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Low-temperature biodegradation of petroleum hydrocarbons (n-alkanes, phenol, anthracene, pyrene) by four actinobacterial strains

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ABSTRACT

In this study we evaluated the ability of four cold-adapted bacterial strains to degrade n-alkanes (C_{12}–C_{22}), aromatic hydrocarbons (phenol) and polyaromatic hydrocarbons (anthracene, pyrene) at low temperatures. All four strains belonged to the phylum *Actinobacteria* and were identified as *Rhodococcus erythropolis* (strain BZ4), *Rhodococcus cercidiphyllus* (strain BZ22), *Arthrobacter sulfureus* (strain BZ73) and *Pimelobacter simplex* (strain BZ91). The strains could grow over a temperature range of 1–30 °C and showed catechol-1,2-dioxyogenase activity. One of the strains, *R. erythropolis* BZ4, degraded all of the compounds tested. The strain utilized n-alkanes and high amounts of phenol (7.5 mM), anthracene and pyrene (50 mg l^{-1}) at 15°C. *P. simplex* BZ91 degraded n-alkanes as well as up to 7.5 mM phenol; phenol degradation was observed at 1–30 °C. *R. cercidiphyllus* BZ22 fully degraded C_{12} (700 mg l^{-1}) at 1–20 °C, while degradation of C_{16} and C_{20} was delayed and lower compared to C_{12} degradation. *A. sulfureus* BZ73 was the best phenol degrader and utilized up to 12.5 mM phenol; phenol degradation occurred over a temperature range of 1–25 °C. Such strains are promising candidates for low temperature (low-energy) treatment of industrial wastewaters contaminated with hydrocarbons.

1. Introduction

Petroleum hydrocarbons are the most widespread contaminants in the environment. Alkanes, aromatic and polyaromatic hydrocarbons are representative fractions of petroleum hydrocarbons. Alkanes can be linear (*n*-alkanes), cyclic (*cyclo*-alkanes) or branched (*iso*-alkanes) and can constitute up to 50% of crude oil, depending on the oil source, but they are also produced by many living organisms. Since alkanes are apolar molecules that are chemically very inert, they are characterized by low water solubility and tend to accumulate in cell membranes (Rojo, 2009). Phenol and phenolic compounds are widely distributed in nature and as environmental pollutants. They are common constituents of many industrial wastewaters such as those produced from crude oil refineries and coal gasification plants. Due to their toxicity to microorganisms, phenolic compounds may often cause the breakdown of wastewater treatment plants by inhibition of microbial growth (Ren and Frymier, 2003), even at relatively low concentrations such as 2 mM (Li and Humphrey, 1989). PAHs are frequent environmental contaminants that occur in coal, oil and tar deposits and are produced as byproducts of fuel burning and by incomplete combustion of carbon-containing fuels. PAHs are of concern as pollutants since they are lipophilic; some compounds are mutagenic, cancerogenic and teratogenic (Samanta et al., 2002).

The capacity of a broad spectrum of microorganisms to utilize hydrocarbons as the sole source of carbon and energy has been recognized very early (Zobell, 1946) and was the basis for the development of biological remediation methods. The ability to degrade hydrocarbons is widespread among soil microorganisms. They may adapt rapidly to the contamination, as demonstrated by significantly increased numbers of hydrocarbon degraders after a pollution event (Margesin and Schinner, 2001; Greer et al., 2010).

The intensity of biodegradation is influenced by several factors, such as nutrients, oxygen, pH-value, composition, concentration and bioavailability of the contaminants. Temperature plays a significant role in controlling the nature and the extent of microbial hydrocarbon metabolism. Bioavailability and solubility of hydrophobic substances with low solubility, such as some aliphatic and polyaromatic hydrocarbons, are temperature-dependent. The decreased volatilization and solubility of some hydrocarbons at low temperature affects toxicity. A temperature decrease also results in a decrease in diffusion rates of organic compounds and in an increase in viscosity, which affects the degree of distribution (Whyte et al., 1998; Rojo, 2009).

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In cold and temperate climatic regions, temperatures of industrial wastewater, groundwater and soil can often decrease to temperature levels around or below 15 °C due to seasonal and/or diurnal fluctuations. The activity of mesophilic degraders is severely limited at this temperature, whereas cold-adapted microorganisms have evolved a series of adaptation strategies that enable them to compensate for the negative effects of low temperatures on biochemical reactions (Feller, 2007; Margesin et al., 2008).

It was the objective of this study to evaluate and to compare the capability of four cold-adapted actinobacterial strains to degrade representative fractions of petroleum hydrocarbons, i.e. alkanes, aromatic hydrocarbons (phenol) and polyaromatic hydrocarbons (anthracene, pyrene) at low temperatures. Strains with the required properties for efficient low temperature biodegradation, i.e., growth and degradation at low temperatures, are useful for the treatment of hydrocarbon-contaminated ecosystems.

2. Materials and methods

2.1. Isolation and identification of strains

Bacterial strains were isolated from petroleum hydrocarbon contaminated alpine soil collected from a former industrial district in Bozen, South Tyrol, Italy. Leakage from heavy oil storage tanks were the main reason for contamination. At the time of sampling, the mean soil temperature in the sampling area was 8–10 °C. The soil contained 13,300 mg hydrocarbons/kg dry soil. 40% and 60% of this contamination consisted of C_{10}–C_{20} and C_{20}–C_{40} hydrocarbons, respectively, which points to a high content of heavy oils.

