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Potential Degradation of Certain Alkanes by *Pseudomonas frederiksbergensis*

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Biodegradation of polluted temperate and cold temperature environments may require the activity of psychrophilic and psychotrophic bacteria, because their low temperature growth range parallels the ambient temperatures encountered in these environments. In this present study a mixed population of microorganisms from an ice sample from Spitzbergen was cultivated by enrichment culture technique which exhibited high efficiency to assimilate and mineralize C_{10} to C_{22} *n*-alkanes as the sole source of carbon and energy at both 4 and 20°C. *Pseudomonas frederiksbergensis* was isolated from this mixed culture, characterized and identified according to the cell wall fatty acids analysis and 16S rDNA sequence. The isolate was psychrophilic, with a growth temperature ranging from 4 to 20°C, an optimum growth temperature of 15°C and an optimal pH of 7.

Key words: Alkanes, Biodegradability, Degradation, GC-MS, Hydrocarbons, Psychrophiles.

Hydrocarbon pollution has become a big problem with the development of the petrochemical industry and installation of numerous petrol stations and underground pipes. Petroleum hydrocarbons of crude oil and refined fossil fuel are the most widespread contaminants in the environment. Accidental spillage of petroleum often causes serious damage to the natural environment (Stberga *et al.* 2006). The prevalence of oil spills into the environment has increased interest in studying microbes involved in biodegradation of hydrocarbons (Zhuang *et al*. 2013). Many different aspects of these communities are being studied, including isolation and identification the microorganisms present to determine the roles they play in the biodegradative processes (Siddique *et al*. 2012). Considerably interest is being devoted in the use of bacterial alkane oxidation systems as biocatalysts for the production of fine chemicals and pharmaceuticals (Whyte *et al*. 1999). Long chain alkanes are often found in oil spills, at old gasworks sites or sites formerly used for wood preservation (creosote spills). Employing biodegradative processes to remove or detoxify pollutants that have found their way into the environment, is proving to be a useful method for pollution abatement. Several studies have shown that a wide range of microorganisms are capable of degrading diesel fuel (Marquez-Rocha *et al*. 2001) and *n*-alkanes (Stberga *et al.* 2006).

In fact, microbial degradation of spilled oil is one of the major routes of the natural removal of contaminants from the environment. Although *n*-alkanes are easily biodegradable, long-chain *n*-alkanes, branched-chain hydrocarbons, and polycyclic aromatic hydrocarbons are difficult to degrade (Hasanuzzaman *et al*. 2007). The problem

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in the biodegradation of hydrocarbons especially long chain alkanes is the very low solubility in the aqueous phase**,** especially at low temperature. Therefore studies were conducted to elucidate conditions such as pH and temperature for the optimal degradation of long chain alkanes compounds. The ability of psychrophilic bacteria to grow at low temperature near 0°C is characteristic that differentiates them from the mesophilic bacteria (Kohshima 2000). In the present study, the isolation of a psychrophilic, *Pseudomon*as *frederiksbergensis* at low temperature from a mixed culture is reported. The mineralization of eicosane by this bacterium is described, as well as its ability to degrade alkanes of variable chain length. Finally, its biodegradative capability of degradation such compounds was described.

MATERIALS AND METHODS

Cultivation and enrichment of the mixed culture

Microorganisms used in the present study were isolated from samples collected in Spitzbergen (largest Norwegian island in the Arctic Ocean) during an expedition by Prof. Trinks (former President of Technical University Hamburg Harburg) and transported to the laboratory to an ambient temperature of 4°C. The sample was used as inoculum for the enrichment culture. This mixed culture cultivated in sterilized mineral salts medium (MSM) (without yeast extract). Medium was composed of 0.3% of KH_2PO_4, 1.28% of Na_2HPO_4, 0.1% of NH_4Cl, 0.05% of NaCl (per liter deionized water, w/v). Enrichment of the mixed culture was carried out as follow, in 100 ml sterilized flask, 18 ml media, 2 ml mixed culture supplemented with 1 mM of decane for initial activation purpose. The pH value was adjusted to 7.0. The flasks were tightly sealed with screw caps and the control flask (without decane as a sole of carbon and energy source) was incubated in parallel. The incubation period was 16 days on a rotary water bath shaker at 4°C and 200 rpm until the growth observed.

