble contaminant particles. Surfactants, which pseudosolubilise contaminants, inhibit this microbial adhesion and biodegradation. Obuekwe et al. (2008) noticed that differential production of exopolysaccharide accounted for the differences in CSH, which in turn affected the ability of the variants to utilize hydrocarbon.

Alkanes, which are proven to be the main components of mineral oil and many fuels, can be degraded by microorganisms depending on the chain length. Kubota et al. (2008) isolated the microorganisms belonging to the *Acinetobacter* genus, that were able to degrade long-chain alkanes, but could neither degrade short-chain alkanes nor cyclic alkanes. According to Wentzel et al. (2007), some of the bacterial species which are capable of degrading long-chain alkanes have been said to be playing a very important role in bioremediation of oil-polluted environments. For this reason, hexadecane, belonging to this class of compounds, has been chosen in our work. Furthermore, the enzymes involved in first step of degradation of hexadecane (and other long chain alkanes) are monooxygenases, which induce the formation of an alcohol either by a terminal or sub-terminal oxidation (Okoh, 2006).

The rapid stress induces activation of regulatory mechanisms on bacteria that ensure the expression of stress response genes when and where they are needed (Hagen et al., 2009). Some of the functional groups of the compounds of diesel oil may be induced to break DNA bonds and modify deoxyriboses (Colak, 2008). The heterocyclic compounds in crude oil are possible mutagen agents for bacteria even if they are not metabolized by microorganisms (Kim et al., 2005).

In the present study we investigated the influence of long-term contact with diesel oil as a sole carbon and energy source on the bacterial strain *Achromobacter* sp. 4(2010) isolated from the environment polluted by diesel oil. The investigation concerned the (i) modification of cell surface properties, (ii) diesel oil biodegradation, (iii) fatty acid profiles and (iv) hexadecane monooxygenase activity. The results were compared with all works performed for *Achromobacter* sp. 4(2010) stored on standard nutrient agar plates. In addition, we examined the impact of selected surfactants (rhamnolipids and saponins) on the above-mentioned features. Our study provides important information on the surface, genetic and enzyme changes of bacteria under prolonged exposure to long-term contact with diesel oil, as a carbon and energy source.

2. Materials and methods

2.1. Chemicals

The hydrocarbons and other chemicals employed in this study were of the highest purity grade, produced by Merck (Germany). Diesel oil was purchased from a petrol station (PKN Orlen, Poland) and was filtrated (Millex, pore size of 0.2 μm; Milipore). For bacterial DNA isolation, a commercial kit (GenElute Bacterial Genomic DNA Kit, Sigma–Aldrich) was used.

Surface active agents used in the experiments were:

- rhamnolipids, anionic surfactant, produced by *Pseudomonas aeruginosa* available on the market as JBR-425 (Jeneil Biosurfactant Company, USA, JBR 425 – content 25% of rhamnolipids),
- saponins, glycosides, non-ionic natural surfactant, extracted from Yucca and Quillaya desert plants, Quillaya Bark, (Sigma–Aldrich, high grade purity, molecular weight 1050),

2.2. Microorganism and growth conditions for the biodegradation test and measurement of cell surface changes

The *Achromobacter* sp. 4(2010) strain was used in the experiments; the strain was isolated from soil contaminated with crude oil. Samples were collected from sites in the Polish Carpathian mountains. This strain was kept on standard nutrient agar plates and was denoted by AS4. However, in order to determine the influence of the presence of diesel oil during a 12 month storage period, this strain was also kept on agar plates with 50 μl of diesel oil as the only carbon and energy source and was denoted by AS4s (subcultured every month to mineral medium plates with diesel oil). All experimental tests were done with the strain after a 12 month storage period.

The mineral salts culture medium used throughout these studies was described previously by Kaczorek et al. (2010). Bacterial inoculum contained 50 ml of mineral salts medium, 50 μl diesel oil, 0.2 g l^{-1} yeast extract and a loop full of cells from an agar plate. After approximately 24 h, 3–5 ml of this liquid cultures was used for the inoculation of the final culture to reach an optical density (OD) of ca. 0.1 (this corresponds to 1×10^8 cells ml^{-1}). Microbial growth was monitored through culture density by measuring absorption spectrophotometrically at 600 nm (Shimadzu, 1601PC, Japan).

2.3. Morphological physiological and biochemical characterization of the isolated strain

The isolated strain was characterized phenotypically and biochemically characterized using standard techniques (Gram staining, colony shape, size and color on nutrient agar plate, catalase and oxidase test, etc.), according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Additional biochemical and physiological characteristics were determined using the API GN 32 system (BioMerieux, Lyon France). Isolation of fatty acids was performed according to Sasser (1990). Analysis of FAMEs was performed using an HP 5890 gas chromatograph (Hewlett Packard, Rolling Meadows, IL, US) equipped with an HP 25 m × 0.2 mm cross-linked methyl-silicone capillary column. The initial oven temperature was 170 °C, increased by 5 °C min^{-1} to 260 °C, the increased by 40 °C min^{-1} and held constant at 320 °C for 1.5 min. Helium was used as the carrier gas. Fatty acid methyl esters (FAMEs) were identified with Sherlock software (TSBA library, version 3.9. Microbial ID, Newark, NJ, USA), based on the actual calibration retention times run prior to sample analysis.

2.4. Phylogenetic analysis

Bacterial DNA was isolated from a pure culture using a commercial DNA kit (GenElute Bacterial Genomic DNA Kit, Sigma–Aldrich). For 16S rRNA gene amplification the Bacteria-specific primers 8F 5′AGTTTGATCATCGCTCAG 3′ and 1492R 5′GGTTACCTTGTTACGACTT3′ were used. Amplification was carried out through a program consisting of initial denaturation at 94 °C for 300 s, 3 cycles at 94 °C for 45 s, 57 °C for 30 s, 72 °C for 120 s; 3 cycles at 94 °C for 45 s, 56 °C for 30 s, 72 °C for 120 s; 3 cycles at 94 °C for 45 s, 55 °C for 30 s, 72 °C for 120 s; 26 cycles at 94 °C for 45 s, 53 °C for 30 s, 72 °C for 120 s; and a final elongation step at 72 °C for 300 s. The nucleotide sequencing of the gene was performed using a Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystem) and AbiPrism®3100 Genetic Analyzer. The MegaBLAST program was used for homology searches with the standard program default. Multiple sequence alignments were performed and the neighbor-joining phylogenetic tree was constructed using CLC Free Workbench 4.5.1 program. The 16S rRNA gene sequence