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Biodegradation of Aliphatic Hydrocarbon Compounds by Bacterial Cultures

Pravinkumar A. Domde[1], Hemant J. Purohit[2], Rajpal Singh Kashyap[3], Shardul S. Wagh[4]
[1]Assistant Professor, Biochemistry Department, Kamla Nehru Mahavidyalaya, Nagpur, Maharashtra, India
[2]Ex-Cheif Scientist G, National Environmental Engineering andResearch Institute (NEERI), Nagpur
[3]Director Research, Central India Institute of Medical Sciences (CIIMS), Nagpur
[4]Assistant Professor, Biochemistry Department, Kamla Nehru Mahavidyalaya, Nagpur

ABSTRACT: In the present study biodegradation of alphatic hycarbon compounds by bacterial cultures has been targeted. Growth curves analysis of *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 during utilization of dodecane, hexadecane, octadecane, eicosane and tetracosane as a sole source of carbon for growth and energy was performed. During this study, the degradation of different aliphatic hydrocarbons was studied as a function of bacterial growth. The hydrocarbon degradation efficacy of different bacterial species (selected on the basis of preliminary screening studies) was determined by indirect method wherein, the change in COD (Chemical Oxygen Demand) was determined after a specific time interval (0 day to 6th day). Presence of aliphatic hydrocarbon degradation capability in the selected four bacterial cultures was substantiated by the PCR amplification of *alk*B genetic loci in three out of four cultures. Successful amplification of *alk*B gene loci in *Bacillus subtilis* PD6, *Bacillus sp.* PD9 and *Bacillus sp.* PD14 indicated that, these cultures are potential aliphatic hydrocarbon degraders and possess required genetic arsenal for degradation of n-alkanes.

KEYWORDS: Aliphatic hydrocarbons, Biodegradation, Bacterial Cultures, COD analysis, *alk*B gene, Growth curve analysis.

INTRODUCTION

Hydrocarbons of natural origin are widespread in the environment but because of the massive utilization of petroleum products, they are nowadays strongly involved in environmental pollution (Alexis Nzila (2018), Domde et. al., 2007). Biodegradation, naturally occurring (natural attenuation) or by engineered bioremediation (Das and Chandran, 2010), is a key process for the decontamination of polluted areas. Microorganisms have developed specific mechanism for the utilization of hydrophobic hydrocarbon compounds (Bouchez-Naıtali *et al.*, 2001). 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. Even more relevant are the alkanes (mainly waxes or paraffins) produced by plants, algae, and other organisms because they are available to bacteria throughout the biosphere (Smits *et al*., 2002).

On an average, saturated and aromatic hydrocarbons together make up 80% of the oil constituents (Widdel and Rabus, 2001). Aliphatic hydrocarbon compounds constitute major component of petroleum oils and contaminate water and soil significantly because of release from automobile vehicular washing. Alkanes are major components of petroleumproducts (Head *et al.*,2006). Due to deliberate or inadvertent release into water bodies they are commonly found in contaminated environments (So *et al.*, 2001). Crude oil is mainly composed of hundreds of different hydrocarbon molecules, mainly alkanes from C1 to C40 straight chain, C6–C8 branched-chain, cyclohexanes, aromatics and compounds containing sulphur, nitrogen and oxygen (Stafford *et al*., 1982, Hadibarata *et al.*, 2009). Since the saturated hydrocarbon fraction is the most abundant in crude oil, its biodegradation is quantitatively most important in oil bioremediation (Head *et al.*, 2006). n-Alkanes are relatively stable due to lack of functional groups, presence of only sigma bonds, nonpolar nature, and low solubility in water.

Aerobic microbial degradation of n-alkanes is known since almost a century, and the mechanisms of degradation, with the enzymes and genes involved, are rather well understood (Head *et al.*, 2006; Throne-Holst*etal*., 2007).

In the present study total five aliphatic hydrocarbon compounds dodecane, hexadecane, octadecane, eicosane and tetracosane were chosen (Bahl and Bahl, 2008).

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Dodecane (also known as dihexyl, bihexyl, adakane 12 or duodecane) is a liquid alkanehydrocarbon with the chemical formula$CH_3(CH_2)_{10}CH_3$ (or $C_{12}H_{26}$), an oily liquid of the paraffin series. It has 355 isomers. It is used as a solvent, distillation chaser, scintillator component. Moreover it is used as a diluent for tributyl phosphate (TBP) in plants reprocessing.

