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Alkane Degradation by *Dietzia* sp. K44 and partial cloning of putative alkane hydroxylase gene

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***Dietzia* sp. K44, a moderately hydrophobic actinobacterium was found to utilize hexadecane at a very slow rate at optimal temperature and pH of 30 °C and 8, respectively. Reduction in the surface tension of the growth medium signified alkane degradation. This was further confirmed by polymerase chain reaction of genomic DNA using degenerate oligonucleotide primers for alkane hydroxylase gene. An amplified product of 550bp was obtained that was cloned into pDRIVE vector. Sequencing of this partial alkane hydroxylase gene from *Dietzia* sp. K44 showed significant amino acid sequence homology with *Rhodococcus* sp. 1BN putative alkane hydroxylase suggesting a similar mechanism of alkane degradation in *Dietzia* and *Rhodococcus* species.**

Keywords: *Dietzia* sp. K44, *alkB* gene, surface tension, biosurfactant.

Alkanes, also known as paraffins, are highly reduced and inert forms of carbon constituting about 20-50 % of crude oil and are also generated by many plants and algae (van Beilen *et al.,* 2003, Whyte *et al.,* 1999). Bacterial oxidation of n-alkanes is a very common phenomenon in soil and water and is a major process in geochemical terms. The estimated amount of alkanes that is recycled per year amounts to several million tons from natural oil seepage and oil spills alone (Rosenberg and Ron, 1996).

Biodegradation of aliphatic hydrocarbons is well established, with several bacteria, yeast, fungi and algae being isolated from contaminated and non-contaminated sites. From a biotechnological perspective, alkane hydroxylases are versatile biocatalysts, which carry out a wide range of useful oxidation reactions. The enzyme system consists of three components: alkane hydroxylase (AlkB), rubredoxin and rubredoxin reductase (van Beilen *et al.,* 1994). Rubredoxin reductase transfers electrons from NADH (nicotinamide adenine dinucleotide) via its co-factor FAD (flavin adenine dinucleotide) to rubredoxin, which transfers electrons to the alkane hydroxylase. Alkanes are usually activated by terminal oxidation to the corresponding primary alcohol, which is further oxidized by alcohol and aldehyde dehydrogenases (Van Beilen *et al.,* 2003). However, most of the work on the alkane degradative genes has been performed on gram-negative bacteria, namely, *Pseudomonas, Acinetobacter* and *Alcanivorax*. The n-alkane oxidizing system has been described in few gram-positive bacteria, mainly in rhodococci (Whyte *et al.,* 2002). At the genetic level, comparison of alkane hydroxylase sequences have shown that four histidine-containing motifs are well conserved and based on two of the motifs, highly degenerate primers have been developed that amplified internal gene fragments of *alkB* homologs from gram-negative as well as gram-positive strains (Smits *et al.,* 1999).

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Dietzia species are gram-positive, mycolic acid containing bacteria belonging in the actinomycetales family. These pigmented bacteria have high GC content and are closely related to *Rhodococcus* species (Rainey *et al.,* 1995). Species belonging to this genus have been isolated from oil contaminated soil (von der Weid *et al.,* 2007). In the present study, we report here that *Dietzia* sp. K44 is able to degrade long-chain (hexadecane) *n*-alkanes. We have cloned partial *alkB* gene from this organism that might be involved in the alkane degradation pathway of this organism.

MATERIAL AND METHODS

Source of organisms

The strain used in this work *Dietzia* sp. K44 was isolated from the soil of Kargil, Jammu and Kashmir, India (MTCC 7402).

Media and Cultivation

Stock cultures of the organism were maintained on nutrient agar medium. Tests for utilization of substrates as sole carbon and energy source were performed using a basal mineral agar medium containing basal mineral medium $Na_2HPO_4.7H_2O$, 28.5 gl^{-1}; KH_2PO_4 3 gl^{-1}; NaCl, 8 gl^{-1}; $NaNO_3$, 1gl^{-1}; C source, 1%.

Determination of the optimum temperature and pH of the bacterium

To determine the optimum temperature of the bacterium, erlenmeyer flasks containing 100 ml nutrient broth containing were inoculated with a preinoculum ($OD_{660} \approx 1$) corresponding to 1 % of the total culture volume and incubated at 10, 20, 30 and 40 ºC and 150 rpm for 3 days. Growth was monitored by determining absorbance at a wavelength of 660 nm at constant intervals.

