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Full Length Research Paper

Hexadecane degradation by bacterial strains isolated from contaminated soils

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A study was undertaken to detect and monitor the degradation of hexadecane by three potential degrading bacteria (*Pseudomonas putida*, *Rhodococcus erythroplolis* and *Bacillus thermoleovorans*) isolated from contaminated soils in Riyadh, Saudi Arabia. The extraction of the bacterial populations from these polluted soils were 5.25 x 10^5, 1.76 x 10^6 and 5.11 x 10^5 cells/ml, respectively with three different colony types of bacterial strains. Microbial population diversity studies were carried out by microbial enumeration identification and determination of growth responses of bacterial isolates in different concentrations of hexadecane. Phenotypic examination of the heterotrophic bacteria belonged mainly to the genus *Pseudomonas*, *Rhodococcus* and *Bacillus*. The mixed populations were capable of degrading hexadecane up to 120 ppm. The biodegradation of hexadecane by *P. putida*, *R. erythroplolis* and *B. thermoleovorans* together, was fast when compared to the biodegradation of hexadecane by each strain separately. This study reported on how to change contaminated soils to a non contaminated state. Healthy soils are essential not only to sustain production of food and fibber for citizens of the world, but also to provide a good quality life.

Key words: Hexadecane, biodegradation, *Pseudomonas putida*, *Rhodococcus erythroplolis*, *Bacillus thermoleovorans*.

INTRODUCTION

Pollution, due to petroleum oil, is now a prevalent ecological hazard and hence, microbial degradation of hydrocarbons remains a topical issue as before (Singh et al., 2009). Contaminations in soil may enter the soil environment purposefully as in a pesticide application. Hexadecane is one of such water immiscible hydrocarbon substrate which forms an important constituent of oil. Soil contaminated with hexadecane has posed a great hazard for terrestrial and marine ecosystems. So far, biodegradation suggests an effective method (Morgan et al., 1989). One major mechanism employed by hydrocarbon degrading organisms to utilize such substrates is the production of biosurfactants. Biodegradation of hexadecane agricultural agrochemicals and other environmental pollutants in natural ecosystems is quite complex as it occurs relatively slow. During biodegradation, hexadecane is used as an organic carbon source by a microbial process resulting in the breakdown of low molecular weight compounds. Based on the facts known from alkane oxidation, it was assumed that the reaction catalyzed by alkane monooxygenase resulted in an alcohol which is further oxidized in the corresponding fatty acid via aldehyde by an alcohol and an aldehyde dehydrogenase, respectively. The presence of primary alcohols and aliphatic fatty acids of the same chain length in cultures grown on hexadecane indicated that the oxidative attack was on one of the methyl groups of the hydrocarbon molecule. This mode of attack is similar to what has been observed in other bacterial systems metabolizing n-alkanes as shown in Equation 1. The fundamental details of the metabolic pathways involving qualitative determination of interme-

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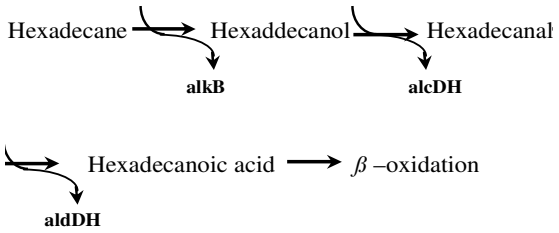

----- Eq. 1

diates in supernatants are well documented (Britton, 1984; Barreto et al., 2010).

In most cases, the initial metabolic attack on n-alkanes is done by a hydroxylase (monooxygenase) enzyme producing the corresponding alkanol (Noordman and Janssen, 2002; Abdel-Megeed, 2004). This assessment includes evaluations of the ease or difficulty of degradation in the ability to achieve total mineralization as well as the environmental conditions necessary for mineralization. Regulatory officials and citizens demand information on the potential degradation products and their potential hazard (Whyte et al., 1999).

Microbial transformations of organic compounds are frequently described using the terms detoxification degradation and mineralization (Singh et al., 2009). The cleanup of soils and ground water contaminated with hydrocarbons is of particular importance in minimizing the environmental impact of petroleum and petroleum products in preventing contamination of potable water supplies. Consequently, there is a growing industry involved in the treatment of contaminated topsoil, subsoil and groundwater (Morgan and Watkinson, 1989).