Hexadecane (also called cetane) is an alkanehydrocarbon with the chemical formula $C_{16}H_{34}$. Hexadecane consists of a chain of 16 carbon atoms, with three hydrogen atoms bonded to the two end carbon atoms, and two hydrogens bonded to each of the 14 other carbon atoms. Likewise Octadecane, Eicosane and Tetracosane consist of 18, 20, 24 carbon, with three hydrogen atoms bonded to the two end carbon atoms, and two hydrogens bonded to each of the 18, 20, 24 carbon atoms respectively.

Alkanes from pentane to hexadecane (an alkane with sixteen carbon atoms) are liquids of higher viscosity, which are less suitable for use in gasoline.

Alkanes from hexadecane upwards form the most important components of fuel oil and lubricating oil. In latter function they work at the same time as anti-corrosive agents, as their hydrophobic nature means that water cannot reach the metal surface. Many solid alkanes find use as paraffin wax, for example in candles. This should not be confused however with true wax, which consists primarily of esters.

Alkanes with a chain length of approximately 35 or more carbon atoms are found in bitumen, used for example in road surfacing. However, the higher alkanes have little value and are usually split into lower alkanes by cracking.

SOURCES OF CONTAMINATION

Aliphatic hydrocarbons enter the environment through various sources. Now a day the common source, found to be mainly responsible for the contamination of soil and water by different types of hydrocarbons, are automobile vehicles repairing service stations. Other sources include contamination of marine and terrestrial environments by oil spillage from oil tankers caused due to accidents, deliberate throwing of crude oil during war situation in the sea as happened during Iraq war (Das and Chandran, 2010, Pritchard, 1993). The petroleum hydrocarbon compounds are often introduced into the environment through contamination by crude oils, refinery products and harbor and offshore activities, including tanker wreckages (Alexis Nzila (2018), Syakti *et al.*, 2009).

In the natural environment, a spill containing only one fraction of oil such as aromatics or alkane compounds is unlikely (Atlas, 1981). However, the study on microbial breakdown of groups of pure *n*-alkanes is important from a scientific point of view since there is lack of information in this field, particularly in the area which deals with specific microorganisms and their relative effectiveness in the breakdown of long chain hydrocarbon (Fayad and Overton, 1995). Hydrocarbons are hydrophobic in nature and exhibits little solubility in the water. Bacteria have evolved a mechanism by which hydrocarbon solubility in the water and thereby bioavailability to the cells has been increased. This mechanism involves the production of biosurfactants by the bacterial cells which increases the dissolution of hydrocarbons in the water and thereby increases its uptake by the cell. This ultimately leads to the degradation of hydrocarbons.

Variety of aliphatic hydrocarbon compounds from C6 to C40 has been found to be present in the petroleum mixtures for example in the engine oil used for two wheelers and four wheelers vehicles. n-Alkane fraction of the petroleum mixture contains n-Hexane, n-Heptane, n-Octane, n-Nonane, n-Decane, n-Undecane, n-Dodecane, n-Tridecane, n-Tetradecane, n-Pentadecane, n-Hexadecane,n-Heptadecane, n-Octadecane, n-Nonadecane, n-Eicosane, n-Heneicosane,n-Docosane, n-Tricosane, n-Tetracosane, n-Pentacosane, n-Hexacosane etc. i. e. upto C40 long n-Tetracontane have been found and all these aliphatic hydrocarbons constitute major portion of oil (Alexis Nzila, 2018). Analysis of the effluent from automatic vehicle washing facilities performed by Paxéus (1996) as a part of charting organic pollutants in the municipal wastewater in Goteborg showed presence of significant amount of C8 to C30 long aliphatic hydrocarbons.

DEGRADATION STUDIES

Various researchers executed degradation studies of n-alkane by employing different aspects. Nieder and James Shapiro (1974) showed that the bacterial strain *Pseudomonas putida* PpG6 could utilize n-alkanes having 6 to 10 carbon atoms for growth which involves single alkane hydroxylase complexEmiliana Pandolfo (2023). Apart from bacteria, fungi are also involved actively in the degradation of n-alkane, for example studies executed by Hadibarata and Tachibana (2009) showed degradation of Eicosane by the fungal strain *Tricodermasp.* S019.