The effect of pH on the growth rate of *Dietzia* sp. K44 was determined in nutrient broth modified by the addition of 1M HCl (for acidic conditions) or 1 M NaOH (for alkaline conditions) over a pH range of 6.0 to 11 (in high-pH broths, precipitated salts were removed by filtration prior to use). All the experiments were performed in triplicates.

Cell Surface Hydrophobicity

The bacterial surface hydrophobicity was measured by the method of Rosenberg *et al.,* 1980 using n-hexadecane as the hydrocarbon phase. Briefly bacteria grown in basal mineral broth were harvested by centrifugation at 5000g for 10 min and resuspended in PUM buffer (22.2 g $K_2HPO_4.3H_2O$, 7.26 g KH_2PO_4, 1.8g urea, 0.2g $MgSO_4.7H_2O$ per liter of distilled water, pH 7.1) to give a OD_{400} of 1.0. For qualitative monitoring of hydrophobicity, equal volume of cell suspension (5 ml) and hexadecane were mixed for each type of cells and were monitored for cell partitioning. For quantitative measurements, bacterial suspensions (1.2 ml) were mixed with various volumes of hexadecane in a glass tube with a vortex mixer for 120 s. Optical density at 400 nm was recorded of the aqueous phase after allowing 15 min for the hexadecane to rise completely. *E. coli*, a hydrophilic strain was considered as a control. The hydrophobicity was expressed as the percentage of the initial absorbance of the aqueous suspension.

Biosurfactant activity

To determine if strain K44 biosurfactant remained associated with cells or was released into the medium, 25 ml of culture medium was centrifuged (12,000 X g, 10 min, 4 °C) and the cell free supernatant was separated from the cell pellet. The latter was resuspended in 25 ml of fresh medium and surface tension of both fractions was determined. All surface tension measurements were performed in duplicate (Whyte *et al.,* 1999)

Surface Tension

The surface tension was measured at room temperature with Kruess K11 Tensiometer (KRÜSS GmbH) equipped with a 2 cm platinum plate.

Cloning of partial *alk* gene and sequencing

Genomic DNA of *Dietzia* sp. K44 was isolated by the 4M GIT cocktail method of Bose *et al.,* 1993. Partial *alk* genes were amplified from genomic DNA using highly degenerate oligonucleotide primers, TS2S (5'- AAY AGA GCT CAY GAR YTR GGT CAY AAG-3') and Deg1RE (5'-GTG GAA TTC GCR TGR TGR TCI GAR TG-3') (Smits *et al.,* 1999). The PCR conditions were 35 cycles consisting of 45 seconds at 94 °C, 45 seconds at 50 °C, and 1 minute at 72 °C, with a final extension step of 10 minutes at 72 °C. About 550 bp of PCR product was further purified using QIAGEN PCR purification kit (QIAGEN, Germany). PCR products was cloned into the pDRIVE cloning vector (QIAGEN,

Germany) and sequenced using T7 promoter (5'-GTA ATA CGA CTC ACT ATA GGG C-3') and M13 forward (5'- GTT TTC CCA GTC ACG AC-3') at The Centre for Genomic Application, New Delhi, India. DNA sequence homology searches of the sequence were performed using BLAST algorithm of NCBI (Altschul etal., 1990).

Nucleotide submission

The GenBank/EMBL/DDBJ accession number for the partial *alkB* gene sequence of strain K44 is AY819733.

RESULTS AND DISCUSSION

Growth Pattern

Strain K44, a gram positive pigmented bacterium, utilizes hexadecane as sole carbon source in basal minimal medium as well as nutrient medium at the pH-temperature optima of 8 and 30°C respectively (Fig. 1 & 2). However, determination of its cellular growth in alkane containing medium by absorbance measurement at 600 nm proved to be difficult due to flocculation of cells.