Therefore, the objectives of the present work are to identify microorganisms capable of degrading hexadecane with views to a future employment in the bioremediation of polluted soils. Understanding the microbial degradation process of hexadecane will increase possibilities of developing models and strategies for removing hydrocarbon from contaminated sites.

MATERIALS AND METHODS

Sample collection

South of Riyadh, Saudi Arabia was the study area of this investigation. The soil samples contaminated by hexadecane were collected from 5 - 10 cm depth of Riyadh agricultural area according to the method described by American Society for Testing and Materials (1998). During operation, sterile plastic bottles were used in collecting the samples after which they were transported and stored at laboratory conditions.

Isolation and culturing conditions

Microorganism isolation was carried out using selective media for the isolation of hexadecane degrading microorganisms as described by Abdel-Megeed (2004). Nutrient broth and mineral salt medium (MSM) were the liquid media, while mineral salt agar was the solid media. The extraction of the hexadecane was carried out according to Deppe (2003). Heterotrophic bacteria containing sample were enumerated by making serial dilutions of the soil extraction liquid sample using nutrient broth as the diluent. Mineral salt agar was employed in determining the density of hexadecane degrading bacteria. In all the growth studies, temperature of incubation was 37°C and aeration was maintained by agitation on a rotary shaker [Cultivation of the cell: (HL-Infos AG, Switzerland), Incubator (Köttermann, Germany)] (200 rpm). The isolated strains were characterized and identified by determination of the cell wall composition. Initial identification was based on the criteria of Bergey's Manual of Determinative Bacteriology. Further classification and identification were performed by King Faisal Specialist Hospital and Research Center by fatty acids analysis and 16S rDNA sequencing.

Degradation capacity of hexadecane by various isolates

Three dominant isolates which grew profusely on the mineral salt hexadecane agar medium were used for this study. The isolates were streak inoculated onto nutrient agar plates and incubated at 20°C for 24 h to check for viability and purity. In order to get the optimal growth temperature at pH 7.0, the temperature was adjusted to 4, 10, 15, 20 and 30°C. The cell growth was measured by cell counting technique in time interval of 24 h. The isolates from the pure plates were inoculated aseptically into 20 ml of sterile nutrient broth in screw-capped flasks. These were incubated at 20°C for 24 h. Serial dilutions of the broth cultures were prepared and 0.1 ml volumes were inoculated onto duplicate plates using the pour plate technique. These were incubated aerobically at 20°C for 24 h. The mean counts on the duplicate plates were used to calculate the number of cell/ml of the original broth culture. Mineral salt medium was prepared in sterile 250 ml capacity cotton stopper flasks.

Triplicate flasks with different concentrations (30, 60 and 120 ppm) of hexadecane were prepared. Hexadecane was mixed properly with the mineral salt medium and 1 ml of the isolates was added to each flask. The flasks were incubated at 20°C for a period of 12 days. To measure microbial growth and pH sample, aliquots were withdrawn at 2 day interval. Serial dilutions of samples were carried out using sterilized mineral salt solution as the diluents. Aliquot of 0.1 ml of samples were inoculated onto duplicate plates employing the pour plate technique. All inoculated plates were incubated aerobically at 20°C for 24 h, after which cell numbers were counted and mean counts were recorded.

Gas chromatography analysis

Hexadecane was analyzed according to the method reported by Tzing et al. (2003) and Abdel-Megeed and Mueller (2009) with a Varian Gas Chromatograph-Mass Spectrometer (GC-MS; model CP-3800 gas chromatograph and Saturn 2200 mass spectrometer Varian Technologies Japan Inc.). Resulting chromatograms were analyzed by Saturn Software GC/MS workstation (version 5.52) to identify hexadecane degradation. All analyses were carried out with the split ratio of 20:1. Helium was used as the carrier gas with a flow rate of 0.8 ml min^{-1}. Injector temperature was set at 250°C. Hexane was used as internal standard and organic solvent.