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In the marine environment (Ramzi H. Amran et al 2022), biodegradation is a promising process for responding to contamination by petroleum hydrocarbons, considering that each bacterial strain produced biosurfactants which facilitated the direct contact between cells and a given pollutant, thereby promoting an increase in solubilisation. Syakti *et al.*,(2009) investigated the possibility for *Corynebacterium sp.* and *Sphingomonas sp.* 2MPII to modify simultaneously the rate and extent of the degradation of n-eicosane and phenanthrene.

Noordman *et al.*, (2002) studied the uptake of hexadecane in presence of biosurfactant rhamnolipid and found that the uptake rate of hexadecane in presence of rhamnolipid by bacterial cells was enhanced, but the extent to which degradation of hexadecane is enhanced by rhamnolipid was found to be dependent on the availability of the substrate.

Whyte *et al.*, (1998) in an investigation, isolated number of psychrotrophic bacteria from environmental samples, obtained from both contaminated and noncontaminated sites across Canada, and which were shown to mineralize a variety of petroleum hydrocarbon components (toluene, naphthalene, and alkanes) at 5°C. One psychrotrophic bacterium, originally isolated from Lake Ontario and tentatively identified as *Rhodococcus sp.* Strain Q15, readily mineralized both shorter-chain ^{14}C-labelled alkanes (dodecane and hexadecane) and longer-chain alkanes (octacosane and dotriacontane) at 23°C.

_alk_B GENE SYSTEM IN BACTERIA

The best-characterized system for alkane degradation is the *alk* system of *Pseudomonas putida* GPo1, sequentially converting alkanes to the corresponding alcohols, aldehydes, carboxylic acids, and acyl-coenzyme A (CoA), which then enter the β-oxidation pathway. Most of these systems catalyze the degradation of relatively short-chain alkanes, and very little is known about enzymes involved in the degradation of Long Chain alkanes (Throne-Holst *et al.*, 2007). Long-chain (LC) alkanes, with chain lengths of >20 C atoms, are environmental pollutants and may also cause problems in recovery, transportation, and processing of crude oil by e.g., clogging pipes.

The *P. putida* GPo1 alkane hydroxylase system consists ofthree components: alkane hydroxylase (AlkB), rubredoxin(AlkG), and rubredoxin reductase (AlkT). AlkB is a non-hemeiron integral membrane protein which carries out the hydroxylationreaction. Rubredoxin transfers electronsfrom the NADH-dependent flavoprotein rubredoxin reductaseto AlkB. The molecular genetics of this enzymesystem has been reviewed by van Beilen *et al.*, (2002,2006).Genes that are closely related to the alkane hydroxylasegene (*alkB*) of GPo1 have been detected in a large fraction ofthe microbial population in oil-contaminated environments and in several fluorescent pseudomonads.

The *alk* genes identified sofar are located in two different regions of the OCT plasmid (Chakrabarty *et al*., 1973).The *alkBFGHJKL* operon encodes the alkane hydroxylase, tworubredoxins, an aldehyde dehydrogenase, an alcohol dehydrogenase,an acyl coenzyme

EXPERIMENTATION AND RESULTS

GROWTH CURVES OF BACTERIAL CULTURES ON ALIPHATIC HYDROCARBONS

Figure 1.1, 1.2, 1.3,1.4 and 1.5 show Growth curves of *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 during utilization of dodecane, hexadecane, octadecane, eicosane and tetracosane as a sole source of carbon for growth and energy respectively. All the selected bacterial cultures showed growth on dodecane, hexadecane, octadecane, eicosane and tetracosane.

The utilization of aliphatic hydrocarbons by selected bacterial cultures, as depicted by Figures 1.1 to 1.5, indicates that, hexadecane and octadecane are favoured substrates for the selected bacterial cultures.

Combined growth curve of all four bacterial cultures for dodecane utilization as shown in the Figure 1.1 suggests that *Bacillus subtilis* PD6 is efficient culture for the utilization of Dodecane whereas *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 was also found to utilize Dodecane as is evident from the increase in OD. *Bacillus sp.* PD9 was found to be least efficient culture for Dodecane utilization.

Growth curve shown in the Figure 1.2 for also depicts the predominance of *Bacillus subtilis* PD6 for hexadecane utilization followed by *Enterobacter sp.* PD11, *Bacillus sp.* PD14 and *Bacillus sp.* PD9.