Hydrocarbon degrading bacterium and substrate selectivity

All chemicals used in this investigation were technical grade from Merk (Germany). The following compounds were tested, decane ($C_{10}H_{22}$), hexadecane ($C_{16}H_{34}$), octadecane ($C_{18}H_{38}$), eicosane ($C_{20}H_{42}$), docosane ($C_{22}H_{46}$), tetracosane ($C_{24}H_{50}$), dotricontane ($C_{32}H_{66}$), and tetracontane ($C_{40}H_{82}$). The liquid compounds $<C_{16}$ were added directly to the media after sterilization by filtration through a filter with a pore size of 0.2 µm. The *n*-alkanes with carbon chain length $>C_{16}$ were dissolved in hexane and then added to the medium under the sterilized hood until the evaporation of hexane. Afterwards the medium was inoculated as mentioned (without yeast extract), whereas 1mM from each of the previously mentioned compounds supplemented with the 2 ml mixed culture after centrifugation to 13×10^3 rpm for 10 min. The pellet was washed three times, suspended in the MSM and incubated as previously mentioned. Bacterial growth was determined by direct cell counting using a Neubauer counting chamber (depth of 0.02 mm and a small square area of 0.0025 mm^2) and a phase contrast microscope (Axiolab, Zeiss, Germany) at a magnification of 400X.

Isolation and characterization of psycrophilic hydrocarbon degrading bacterium

Isolation and characterization of the hydrocarbon degrading bacteria were carried out by taking 1ml from each flask that exhibited high turbidity, then diluted from 10^{-1} to 10^{-10}. By streak method technique the culture with appropriate dilutions was plated on agar mineral salt media containing eicosane crystals. The preparation of agar dishes was performed according to the method described by Kiyohara *et al.* (1982). Agar mineral salt media dishes were sealed with tape and incubated for at least 14 days at 4°C. After the incubation period, the single colonies were picked and cultivated again in liquid mineral salt media for at least 10 days. This procedure was repeated until getting identical colonies.

Determination of the growth rate $(\mu)d^{-1}$

In time interval of 2 days, 10 µl from each flask were taken and by the aid of Petrhoff Hauser counting chamber the cell counting was determined. The growth rate $(\mu)d^{-1}$ was calculated according to the growth curve. The growth rate $(\mu)d^{-1}$ was determined according to Herbert and Burill (1997). The pure strain has been maintained at -70°C for further experiments.

$$\text{Growth rate } \mu(d^{-1}) = \ln\left(\frac{\log x2 - \log x1}{T2 - T1}\right)$$

where: x_1 and x_2 are growth values at time t_1 and t_2, respectively.

ln2 = 2.2036 and µ = ln2.TD

Characterization of the hydrocarbon degrading bacterium

The isolated strains were characterized and identified depending on the cell wall composition, substrate selectivity and the growth temperature. Further classification and identification was performed by Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ (Germany) according to the fatty acids analysis and 16s rDNA sequence.

Determination of pH and temperature optima

The pH values in MSM supplemented with 1 mM eicosane and 2 ml bacteria to final volume of 20 ml in 100 ml sterilized flasks were adjusted to 3.0; 3.5, 4.0; 4.5; 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5. The flasks were incubated on a rotary water bath shaker at 4°C and 200 rpm. In time interval of 2 days, 10 µl from each flask was taken to determine the cell numbers. Depending on the optimal pH; the temperature values were adjusted to 4, 10, 15, 20 and 30°C with previously mentioned procedures and conditions. Determination of the cell growth was measured by cell counting technique. The pH values were measured during the growth of the pure strain on eicosane for monitoring the changes during growth.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis and determination of the eicosane concentration