RESULTS AND DISCUSSION

Identification and characterization of the isolates

Three bacterial isolates were identified on the basis of their cultural and biochemical characteristics and with

Table 1. Morphological and physiological properties of *P. putidas*, *B. thermoleovorans* and *R. erythropolis*

Property	*P. putida*	*R. erythropolis*	*B. thermoleovorans*
Motility	-	+	+
Gram reaction	-	+	-
Adipate	-	+	+
Caprate	+	+	+
Arabinose	+	+	+
m-inositol	+	+	+
Catalase	+	+	-
n-acetylglucosamin	+	+	+
Pigment fluorescent	+	-	-
D-xylose	+	+	-
Malat	+	-	-
Maltose	-	-	-
Mannitol	+	+	+
Shape of the cell	rods	short rods	coccus
NO_2 from NO_3	-	-	-
Oxidase	+	+	+
Phenylacetate	+	+	+
Pigment fluorescent	+	-	+
Sorbitol	+	+	+

+: Growth; -: no growth.

reference to Bergey's Manual of Determinative Bacteriology (9th edition). The bacterial isolates were *Pseudomonas putida* (GPo1), *Rhodococcus erythroplolis* (DMS 43066) and *Bacillus thermoleovorans* (IHI-91). Further, identifications and characterization by 16S rDNA sequence and fatty acids analyses were performed (Table 1).

Effect of hexadecane on the mixed culture

The standard cultures of the isolates *P. putida, R. erythropolis* and *B. thermoleovorans* were found to be 5.25 x 10^5, 1.76 x 10^6 and 5.11 x 10^5 cell/ml, respectively in the extraction sample. According to the preliminary experiment carried out using different concentration (30, 60 and 120 ppm) of hexadecane, the mixed culture exhibited high efficiency of assimilating the hexadecane. The bacterial growth reached its maximum value of 5.66 x 10^7 cell/ml. Fluctuations in bacterial counts expressed as number of bacteria cells per ml, over the incubation period of 12 days in mineral salt medium containing hexadecane as sole carbon source, was monitored (Figure 1). No growth or degradation occurred after 8 days. The maximum concentration of hexadecane utilized by the mixed culture was 120 ppm.

Taking into the account the existence of the heterotrophic bacteria that ranged from 4.20 x10^4 to 9.86 x 10^5 cell/ml in the mixed culture, the indigenous microbial communities are likely to contain microbial populations of different taxonomic characteristics which are capable of degrading the contaminating chemicals (Barth and Atlas, 1977). This observation is in line with reports of Marty et al. (1979) that there was an increase in heterotrophic bacterial population in the presence of dispersant agents. Also, with a full agreement from Calomiris et al. (1976), an important fact is that the medium employed for isolation of hexadecane degrading bacteria may have a significant selective effect on the bacterial population that are sampled. The mixed population is capable of degrading hexadecane up to 120 ppm. Similar patterns of hexadecane degradation (in which the preferred degradation occurs for hexadecane) have been observed with cells of *Flavobacterium* sp. ATCC 39723 (Steiert et al., 1987) and cell extracts from *Arthrobacter* sp. This strongly suggests the complete degradation of hexadecane without the accumulation of inhibitory or toxic metabolites for these microorganisms. This is considered to be a very important issue in taking into account the degradation processes.

For *B. thermoleovorans*, there was a steady increase in the cell number from 8.1 x 10^5 to 1.5 x 10^8 cell/ml within 9 days. From the 9th day, there was a gradual decrease in cell number and by the 12th day, 3.9 x 10^7 cell/ml was recorded. From microbiological point of view, the delay in the *Lag* phase of *B. thermoleovorans* was expected since a number of different chemical reactions are involved in hexadecane utilization (Figure 2).