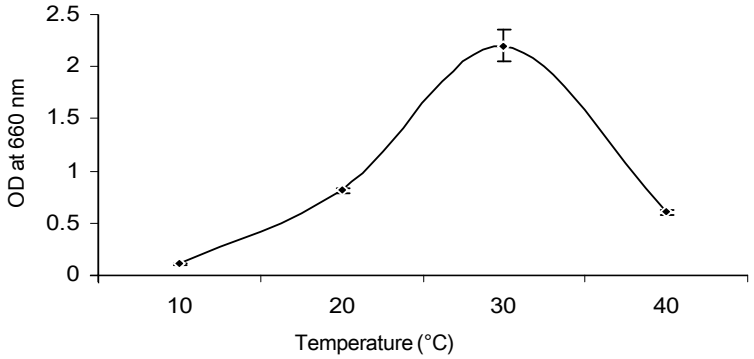

Fig. 1. Effect of temperature on the growth of *Dietzia* sp. K44

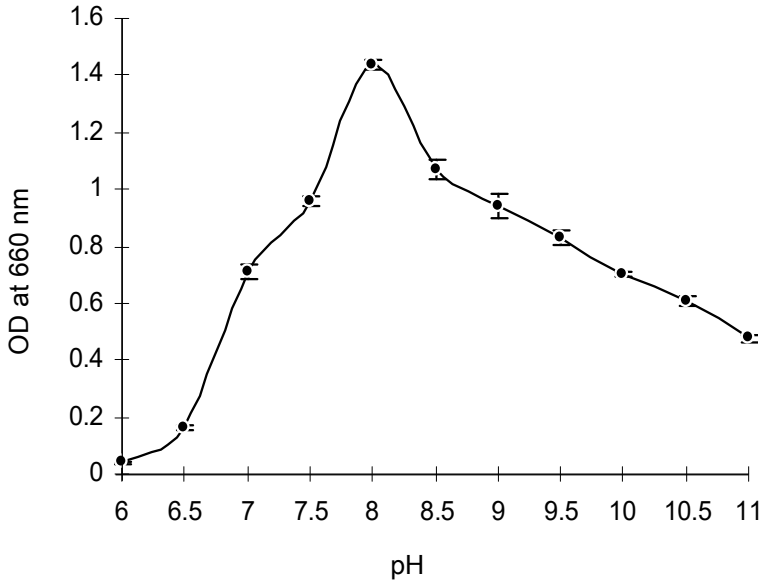

Fig. 2. Effect of pH of the medium on the growth profile of *Dietzia* sp. K44

Hydrophobicity tests

Hydrophobicity assays indicated the relative cell surface hydrophobicity of strain K44 grown on basal mineral medium containing 5% hexadecane as carbon and energy source, as measured by MATH test, to be 48. 84 % ± 3.26 (mean ± standard deviation). Cells of the strain were found to form emulsion at the oil-water interface, reaffirming their moderately hydrophobic nature. However, affinity for hydrocarbons varies among hydrocarbon degrading bacteria, with some hydrocarbon degrading bacteria showing lesser affinity than their non-hydrocarbon degrading counterparts (Rosenberg *et al.,* 1980). Hence the ability to degrade hexadecane was demonstrated by measuring surface tension of the growth medium.

Surface tension analyses of *Dietzia* sp. K44

Cells growing on BSM containing 1 % and 5% hexadecane respectively were measured for decrease in surface tension. During growth at 30 °C, the surface tension of medium containing 1 % and 5 % hexadecane were found to be 61. 33 ± 1. 124 mN/m and 60.67 ± 1.75 mN/m respectively. However, a decrease in the surface tension of the medium was observed following seven days incubation with strain K44 at 30 °C, substantiating alkane degradation by this bacterium. Surface tension of the medium was found to reduce to 33. 867 ± 1.137 mN/m and 40. 9 ± 2.29 mN/m respectively (Fig 3). Microorganisms that decrease surface tension of cultivation media by more than 10mN/m are considered promising producers of surfactants (Francy *et al.,* 1991). Biosurfactants are biphilic surface-active compounds consisting of a hydrophilic (polar) moiety and a hydrophobic (non-polar) moiety that are produced mainly by microorganisms as secondary metabolites. They tend to bind to each other, interact with surfaces of various polarities, adsorb at water-air or water-oil boundaries, cause wetting of hydrophobic surfaces, form structures analogous to lipid films or membranes, and reduce the surface and interfacial tension of solutions. However, their role in the cell are poorly studied with the following functions attributed to them; transport of hydrophobic water-insoluble compounds into the cell, formation of biofilms, and adhesion of cells to various surfaces (Fiechter, 1992).

Surface tension of hexadecane grown bacterial cells was measured after separating them from the culture supernatant. A reduction in the surface tension was observed in cell pellets