In order to determine the range of the substrate utilized by bacteria and to estimate the biodegradation, in vials of 7 ml were tightly sealed with Teflon rubber and an aluminium cap (ICT-ASS-Chem. Handels, GmbH) containing 2 ml MSM supplemented with 10 µl pure culture and 1 mM eicosane. The small vials were incubated on a rotary water bath shaker at optimal conditions and 200 rpm. The control vial was incubated in parallel. In time interval of 2 days each vial was introduced to GC-MS analysis by centrifugation the whole vial for 10 min at 13×10^3 rpm. The supernatant was collected and the rest of the eicosane in each sample was extracted by hexane with a ratio of 1:1 and centrifuged again three times for 10 min at 13×10^3 rpm. The eicosane containing apolar phase was subjected to GC-MS for quantitative and qualitative analysis.

GC-MS parameters for eicosane degradation analysis

The samples were analyzed by the aid of GC-MS (HP-G1800 AGCD system). Both inlet and detection temperature were 280°C, a temperature program consisting of an initial oven temperature of 150°C and the final temperature was 220°C, heating a rate of 15°C/min to 280°C with helium as gas carrier at 0.37 atm.

Determination of the substrate spectrum

The following aromatic and aliphatic hydrocarbons were tested as a sole of carbon and energy sources, 2,4-dichlorophenol, phenol, 2-chlorophenol, toluene, decane, naphthalene, dodecane, tetradecanol, hexadecane, heptadecane, octadecanol, octadecane, eicosane, docosane, docosanol, tetracosane, dotricontane and tetracontane.

RESULTS

Isolation and characterization of psychrophilic hydrocarbon degrading bacterium

A mixed population sample of microorganisms from Spitzbergen was enriched and cultivated on several compounds of long chain alkanes. It exhibited high efficiency to assimilate and mineralize *n*-alkanes from C_{10} to C_{22} particularly decane ($C_{10}H_{22}$), hexadecane, ($C_{16}H_{34}$), eicosane ($C_{20}H_{42}$), and docosane ($C_{22}H_{46}$), as a sole of carbon and energy source in mineral salt medium without yeast extract from 4 and 20°C. The alkane biodegradative strain was isolated on mineral salt medium. It was identified as a member of the genus *Pseudomonas* (*P*) with physiological characteristics based on the cell morphology. The aerobic, gram negative, rod shaped bacterium was 0.5-0.8 µm in diameter and 1.5-3.0 µm long (Fig. 1).

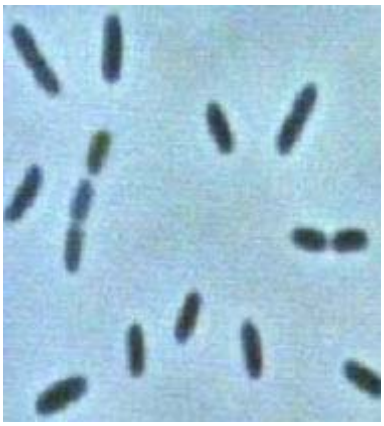

Fig. 1. *P. frederiksbergensis* growth on LB medium

The colonies are smooth and pale yellowish on complex media. During the growth on eicosane milky solution with the presence of the detergent production and some secretions were observed around the colonies on the mineral salt medium (Fig. 2).

On the other hand, these secretions could not be observed on LB medium and this may be somehow evidence that these secretions may have a potential effects helping in substrate availability.

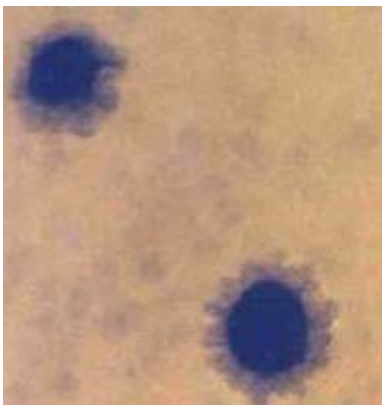

Fig. 2. *P. frederiksbergensis* growth on MSM medium

The strain was oxidase and catalase positive, rods and produced dark pigments in the middle of the colonies during the growth on eicosane MSM as a sole of carbon and energy sources. Growth took place from 4 to 20°C with optimal temperature of 15°C. There was not any growth observed at 37°C. *P. frederiksbergensis* was considered obligate psychrophiles according to the classification of Nedwell and Rutter (1994). Cells varied morphologically depending on medium type and culture age. Additional analysis and identification were carried out by DSMZ. Fatty acids compositions indicated that the isolate belongs to the genus of *Pseudomonas* with 99.8% of DNA sequence identity to *P. frederiksbergensis*.