By the 6th day, there was a steady rise from 4.1 x 10^7 cell/ml till the 9th day where there was a final decrease on the 12th day. There was a fall in bacterial growth estimated by 3.9 x 10^7 cell/ml and the substrate was

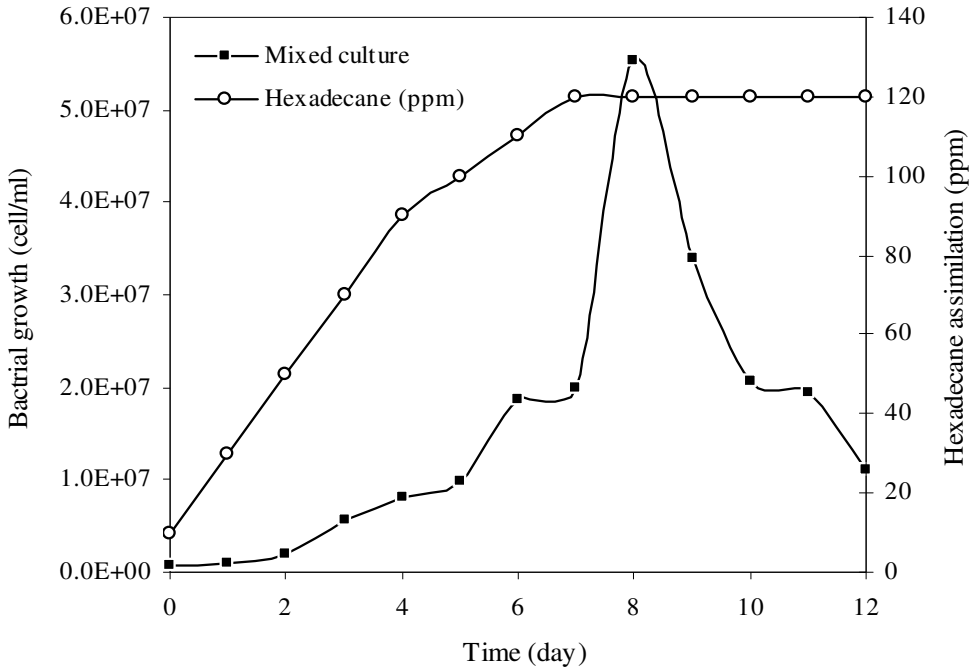

Figure 1. Biodegradative capability of the mixed culture grown on hexadecane.

