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Microbial degradation of *n*-hexadecane in mineral salt medium as mediated by degradative enzymes

Shweta Mishra, S.N. Singh *

Environmental Science Division, CSIR–National Botanical Research Institute (NBRI), Lucknow 226001, Uttar Pradesh, India

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ABSTRACT

In the present study, *n*-hexadecane degradation in MSM was investigated by three bacteria identified as *Pseudomonas aeruginosa* PSA5, *Rhodococcus* sp. NJ2 and *Ochrobactrum intermedium* P2, isolated from petroleum sludge. During 10 days of incubation, *n*-hexadecane was degraded to 99% by *P. aeruginosa* PSA5, 95% by *Rhodococcus* sp. NJ2 and 92% by *O. intermedium* P2. During degradation process, the induction of catabolic enzymes alkane hydroxylase, alcohol dehydrogenase and lipase were also examined. Among these enzymes, the highest activities of alkane hydroxylase (185 μmol mg^{-1} protein) and alcohol dehydrogenase (75.78 μmol mg^{-1} protein) were recorded in *Rhodococcus* sp. NJ2 while lipase activity was highly induced in *P. aeruginosa* PSA5 (48.71 μmol mg^{-1} protein). Besides, accumulation of *n*-hexadecane in inclusion bodies was found to be maximum 60.8 g l^{-1} in *P. aeruginosa* PSA5, followed by *Rhodococcus* sp. NJ2 (56.1 g l^{-1}) and the least (51.6 g l^{-1}) was found in *O. intermedium* P2. Biosurfactant production by bacterial strains was indicated by the reduction in surface tension and induction of cell surface hydrophobicity and pseudosolubilization which facilitated *n*-hexadecane degradation.

1. Introduction

Petroleum hydrocarbons are major environmental pollutants as a result of terrestrial and freshwater runoff, refuse from coastal oil refineries, off shore oil production, shipping activities and accidental spills (Arulazhagan et al., 2010). Alkanes are saturated hydrocarbons representing the main constituents of mineral oil and among the alkanes, *n*-hexadecane is a major component. The solubility of *n*-hexadecane in water is 5.21×10^{-5} mg/L at 15 °C and has high partitioning co-efficient 9.1logK_{ow} (Eastcott et al., 1988; Stroud et al., 2007). Besides, being high inflammable, alkanes are the least reactive class of organic compounds. The success of bioremediation is dependent on the inherent biodegradability of the pollutant, the accessibility of the pollutant to degrading microorganisms and on the optimization of biological activity (Yousefi Kebria et al., 2009). Low molecular weight alkanes are biodegraded rapidly as compared to multiple branched and long chain alkanes (Whyte et al., 1998). There are two main factors responsible for rapid degradation of *n*-alkanes from petroleum mixtures by microorganisms: (1) the activity of metabolic enzymes for the oxidation of *n*-alkanes and (2) the transport of alkanes into the cells (Sikkema et al., 1995).

A wide range of microorganisms have been reported to be involved in the degradation of aliphatic and aromatic hydrocarbons. However, Jimenez et al. (2011) have suggested that α and γ-Proteobacteria are the predominant oil-degrading taxonomic groups. The *Thalassospira* and *Roseobacter* genera may be important to aliphatic hydrocarbon degradation whereas *Mesorhizobium* and *Muricauda* are related to PAH degradation.

Biodegradation of aliphatic hydrocarbons by microbes can be enhanced by increasing their bioavailability through solubilization or emulsification or by adherence and uptake of oil directly from the oil–water interface. Extracellular biosurfactants or bioemulsifiers produced by microbes play an important role in enhancing the cell uptake by pseudosolubilization, and increase the interfacial area by lowering the surface tension for mass transfer, where *n*-alkane droplets complex with biosurfactant and then complex gets dissociated within a hydrophobic compartment of the cell wall (Beal and Betts, 2000). Several bacteria belonging to the genera *Acinetobacter, Rhodococcus and Pseudomonas* have been reported for the direct uptake of aliphatic hydrocarbons, which is facilitated by changing the structure of their outer membrane and by enhancing the cell surface hydrophobicity (Van Hamme and Ward, 2001).

