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Bacteria-mediated aerobic degradation of hexacosane *in vitro* conditions

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HIGHLIGHTS

- Hexacosane degradation by two bacteria in isolation and combination.
- No report till date is available on bacterial degradation of hexacosane.
- Involvement of alkane hydroxylase & alcohol dehydrogenase in hexacosane degradation.
- Impact of cell surface hydrophobicity, surface tension and emulsification index.
- A drop in medium pH indicates formation of acidic intermediates.

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ABSTRACT

In vitro degradation of hexacosane ($C_{26}H_{54}$), a HMW *n*-alkane, was studied in MSM by two bacterial strains i.e., *Pseudomonas* sp. BP10 and *Stenotrophomonas nitritireducens* E9, isolated from petroleum sludge, in isolation and combination. The results revealed that both the strains were able to metabolize hexacosane by 82% in isolation and 98% in their consortium after 7 days. An enhancement of 16% in hexacosane degradation by the consortium indicated an additive action of bacterial strains. However, in control, a degradation of 21% was attributed to abiotic factors. During incubation with hexacosane, both the bacteria continued to multiply in isolation and consortium, which reflected that hexacosane was utilized by bacteria as a carbon and energy source. Activities of alkane hydroxylase and alcohol dehydrogenase were differentially expressed in isolation and combination, indicating their involvement in hexacosane degradation. Enhanced cell surface hydrophobicity and emulsification index and reduced surface tension also supported the degradation process.

1. Introduction

Alkanes are found highly abundant in the environment due to extensive use of petroleum fuels and their products. These are generally classified into linear (*n*-alkanes), cyclic (cyclo-alkanes) or branched (iso-alkanes). Low molecular weight alkanes are usually volatile in nature and easily degradable, while high molecular weight alkanes are highly persistent in the environment. As alkanes are aliphatic compounds and chemically very inert (Labinger and Bercaw, 2002), their metabolism by microbes faces challenges of water solubility and accumulation in the cell membranes and the energy to activate the molecule.

A number of microorganisms, including bacteria, filamentous fungi and yeasts, have been reported to metabolize alkanes through different degradative pathways (van Beilen and Funhoff, 2005; Wentzel et al., 2007) However, some recently characterized bacterial species, called hydrocarbonoclastic bacteria (HCB), are highly specialized for hydrocarbon degradation and play an important role in the removal of hydrocarbons from polluted and non-polluted environments (Harayama et al., 2004; Head et al., 2006; Yakimov et al., 2007; Wang et al., 2010). Rojo (2009) observed that direct uptake of alkane molecules from the water phase was only possible for low molecular weight alkanes which are soluble and easily transported into cells. For medium-and long-chain *n*-alkanes, microbes find access to these compounds as facilitated by the hydrophobic cell surface or biosurfactant produced. Biosurfactants have been reported to increase the uptake and assimilation of hexadecane in the liquid medium (Beal and Betts, 2000; Noordman and Janssen, 2002).

A key process for alkane degradation is oxygenation of terminal methyl group (Rehm and Reiff, 1981). Since alkane-degrading bacteria possess multiple genes for alkane hydroxylases, they are highly capable of degrading a wide range of alkanes (van Beilen

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et al., 2002). Alkane degradation is initiated by alkane hydroxylase to transform alkane to alkanols. Usually, there are three types of alkane hydroxylases which degrade short, medium and long chain alkanes (van Beilen and Funhoff, 2007). Methane monooxygenase usually hydroxylates gaseous short-chain alkanes (C_1–C_4), while medium chain alkanes (C_5–C_{16}) are oxidized by membrane-bound non-heme alkane monooxygenase (Alk-B) (van Beilen et al., 1994) and cytochrome P450 monooxygenase. As compared to other bacteria, *Stenotrophomonas maltophilia* strain M2, *S. maltophilia* strain Q2 and *Tsukamurella tyrosinosolvens* strain Q3 could degrade hexadecane two times faster due to high emulsification activity and presence of Alk-B gene (Tebyanian et al., 2013). Besides, other bacteria belonging to the genera *Acinetobacter*, *Rhodococcus* and *Pseudomonas*, have been reported for the direct uptake of aliphatic hydrocarbons, as facilitated by changing the structure of their outer membrane and by enhancing the cell surface hydrophobicity (Van Hamme and Ward, 2001).

Hexacosane ($C_{26}H_{54}$, Mol Wt. 366.71), a long chain *n*-alkane which is invariably found in the crude oil, is very persistent to microbial degradation. However, a few reports of hexacosane degradation by bacteria are available. Mohanty and Mukherji (2008) found two naturally occurring bacterial cultures, *Exiguobacterium aurantiacum* and *Burkholderia cepacia*, highly capable of degrading a wide range of *n*-alkanes including hexacosane in non-aqueous phase liquid (NAPL). da Cruz et al. (2011) observed degradation of hexacosane by 99%, 64% and 44% by aerobic (*Bacillus*, *Brevibacterium*, *Mesorhizobium* and *Achromobacter*), anaerobic facultative strains (*Bacillus* and *Acinetobacter*) and mixed consortium (*Stenotrophomonas*, *Brevibacterium*, *Bacillus*, *Rhizobium*, *Achromobacter* and uncultured bacteria), respectively, in petroleum oil deep sea reservoirs. Very recently, biodegradation of hexacosane was also observed by Elango et al. (2014) in oil in sand aggregates by *Mycobacterium* sp. in coastal environment.