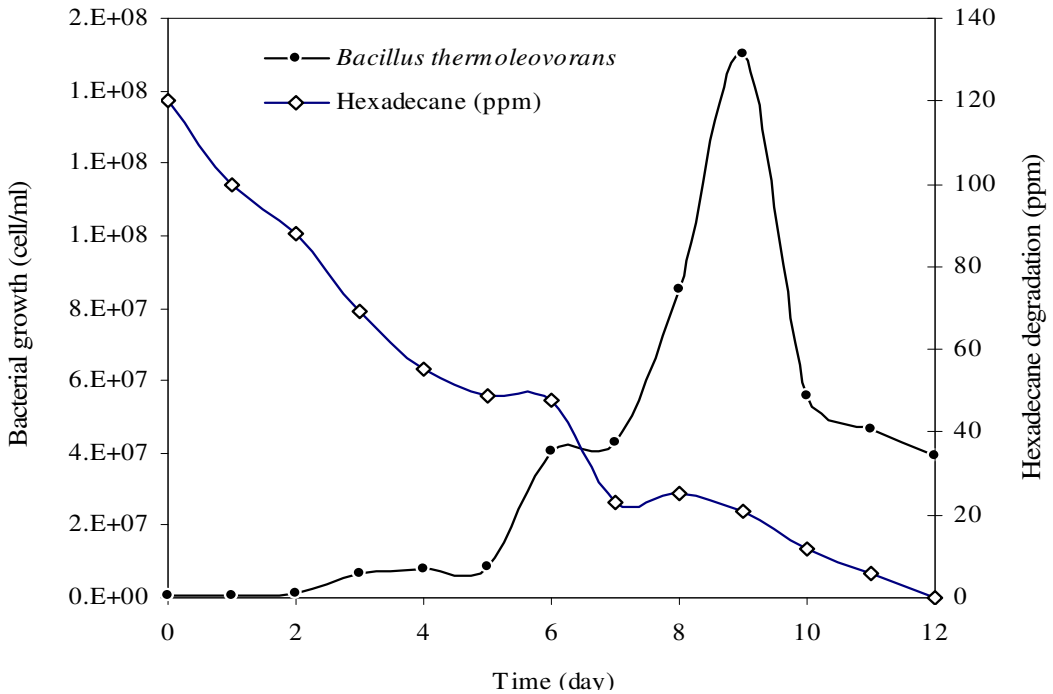

Figure 2. Growth and biodegradation of hexadecane by *B. thermoleovorans.*

totally assimilated. However, the pH was steady within this period and ranged from 6.56 to 8.38. For *P. putida*, he cell number had a steady increase from 8.25 x 10^5 to 1.02 x 10^8 cell/ml by the 9th day and this was followed by a gradual decrease till it reached 2.10 x 10^6 cell/ml by the 12th day (Figure 3). The pattern observed was oscillatory with a final count of 2.10 x 10^6 cell/ml by the 12th day. The pH however rose steadily within this period. One can notice that after the 3rd day, they began to utilize hexadecane. Hundred percent of the hexadecane concen-