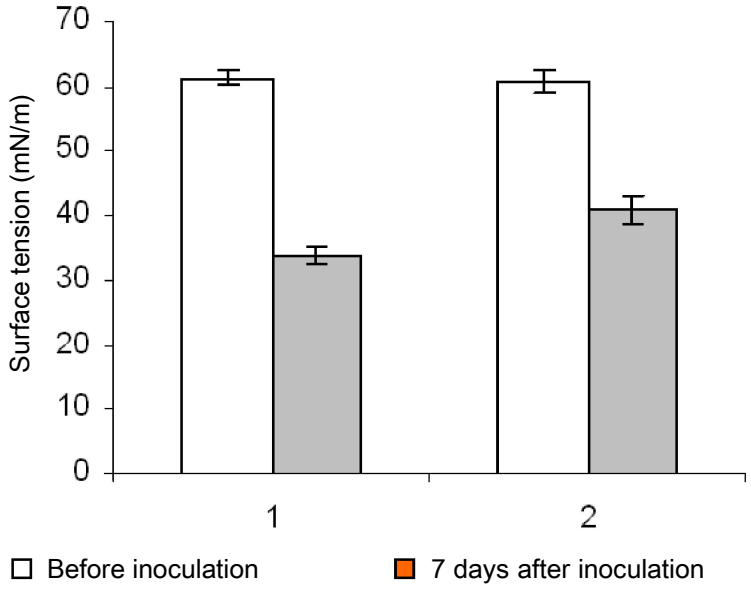

Fig. 3. Comparison of the reduction in surface tension of BSM containing (1) 1% hexadecane and (2) 5% hexadecane, after incubation with *Dietzia* sp. K44

resuspended in fresh minimal medium, but not in the cell free supernatant, signifying that the biosurfactant activity was related with the cell envelope and was not released into the medium (data not shown).

Identification of partial *alkB* gene

PCR amplification using degenerate oligonucleotide primers for *alkB* gene yielded an amplified product of 550bp (Fig. 4). Sequencing of the PCR product of showed 64% amino acid sequence homology with *Rhodococcus* sp. 1BN putative alkane hydroxylase.

Dietzia sp. K44 is phylogenetically closely related to the genus *Rhodococcus* (Rainey *et al.,* 1995). Members of this genus have been recognized as ideal candidates for the biodegradation of hydrocarbons (Beard and Page, 1998) and are known to produce cell-bound biosurfactants during growth on alkanes, and these biosurfactants have been identified as trehalose mycolate lipids (Kretschmer *et al.,* 1982 and Ristau and Wagner, 1983). Although the biosurfactants(s) produced by strain K44 are yet to be characterized, their nature could be similar to trehalose mycolate lipids.

Recent years have seen a growing attention towards alkane hydroxylase genes as markers to predict the potential of different environments for oil degradation (van Beilen, 2003). Sequencing of PCR amplicon obtained using degenerate oligonucleotide primers for *alk* gene showed significant amino acid sequence homology with *Rhodococcus* sp. 1BN putative alkane hydroxylase. Recently, *Rhodococcus* srains NRRL B-16531 and Q15 were found to possess multiple alkane monoxygenase gene homologs (Whyte *et al.,* 2002). Hence, it could be hypothesized that *Dietzia* sp. K44 might posses' alkane monoxygenase system(s) similar to *Rhodococcus*.

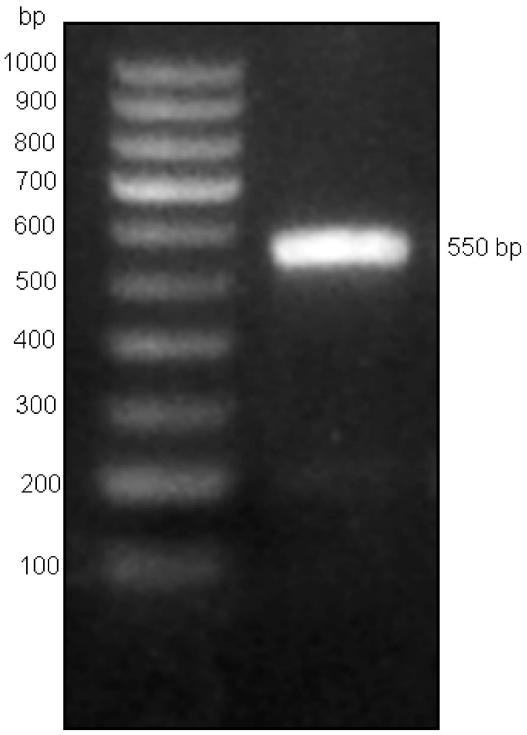

Fig. 4. Agarose gel (1%) analysis of the PCR amplified partial *alkB* gene of 550bp.
Lane 1 DNA molecular weight marker,
Lane 2: PCR product derived with primer pairs TS2S/Deg1RE

Conclusion

Dietzia sp. K44 has been found to degrade hexadecane in minimal medium. The partial *alkB* gene obtained from the bacterium could be used as a probe to fish out genes coding for alkane hydroxylase of the organism and thus, give a more comprehensive overview of its potential to degrade hexadecane.

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