Microorganisms follow different pathways for the degradation of alkanes like monoterminal, diterminal and subterminal. Members of *Rhodococcus* group possess all three pathways and the products of these pathways are primary alcohols, monocarboxylic fatty acids, secondary alcohols and ketones. Terminal oxidation was discovered as a major pathway for *n*-alkane degradation (Van Beilen et al., 2003) and subsequently confirmed by (Wentzel et al., 2007).

Biodegradative enzymes are often encoded on plasmids. Most studies on plasmid encoded pathways of hydrocarbon degradation have been limited to members of the *Pseudomonas* sp. (Kostal et al.,

* Corresponding author. Tel.: +91 522 2297823; fax: +91 522 2205836.
E-mail address: drsn_s@rediffmail.com (S.N. Singh).

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1998). In *Acinetobacter* sp. *and Rhodococcus* sp. strain Q15, the alkane degradative systems were found to be located on the chromosome (Singer and Finnerty, 1984; Watkinson and Morgan, 1990). Many enzymes, reportedly involved in the degradation of alkanes for example, linear, medium and long chain alkanes, are oxidized by alkane monooxygenase or cytochrome P450s (Van Beilen and funhoff, 2007). Alkane degrading bacteria also possess multiple genes for alkane hydroxylase and are capable of utilizing versatile alkanes (Van Beilen et al., 2002). In addition, the bacterial genus *Pseudomonas* is an inexhaustible producer of a number of extracellular enzymes, including lipase which degrades the triacylglycerides produced during alkane degradation (Ueno et al., 2006). Dioxygenase systems also exist in some microorganisms, where the *n*-alkane is initially oxidized to the corresponding hydroperoxide and then transformed to the corresponding primary alcohol (Watkinson and Morgan, 1990) or to the corresponding aldehyde, as originally postulated by Finnerty (1977) and also demonstrated by Sakai et al. (1996) in *Acinetobacter* sp. strain M-1.

In the present investigation, the role of biosurfactant producing bacteria in *n*-hexadecane degradation through involvement of key metabolic enzymes was studied.

Besides, cell surface hydrophobicity, pseudosolubilization and accumulation of intracellular hydrocarbon were also probed in relation to *n*-hexadecane biodegradation.

2. Methods

2.1. Isolation and screening of different bacterial strains

The bacterial isolates designated as PSA5, P2 were isolated from petroleum sludge of Barauni oil Refinery (Barauni, Bihar, India) by enrichment method and NJ2 (supplied by NEIST). For the isolation, 250 ml Erlenmeyer flasks containing 100 ml mineral salt medium (MSM) with 10 g petroleum sludge was incubated in orbital shaker set at 37 °C and 150 rpm for 1 week. After two transfers into the same medium, bacteria were isolated by serial dilution plating method on nutrient agar media. Screening of the bacterial isolates for their ability to utilize *n*-hexadecane was carried out on the basis of their growth as measured by UV–Vis spectrophotometer at 600 nm. Further, substrate utilization was confirmed by estimation of residual *n*-hexadecane in the medium by Agilent Gas Chromatograph (7890A) with FID using capillary BP5 column (5% phenyl methyl polysiloxane column, 30 m × 0.32 mm × 0.25 μm). Both injection and detector temperature were maintained at 280 °C. Initial oven temperature was maintained 80 °C for 2 min and then increased to 300 °C with 10 °C increase per min.

2.2. Growth of bacterial isolates in MSM with (1%) n-hexadecane

The growth of bacterial strains (PSA5, P2 and NJ2) was measured in sterile 10 ml MSM, pH 7.2 containing (1%) *n*-hexadecane as the carbon source in 100 ml Erlenmeyer flasks. All cultures in flasks were incubated in dark in an orbital incubator set at 37 °C and 150 rpm for 10 days. Control flask without *n*-hexadecane was incubated in the same conditions to serve as reference for the growth. Growth of bacterial strains in liquid media was measured by UV–visible Spectrophotometer at 600 nm at 2 days intervals.