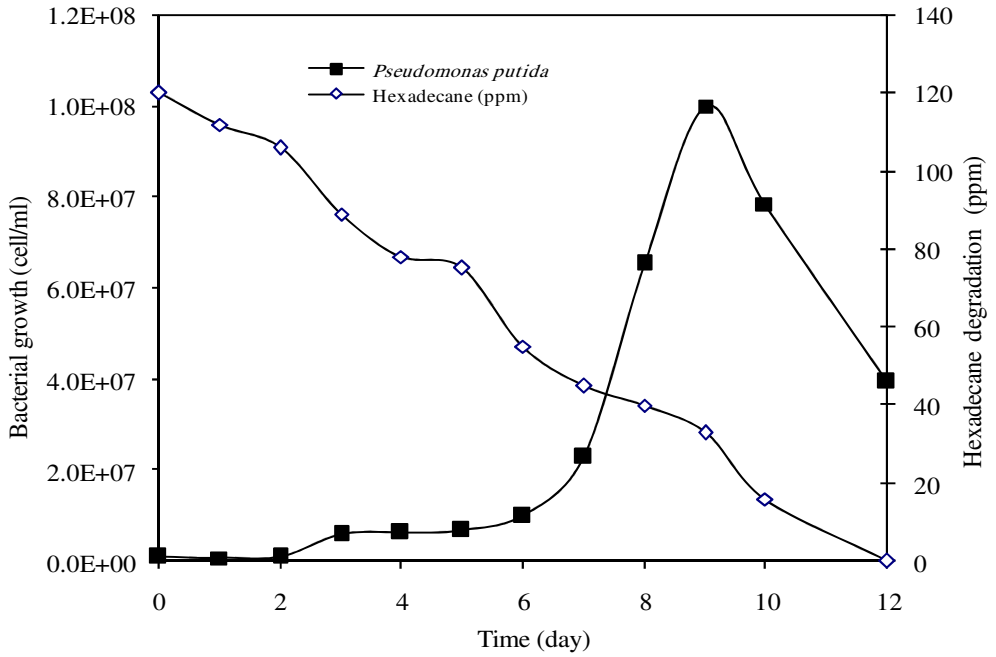

Figure 3. Growth and biodegradation of mineral oil by *P. putida.*

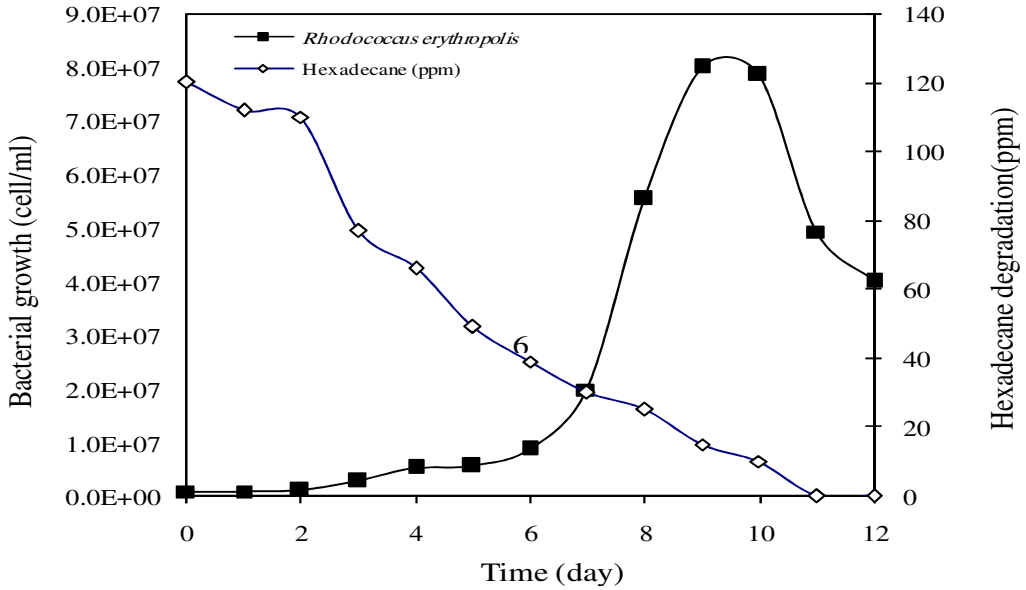

Figure 4. Growth and biodegradation of hexadecane by *R. erythropolis.*

tration was assimilated after 12 days. A similar trend in pH to the previous strain was observed during the growth pattern.

For *R. erythropolis*, the cell number had a steady increase from 8.1 x 10^5 to 7.9 x 10^7 cell/ml by the 10th day (Figure 4). The pH rose steadily to 7.64 by the 12^{th} day, while the cell number rose till the 9^{th} day. After the 9th day, there was a gradual fall in cell number. By the 12^{th} day, there was a fall in bacterial growth estimated by 4.02 x 10^7 cell/ml. It was clear that the mixed culture exhibited high efficiency compared to the isolates in the assimilation of hexadecane (Figure 5). Moreover, if a comparative study was held between the isolates and the mixed culture, it was concluded from Figure 6 that about 75% (90 ppm) of hexadecane was actually degraded after the 2^{nd} day and 100% of hexadecane (120 ppm) was

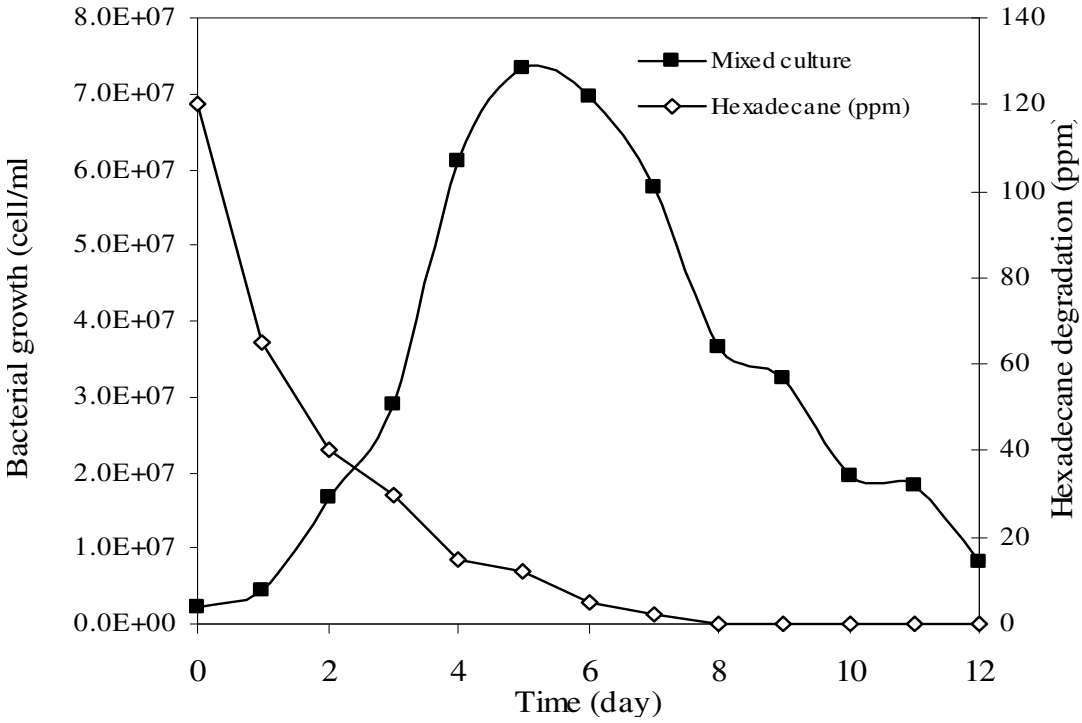

Figure 5. Cometabolite and biodegradative capability of *B. thermoleovorans*, *P. putida and R. erythropolis.*