In our study, an *in vitro* degradation of hexacosane was investigated by two strains of the bacteria separately and in combination over a period of 7 days incubation. Besides, the role of two enzymes involved in degradation process was also ascertained. The bacterial degradation of hexacosane in MSM was directly linked to cell surface hydrophobicity and emulsification index.

2. Methods

2.1. Materials

Minimal salt medium (MSM), nutrient agar (NA) and nutrient broth (NB) were procured from Hi-media, while hexacosane, folin–phenol reagent, NADH, NAD^{+}, bovine serum albumin (BSA) were purchased from Sigma–Aldrich. However, hexane (HPLC grade) was obtained from Qualigen.

2.2. Isolation and screening of different bacterial strains

Ten bacterial strains were isolated from petroleum hydrocarbon-contaminated soil through enrichment method and grown on MSM agar plate (composition: 7 g dipotassium phosphate, 2 g monopotassium phosphate, 0.5 g sodium citrate, 1 g ammonium sulfate, 0.1 g magnesium sulfate in 1 L medium (pH 7.0 ± 0.2) supplemented with 25 and 50 ppm hexacosane. Petri plates were incubated at 37 °C for 5 days in an incubator. Among these strains, six bacterial strains, designated as BP8, E9, PSM10, BP10, E3 and PSA5, were selected based on their better growth performance. These strains were further screened based on their degradation ability of hexacosane in MSM broth. For this purpose, these strains were incubated in 20 ml of MSM broth contained in 100 ml Erlenmeyer flask and supplemented with 50 ppm of hexacosane for7 days in an orbital shaker set at 37 °C and 180 rpm. After 7 days of incubation, residual amount of hexacosane was extracted with hexane (20 ml) thrice and concentrated to 2 ml on rota evaporator. Samples were analyzed using a Gas chromatograph (Agilent 7890A) with FID detector and a capillary BP5 column (5% phenyl methyl polysiloxane column, 30 m × 0.32 mm × 0.25 μm). Both injection and detector temperatures were maintained at 280 °C. Initial oven temperature was maintained at 80 °C for 2 min and then elevated to 300 °C gradually with 10 °C increase per minute.

2.3. Experimental set up

2.3.1. Preparation of inoculum

Two selected bacterial strains and their consortium were first grown in nutrient broth (composition: 5 g of peptic digest of animal tissue, 5 g of sodium chloride, 1.5 g of beef extract and 1.5 g of yeast extract in 1 L of media) separately, for 48 h in an orbital shaker set at 37 °C and 150 rpm. Bacterial cells were harvested by centrifugation at 5000 rpm at 4 °C for 10 min. Pellet of bacterial cells was washed with MSM twice and again re-suspended in 20 ml MSM. The absorbance (OD_{600}) of bacterial cells was measured with an UV–Vis spectrophotometer and adjusted to OD ~1.

2.3.2. Bacterial degradation of hexacosane

For hexacosane degradation study, 20 ml of MSM with 50 ppm hexacosane as a substrate were prepared in 100 ml Erlenmeyer flasks and then sterilized in an autoclave set at 121 °C and15 lb/square inch. Then after, bacterial strains (BP10 and E9) and their consortium (by mixing half of each bacterial inoculum) were inoculated separately in flasks with three replicates. The control flasks were also prepared in the same manner, but without bacterial culture. All flasks were incubated in an orbital shaker set at 37 °C and 150 rpm for 7 days. After every 24 h intervals, hexacosane depletion in MSM was measured and specific degradation rate was calculated by the formula as below:

$$dx/x_0 \cdot dt$$

where, dx = change in concentration of substrate; x_0 = substrate concentration; dt = time interval.

2.3.3. Degradation kinetics

As degradation of hexacosane fits to first order reaction kinetics, it can be expressed by a formula as below:

$$\mathrm{In}_{C_t} = \mathrm{In}_{C_0} - Kt$$

where, C_t is the residual hydrocarbon concentration at any time, C_0 is the initial hydrocarbon concentration; K is the speed constant which reflects the degradation rate and t is time (day). The half-life period of hexacosane was also calculated by the formula as below:

$$t_{1/2} = \ln 2/k$$

where, k is the biodegradation rate constant (day^{-1}).

2.3.4. Extraction of hexacosane and analysis

Residual amount of hexacosane after degradation by isolated bacteria and their consortium, was extracted from MSM by liquid–liquid extraction (1:1 v/v MSM:Hexane) method. Samples were extracted three times with hexane and pooled together. The extracts were concentrated by evaporation and remaining hexacosane was measured with a Gas chromatograph (Agilent 7890A).

2.4. Growth of bacterial isolates in MSM with hexacosane

For determination of bacterial growth, enzyme assay, protein and other properties of bacteria, 100 ml Erlenmeyer flasks were prepared in a similar way as for the hexacosane degradation study