Isolation of bacterial strains was done on mineral medium agar containing diesel oil as previously described (Margesin et al., 2003b). Four bacterial strains, BZ4, BZ22, BZ73 and BZ91, were selected on the basis of their ability to grow well in the presence of petroleum hydrocarbons. Strains were routinely cultured on R2A agar and maintained as a suspension in skimmed milk (10%, w/v) at −80 °C. Cell morphology was examined by phase-contrast microscopy (× 1000). The Gram-reaction was tested by Gram-staining. API strips (API 20 NE, API Coryne, API ZYM; bioMérieux) were used to determine physiological and biochemical characteristics as well as enzyme activities. Ligninolytic activity was evaluated on MM agar plates containing 0.4% (w/v) lignosulfonic acid sodium salt (Margesin et al., 2002) after 7 days at 15 °C. Growth under anaerobic conditions was assessed as described (Zhang et al., 2012b).

Genomic DNA of the bacterial strains was extracted using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories). The 16S rRNA genes were amplified as described earlier (Zhang et al., 2010). The 16S rRNA gene sequences were submitted for comparison and identification to the GenBank databases using the NCBI Blast N algorithm, the EMBL databases using the Fasta algorithm and the Ribosomal Database Project II (RDP) using its Sequence Match (Cole et al., 2005). GenBank accession numbers (National Center for Biotechnical Information, NCBI) for the 16S rRNA gene sequences of strains BZ4, BZ22, BZ73 and BZ91 are HQ588862, HQ588861, HQ588859 and HQ588857, respectively.

2.2. Growth temperature range of bacterial strains

Suspensions of bacterial cells (pre-grown on R2A agar plates at 15 °C) in 0.9% NaCl were used to inoculate R2A agar plates that were incubated at 1, 5, 10, 15, 20, 25, 30 and 37 °C, using two replicates per strain and temperature. Growth was monitored up to an incubation time of 7–21 days.

2.3. Biodegradation of hydrocarbons

The ability of the strains to degrade aerobically n-alkanes, phenol or polyaromatic hydrocarbons was determined in 100-ml Erlenmeyer flasks containing 10 ml of pH-neutral phosphate-buffered mineral medium without yeast extract (MM) (Margesin and Schinner, 1997) amended with the hydrocarbons to be tested as the sole carbon source. The medium was inoculated with a suspension of microbial cells (pre-grown in medium with the hydrocarbon to be degraded) prepared in 0.9% NaCl to give an initial OD_{600} of 0.05 after inoculation. Abiotic losses were monitored in sterile hydrocarbon-containing medium. All tests were performed with three replicates; the standard deviations obtained were ≤15%.

2.3.1. Biodegradation of n-alkanes

MM was amended with a mixture of the n-alkanes C_{12}, C_{16}, C_{18}, C_{20} and C_{22} (each 700 mg l^{-1}) as the sole carbon source. C_{18}, C_{20} and C_{22} are solid at 20 °C and were thus solubilized in a water bath at 55 °C before addition to the medium. Recovery of n-alkanes was determined immediately after contamination of the medium ($t = 0$). Erlenmeyer flasks with screw caps were used in order to minimize abiotic loss by volatilization. After an incubation period of 7 and 15 days at 15 °C and 150 rpm, the residual concentration of the individual n-alkanes was quantified by gas chromatography after extraction with heptane (DIN EN ISO 9377-2).

The best n-alkane degrader, strain BZ22, was selected to evaluate the effect of temperature on n-alkane degradation. The strain was cultured at 1, 10, 20 and 30 °C with a mixture of the n-alkanes C_{12}, C_{16} and C_{20} (each 700 mg l^{-1}) as the sole carbon source and the residual n-alkane concentration was determined after 4, 7, 11, 14 and 28 days of incubation (Fig. 2).

2.3.2. Biodegradation of phenol

Fed-batch cultivation with increasing phenol concentration was used to determine the highest amount of phenol that could be degraded by the tested strains (Fig. 3). Strains were grown at 15 °C

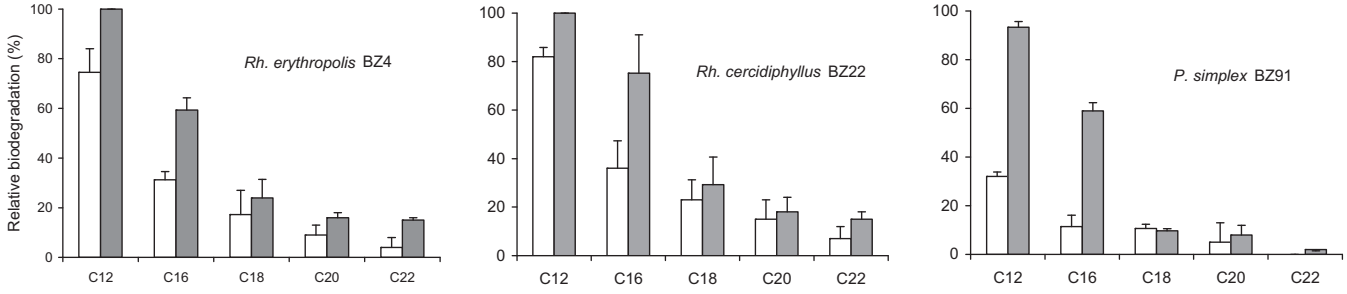

Fig. 1. Relative n-alkane degradation after 7 days (white colums) and 15 days (gray colums) at 15 °C by three actinobacterial strains. The initial concentration (100%) of each of the n-alkanes was 700 mg l^{-1}. Data are mean values and standard deviations of three replicates.