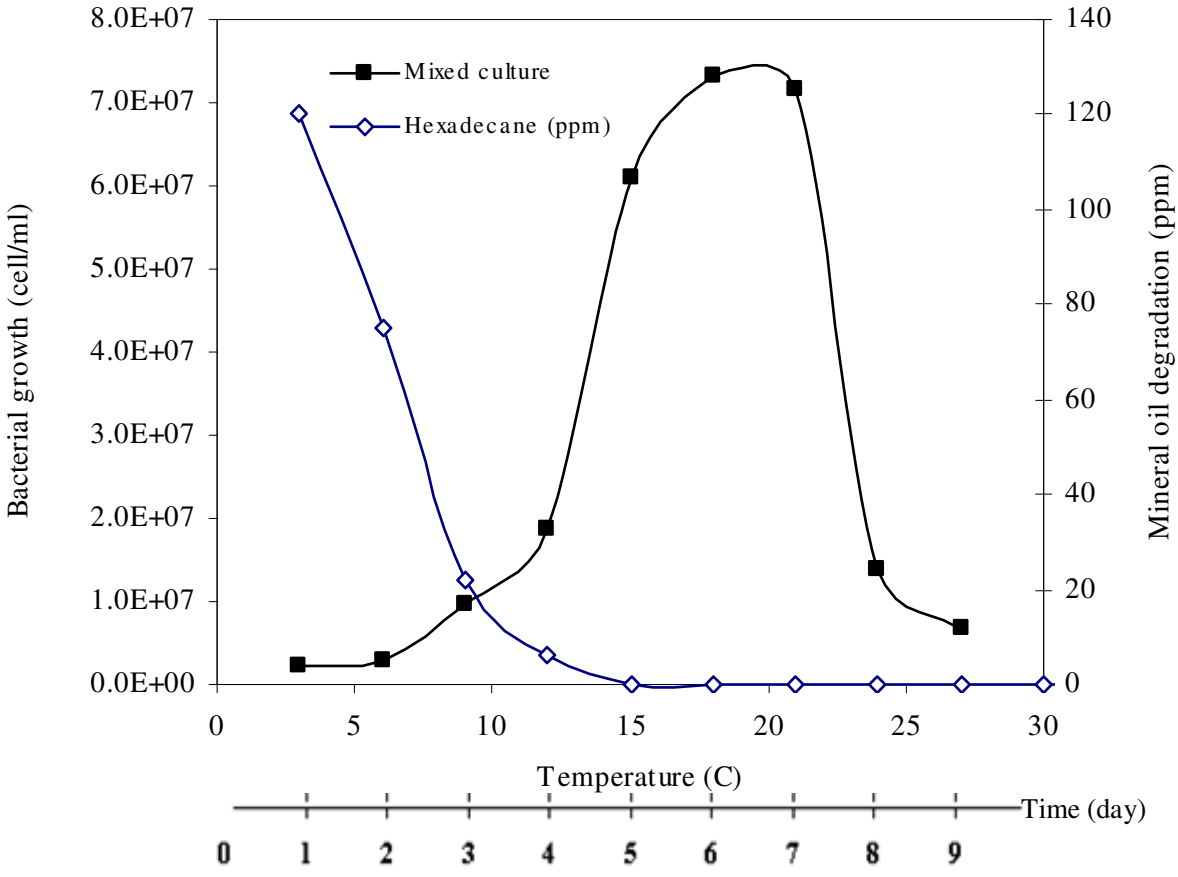

Figure 6. Temperature dependence of mineral oil degradation by the mixed cultures.