Mineralization of eicosane at 4°C by *P. frederiksbergensis*

The mineralization of eicosane by *P. frederiksbergensis* was very slow at 4°C. The concentration of eicosane almost vanished and utilized by the bacteria as a sole of carbon and energy source in 25 days at least (Fig. 3). After the 7th day *P. frederiksbergensis* began to assimilate the available part of eicosane in the medium.

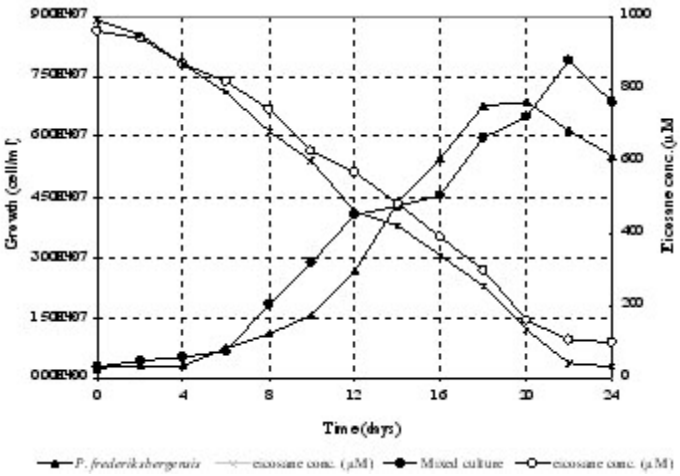

Fig. 3. Growth and biodegradation of eicosane by the mixed culture, *P. frederiksbergensis* and the mixed culture at 4°C

Optimal conditions for the growth and biodegradation by *P. frederiksbergensis*

The optimal pH and the temperature for the growth for *P. frederiksbergensis* were 7.0, 15°C, respectively (Figs. 4, 5). Assimilation and mineralization of the eicosane was promising at these optimal conditions (Fig. 6). It was clear that, the growth rate $\mu(d^{-1})$ was ca. 0.175 and after the optimization of the pH and temperature as essential factors for growth requirements the growth rate $\mu(d^{-1})$ increased to be 0.37.

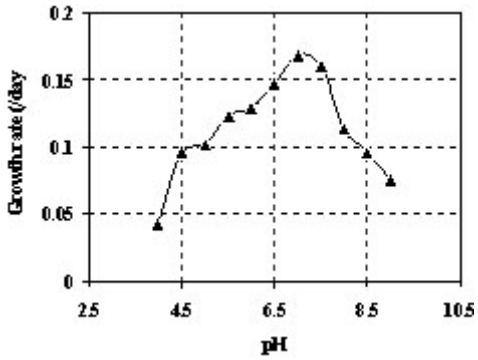

Fig. 4. pH dependence of eicosane biodegradation by *P. frederiksbergensis*.

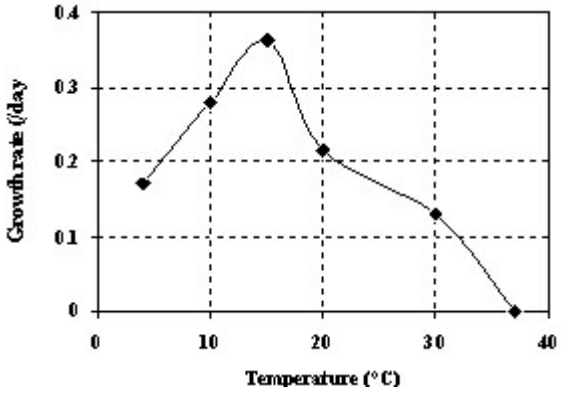

Fig. 5. Temperature dependence of eicosane biodegradation by *P. frederiksbergensis*