2.3. Protein estimation

For the estimation of protein, cells of the bacterial isolates (PSA5, P2 and NJ2), grown in MSM with 1% *n*-hexadecane, were harvested, suspended and washed in potassium phosphate buffer pH 7. They were further sonicated and centrifuged at 20,000 rpm at 4 °C for 25 min. The supernatant was subsequently stored at 2 °C and the protein was measured by Lowry method (1951) at 660 nm by UV–visible spectrophotometer using BSA (bovine serum albumin) as a standard.

2.4. Assay of alkane hydroxylase

Cells were harvested by centrifugation at 5000 rpm, resuspended in 20 mM Tris–HCl buffer (pH7.4), disrupted using ultrasonic disintegrator (Fisher model 300), and centrifuged for 10 min at 8000 rpm. The cell-free supernatant was assayed for alkane hydroxylase activity. The reaction mixture contained 20 mM Tris–HCl and 0.15% CHAPS buffer (pH7.4), 0.1 mM NADH, 10 μl of *n*-hexadecane solution (1% hexadecane in 80% DMSO), and 50 μl crude extract in 1 ml volume. To start the reaction, 10 μl of hexadecane solution was added to the reaction mixture. Enzyme activity was measured by a decrease in absorbance at 340 nm of NADH on spectrophotometer. One unit of alkane hydroxylase activity corresponded to amount of enzyme which oxidized 1 mmol NADH per minute.

2.5. Assay of alcohol dehydrogenase

Cells were harvested by centrifugation at 5000 rpm, resuspended in 10 mM potassium phosphate buffer (pH 7.0), disrupted using ultrasonic disintegrator and centrifuged for 10 min at 8000 rpm .The cell-free supernatant was assayed for alcohol dehydrogenase activity. The reaction mixture contained 1 M Tris–HCl buffer pH 8.8, 4 mM NAD, ethanol (99% pure) and 50 μl crude extract in 1 ml volume. Enzyme activity was measured by an increase in absorbance at 340 nm of NAD on spectrophotometer. One unit of alcohol dehydrogenase corresponded to amount of enzyme which reduced 1 mM NAD per minute.

2.6. Determination of extracellular lipase

To determine the extracellular lipase activity, all the three bacterial isolates (PSA5, P2 and NJ2) were grown in MSM with 1% *n*- hexadecane as the sole carbon source for 10 days in an orbital shaker set at 37 °C and 150 rpm. The cultures were centrifuged at 10,000 rpm for 30 min at 4 °C at every 2 days interval and the supernatant was filtered using a 0.22-μm filter and the cell-free supernatant was used for the lipase assay. The lipase activity was determined by titrimetric assay method. In the titrimetric method, consumption of 1 ml of 0.01 N NaOH was equivalent to 10 μM of fatty acid liberated from *n*-hexadecane. One unit of lipase activity (U) is 1 μM of fatty acid released per min. Lipase activity was expressed in U/ml.

2.7. Biodegradation of (1%) n-hexadecane

To measure the potential of bacterial isolates for substrate degradation in the medium, the test strains (PSA5, P2 and NJ2) were first grown in nutrient broth for 48 h in an orbital shaker set at 37 °C and 150 rpm. Cell biomass was harvested by centrifugation at 4 °C and 5000 rpm for 10 min. Cells obtained were resuspended in 10 ml MSM in a 100 ml Erlenmeyer flask containing 100 μl (1%) *n*-hexadecane as a substrate. The cultures were shaken in orbital shaker set at 37 °C and 150 rpm for 10 days. Residual substrate in the media was extracted with 10 ml of *n*-hexane. The extracts were pooled and concentrated by evaporation and the biodegradation of (1%) *n*-hexadecane was measured by Agilent Gas chromatograph (7890A) with FID using capillary BP5 column (5% phenyl methyl polysiloxane column, 30 m × 0.32 mm × 0.25 μm). Both injection and detector temperature were maintained at 280 °C. Initial oven