utilized after 5 days. On the other hand, after the 5th day, there was a growth reduction by which the substrate was consumed as well. The biodegradation of hexadecane was fast comparing to the biodegradation of each strain separately. They would have great application in bioremediation of hexadecane contaminated sites. Bioaugmentation treatment with such a wide spectrum of degraders would be the preferable choice of treatment over many others. The biodegradation increased fast and the metabolism was high enough to maintain the stability of the bacterial activity. It was also observed that the first day of incubation was the most important and critical stage for the biodegradation of the mixture. Results obtained in this study are similar with results obtained from soil samples at Hokaiddo and Japanese Costal water (Tanaka et al., 1993; Singh et al., 2009; Chen et al., 2009). The behavioral patterns of the hexadecane-utilizing bacteria in media containing different concentrations of hexadecane present an interesting observation.

To carry out the bioaugmentation successfully, it would be necessary to select bacteria having a high capacity and the versatility to degrade the many components of petroleum products. Consequently, bacteria that are able to grow on this carbon source would easily acquire the ability to degrade a wide variety of hydrocarbon components in different petroleum products. Therefore, bacterial screening was conducted using hexadecane as a sole carbon source. Among the three isolated candidates, strains *P. putida* and *B. thermoleovorans* appeared to be the best two degraders. Indeed, the mixed culture was capable of degrading an excess amount of hexadecane (120 ppm) present in the media in an efficient manner. It was observed that the mixed culture utilized hexadecane effectively. As such, *Rhodococcus erythropolis* would be a unique strain possessing the ability to degrade a wide spectrum of hydrocarbons. Currently, evaluation of *B. thermoleovorans,* regarding its ability to decontaminate soils from hexadecane at laboratory scale, is in progress.

Temperature dependence of growth by mixed culture grown on hexadecanes

Another interesting observation during the growth of mixed culture on hexadecane was that the increase in the temperature value enhanced the biodegradation of the hexadecane (Figure 6).

It was also observed that the degradation efficiencies were dependent on the temperature fluctuations. Additionally, the increase in temperature makes the biological membranes to have more fluid due to the increased vibrational activity of the fatty acid chains in the phospholipid bilayer (Merino and Bucalá, 2007). This increase in the rate of fluidity helps in increasing the rate of substance uptake from a cell's surrounding medium.

The ability of the mixed culture to function well at high temperature depends to a great degree on the availability of the substrates. Compared to the biodegradation of three strains separately, the mixed culture could assimilate the whole amount of hexadecane within the first five days. The results conform to Colwell and Walker (1977) and Barreto et al. (2010) report on two major microbial responses to hexadecane which was an increase in microbial biomass.

In fact, when the bioremediation of hexadecane takes place, the following should be considered. Firstly, oxygen is required because biodegradative pathways are aerobic processes. Secondly, many microorganisms are capable of aliphatic hydrocarbons degradation. Thirdly, soil normally contains an adequate inoculum of natural organisms for bioremediation (Kadeeb, 2007). Friendly soil microbes may be too efficient in breaking down acting chemicals before pesticides have had a chance to protect crops. The tiny soil creatures are adapting so successfully to the new wave of non-residual chemicals, in that the chemicals may disappear before they can kill the pests. The beneficial microbes are literally eating the poison and the more pesticide applied, the quicker they devour it.

Conclusion

An enabling environment facilitates the degradation of hexadecane by microorganisms; however, pH, temperature and other growth factors required by the organisms should be optimal. Microbial degradation of hexadecane and its derivatives is an important field of biotechnology research because of the impact of oil spills in the environment.

Further prospects

Knowledge obtained from this study could help in understanding the biodegradation of hexadecane in contaminated sites as well as to design efficient biocatalyst allowing transformation of oil fractions into valuable compounds. The isolation of pure strains from such a consortium has also been achieved; likewise, its hexadecane degradation ability has been confirmed and the different effects of hexadecane on their degrading capacity have been shown. Preliminary identification of these strains has been carried out and further work still continues on their characterization. More research is necessary to understand the fundamental mechanisms of enhancement and inhibition in the microbial degradation of super high concentration of toxic compounds. Consequently, these micro-organisms could be used very effectively for *in situ* bioremediation in an environment which is highly contaminated with hexadecane. However, further research could be carried out on these isolates on genetic manipulation for improvement and exploitation as bioremediation vehicles.

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