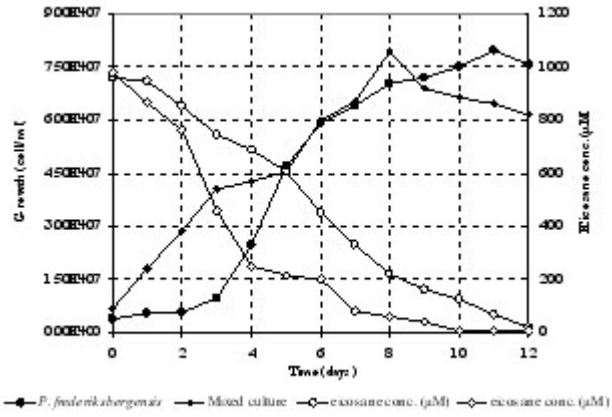

Fig. 6. Growth and biodegradation of eicosane by *P. frederiksbergensis*, and the mixed culture at optimal growth conditions

Growth characteristics of *P. frederiksbergensis* on eicosane and docosane crystals

The growth of *P. frederiksbergensis* on eicosane crystals on MSM is presented in Fig. 7a. After the growth of *P. frederiksbergensis*, clear dark zones could be observed in Fig. 7b. Microscopic examination showed the direct interfacial accession represented in the close direct contact of the cells to the crystals. This led to the increase in the bioavailability and subsequent biodegradation of the eicosane crystals. Microscopic examination showed that most bacterial cells were found around the eicosane crystals or as aggregates in the hydrophobic phase. One of the observations that should be taken into consideration was, that during the subcultivation

of the pure strain the capabilities of mineralizing and utilizing *n*-alkane were not lost. Moreover, the subcultivation on complex medium and cultivation again on eicosane MSM did not affect the activity and efficiency of *P. frederiksbergensis*.

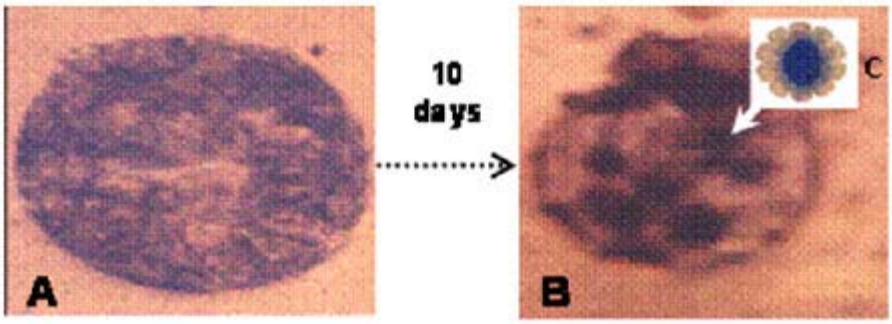

Fig. 7 . Growth characteristics of *P. frederiksbergensis* on eicosane crystals. Eicosane crystal before (A) and after (B) the growth of the bacteria. (C) refers to the single colony of *P. frederiksbergensis*

Substrate spectrum and biodegradative capabilities of *P. frederiksbergensis*

Many compounds of different chemical groups were tested as sole of carbon and energy sources. Among these compounds, *P. frederiksbergensis* showed tendency towards medium chain as decane, hexadecane, heptadecane, long chain alkane as docosane and some alcohols as docosanol, octdecanol. The biodegradative capability of utilizing mixture of decane ($C_{10}H_{22}$), hexadecane ($C_{16}H_{34}$) and eicosane ($C_{20}H_{42}$) as sole of carbon and energy sources was examined. The degradation experiments were carried out using 1 mM of each compound. 100% of decane was degraded after 9 days and hexadecane after 12 days. When about 90% of decane and 60% of hexadecane were consumed, the degradation of eicosane started (Fig. 8). It was observed that only 5% of eicosane was degraded after 6 days. The biodegradation of eicosane was slower compared to the biodegradation without mixing with decane and hexadecane.

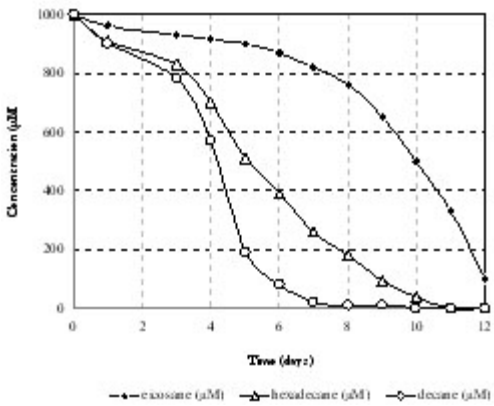

Fig. 8. Time course of the biodegradation of a mixture decane, hexadecane and eicosane by *P. frederiksbergensis*

DISCUSSION

Identification and characterization of the psychrophilic isolate

*Pseudomona*s *frederiksbergensis* was isolated from this mixed culture, characterized and identified according to the cell wall fatty acids analysis and 16S rDNA sequence. It was classified under the obligate psychrophiles organisms since the optimal temperature for the growth and the biodegradation was 15°C in accordance with Whyte *et al*. (1999). In addition, the growth temperature ranges from 4 to 20°C.

In fact, during the growth of the hydrocarbon degrading psychrophilic bacteria on long chain alkanes, many substrate physical parameters such as density and viscosity are altered and shifted to be more available to bacteria (Boseker *et al*. 1989). Furthermore, at low temperatures, the viscosity of oil decreases, reducing the degree of oil spreading in aqueous phase (Witholt *et al.* 1990). Additionally, at or below 4°C the obligate psychrophiles always outgrew the facultative psychrophiles whilst at higher temperatures the position was reversed. This obligate psychrophile appears to be more completely adapted to cold environments, although the biochemical basis of obligate psychrophiles is still poorly understood. The degradation by the mixed culture was faster compared to the pure strain. The degradation of eicosane by *P. frederiksbergensis* and the mixed culture was rather similar and this reflects the similarity in the physiological adaptation in utilizing these hydrophobic substrates.

Moreover, the increase of the growth rate $\mu(d^{-1})$ ~ 2 times can be related to the temperature which is an important physical factor that may increase metabolic rates as well as chemical reactions. In the marine environment, an increase of 10°C would double the rate of abiotic chemical reactions (Morita 1966; Whyte *et al*. 1999). However, the increased of temperatures make the biological membranes more fluid due to increased vibrational activity of the fatty acid chains in the phospholipid bilayer (Meer *et al*. 1992). This increased fluidity has been shown to raise the rate of substance uptake from a cell' surrounding medium. In contrast, a decrease in temperature has been found to decrease metabolism and depuration (Whyte *et al.* 1996). Therefore, in order to optimize the biodegradation activity of psychrophiles in contaminated sites, it is essential to develop a basic understanding of their physiology and ecology as well as the genetics and biochemistry of their catabolic pathways (Kohshima 2000).

On the other hand, temperature is a prime consideration, and cold environments generally demonstrate less or lower, biodegradation due to the higher viscosity of the hydrocarbon. The ability of P. frederiksbergensis to function well at low temperature depends a lot on the substrates availability. When the bacteria are grown in optimal temperature the transport of the substrates will be ideal through the membrane, hence the growth rate $\mu(d^{-1})$ increased. It can be observed that the growth over the maximum growth temperature, the transport of the substrates is impaired. This fact can be simply explained that near the maximal activity the intracellular enzymes are being inactivated (Varanasi *et al*. 1981).

Degradation of long chain alkanes at low temperature

The substrate concentrations were quantified by gas chromatography mass spectrometry (GC-MS) analysis and 1mM of the substrate was actually degraded within the first 12 days. There was no growth occurred during the cultivation on dotricontane ($C_{32}H_{66}$), and tetracontane ($C_{40}H_{82}$) was observed. The bioavailability of long chain alkanes compounds is the critical factor controlling the growth and the biodegradation rate. This could be simply explained that alkanes generally are insoluble in polar solvents such as water because the polar molecules of the water are not attracted to the non-polar alkanes molecules, hence *P. frederiksbergensis* could utilize and assimilate the substrates as sole of carbon and energy source by releasing biosurfactants in the medium. This psychrophilic organism may be better suited for in situ bioremediation of cold contaminated sites. It was clear that *P. frederiksbergensis* degrades short, medium chain alkanes (C_{10} to C_{16}) and long chain alkanes (C_{20} to C_{22}) more readily than longer chain alkanes as (C_{32} to C_{40}) which is common feature of many other alkanes degradative microorganisms. The mineralization of longer chain alkanes, particularly C_{32} to C_{40} was rather difficult and this was due to the low bioavailability of these compounds at low temperature. This explained the lack of growth of *P. frederiksbergensis* on the previous compounds and attributed to their low bioavailability. The ability of *P. frederiksbergensis* to oxidize various *n*-alkanes may indicate that this strain might possess more than one alkane hydroxylase, one with specificity for short chain, the other for long chain alkane. Different alkane hydroxylases in one organism may be responsible for oxidation of different range of alkanes with overlapping substrates ranges (Van Beilen *et al.* 2002).

Therefore, successful bioremediation strategies could require the application of cold

active solubilizing agents to increase the bioavailability of long chain alkanes. The pattern of substrate utilization by *P. frederiksbergensis* at the lower temperature was similar to that observed at 15°C indicating that this organism may be physiologically better adapted for assimilation of these substrates at higher temperatures.

In fact, the isolate in this study showed a valuable, effective and stable degradation capacity from C_{10} to C_{22} at temperature range of 4 to 20°C. The major challenge to meet was the activity assay for the oxidation of alkanes. In the crude extract the activity was determined by the decrease of the substrate by GC-MS. Although the ability to grow exclusively with decane, but not hexadecane, was found to be specific for gram-negative bacteria (Vomberg and Klinner 2000), P. frederiksbergensis represented an exception from this fact. During the growth of P. frederiksbergensis on mixture of n-alkanes, the uptake proceeded sequentially in order of increasing molecular weight. The biodegradation increased slowly even though the metabolism was high enough to maintain the bacterial activity stable. It was also observed that the first 6 days of incubation were the most important and critical stage for the biodegradation of the mixture. These results confirmed that this strain has the ability to oxidise alkanes of various chain length and indicated that P. frederiksbergensis possessed different alkane hydroxylase (Otto 2001).

Medium acidification during the growth of P. frederiksbergensis on eicosane

One of the interesting observations, that the pH value was dropped during the growth of P. frederiksbergensis (Siddique *et al.* 2002). This greatly contributed in the medium acidity. This could be explained that the isolated strain has the capability of oxidizing the eicosane to corresponding alcohol which was oxidized to aldehyde and further more to fatty acid. The catabolism of eicosane released some of the fatty acids containing H^+ in the growth medium and this caused medium acidification.

Environments can also challenge the biodegrdative strains due to their non-optimal pH. Higher rates of biodegradation have frequently been observed near neutral pH. It is clear that the increase in cell number especially the exponential growth phase took place after the 5th day till the 7th day and afterwards and because of the medium acidification, the bacteria reached the stationary growth phase quickly.

CONCLUSIONS

Microbial processes in the various kinds of aerobic and anaerobic systems for treating industrial, agricultural and municipal wastes are very important because these systems represent the first point of discharge of many chemicals into environment. Cold-adapted microorganisms as *P. frederiksbergensis* play a significant role in the in situ biodegradation of hydrocarbons in cold environments, where ambient summer temperatures often coincide with their growth temperature range. The effective and stable degradation capacity of this isolate in utilizing and degrading *n*-alkane (C_{10}-C_{22}) reflected their potential in biotechnological application at low temperature bioremediation of petroleum contaminated marine sites.

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REFERENCES

1. Bosecker, K, Teschner M, Wehner H., Biodegradation of crude oils. *Schriftenr der Wasser Boden Lufthyg* 1989; **80**: 91-117.
2. Hasanuzzaman, M, Ueno A, Ito H, Ito Y, Yamamoto Y, Yumoto I, Okuyama H., Degradation of long-chain *n*-alkanes (C_{36} and C_{40}) by *Pseudomonas aeruginosa* strain WatG. *International Biodeterioration and Biodegradation* 2007; **1**: 40-43.
3. Herbert RA, Burill CR., Growth characteristics of an obligatory psychrophilc *Vibrio* sp. *Archives of Microbiology* 1997; **113**: 215-220.
4. Kiyohara H, Nago K, Yanak K., Rapid screen for bacteria degrading water insoluble hydrocarbon on agar plates. *Applied and Environmental Microbiology* 1982; **43**: 454-457.
5. Kohshima S., Psychrophilic organisms in snow and ice. *Biological Sciences in Space* 2000; **4**: 353-362.
6. Marquez-Rocha FJ, Hernandez-Rodri V, Lamela MT., Biodegradation of diesel oil in soil by a microbial consortium. *Water, Air and Soil*

Pollution 2001; **128**: 313-320.

7. Meer J, De-Vos W, Harayama S, Zehhnder A., Molecular Mechanisms of genetic adaptation to Xenbiotc compounds. *Microbiology and Molecular Biology Reviews* 1992; **65**: 677-694.
8. Morita RY., Marine psychrophilic bacteria oceanoger. *Marine Biology* 1966; **4**: 105-121.
9. Nedwell BD, Rutter M., Influence of temperature and growth rate and composition between psychrtolerant antractic bacteria, low temperature diminishes affinity for substrate uptake. *Journal of Applied Microbiology* 1994; **60**: 1984-1989.
10. Otto K., Characterization of thermostable alkane axidizing system. PhD thesis, Technical University of Hamburg-Harburg, 2001; 116.
11. Siddique T, Okeke BC, Arshad M, Frankenberger WT., Temperature and pH effects on biodegradation of hexachlorocyclohexane isomers in water and soil slurry. *Journal of Agricultural and Food Chemistry* 2012; **18**: 5070-5076.
12. Stberga L O, Jonssonb PA, Lundstrom SU., Accelerated biodegradation of *n*-alkanes in aqueous solution by the addition of fermented whey. *International Biodeterioration and Biodegradation* 2006; **57**: 190-194
13. Van Beilen JB, Smits TH, Whyte L, Schorcht S, Röthlisberger M, Plaggemeier T, Engesser K, Witholt B., Alkane hydroxylase homologues in gram-positive strains. *Environmental Microbiology* 2002; **4**: 676-682
14. Varanasi U, Gmur DJ, Reichert WL., Effect of environmental temperature on naphthalene metabolism by juvenile starry flounder (*Platichthys stellatus*). *Archives of Environmental Contamination and Toxicology* 1981; **2**: 203-214
15. Vomberg A, Klinner U., Distribution of *alk*B genes within *n*-alkane degrading bacteria. *Journal of Applied Microbiology* 2000; **89**: 339-348
16. Whyte LG, Bourbonnie L, Greer CW., Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (*alk*) and naphthalene (*nah*) catabolic pathway. *Applied and Environmental Microbiology* 1999; **63**: 3719-3723
17. Whyte LG, Greer CW, Innis M., Assessment of the biodegradation potential of psychrophilic microorganisms. *Canadian Journal of Microbiology* 1996; **42**: 99-106
18. Whyte LG, Slagman SJ, Pietrantonio FL, Bourbonnie RE, Koval S Lawrence, F, Innis J R, and Greer CW., Physiological Adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. *Applied and Environmental Microbiology* 1999; **7**: 2961-2968
19. Witholt B, Smet MJ, Kingma J, Van Beilen JB, Kok M, Lageveen RG, Eggink G., Bioconversions of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors, background and economic potential. *Trends in Biotechnology* 1990; **8**: 46-52
20. Zhuang WQ, Tay JH, Maszenan AM, Tay ST Isolation of naphthalene-degrading bacteria from tropical marine sediments. *Water Science and Technology* 2013; **47**: 303-308.