In case of growth curve analysis of all the four bacterial cultures for octadecane utilization (Figure 1.3), *Bacillus subtilis* PD6 was found to be efficient culture compared to all other cultures. *Bacillus sp.* PD14 also showed significant increase in the

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growth within 48h and attained stationary phase afterwards with little increase thereafter. *Enterobacter sp.* PD11 appeared to be the average in terms of octadecane utilization whereas *Bacillus sp.* PD9 was found to be least efficient culture compared to three cultures.

Figure 1.4 shows combined growth curves of all four bacterial cultures in presence of Eicosane. *Enterobacter sp.* PD11 was an efficient Eicosane user. Eicosane and Tetracosane are solids at room temperature and remain in that state when added in the growth medium. Hence increase in OD by all the bacterial cultures was low. *Enterobacter sp.* PD11 showed maximum growth at 96 h with rapid to steady increase in the OD from 24 h to 72 h. *Bacillus sp.* PD9 also exhibited rapid increase in OD in 24 h attaining maximum growth at 72 h but it showed sudden decrease in OD after 72 h. *Bacillus subtilis* PD6 was found to follow normal growth pattern in case of eicosane while *Bacillus sp.* PD14 appeared to be least efficient culture for Eicosane utilization.

In case of Tetracosane (Figure 1.5) all the four cultures showed very little growth which was evident from the OD measured. Tetracosane is also insoluble as well as in solid state when added in growth medium resulting in nonavailability to the bacteria. *Bacillus sp.* PD9 and *Bacillus sp.* PD14 utilized tetracosane efficiently, followed by *Enterobacter sp.* PD11 and *Bacillus subtilis* PD6.

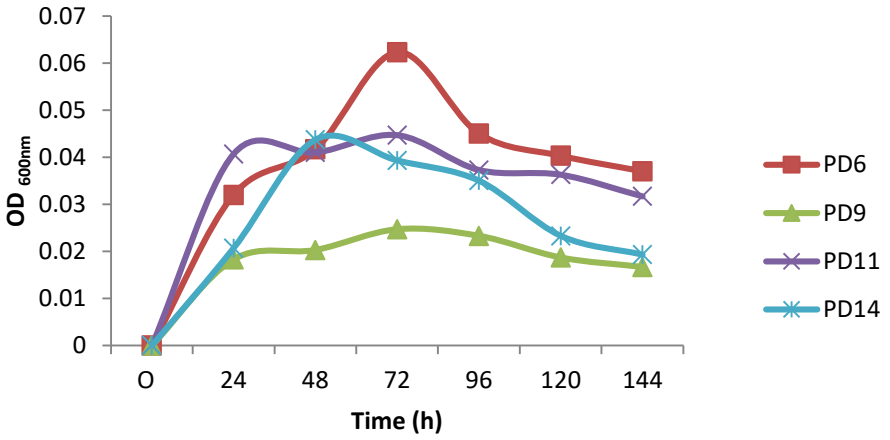

Figure 1.1 : Growth curves of bacterial isolates in presence of dodecan

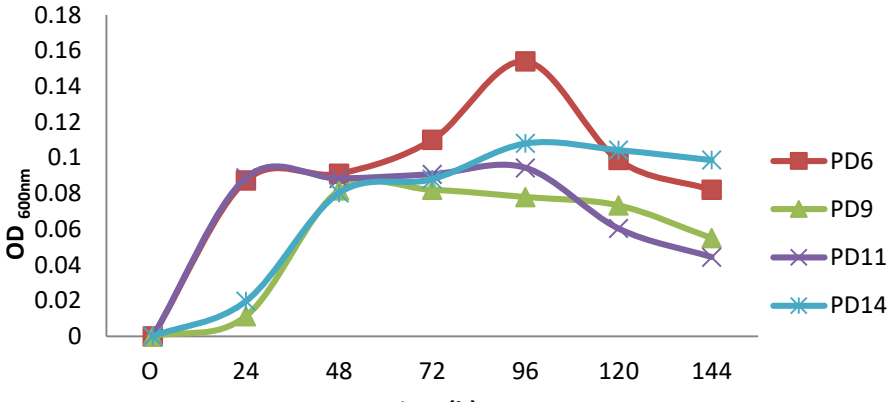

Figure 1.2: Growth curves of bacterial isolates in presence of Hexadecane

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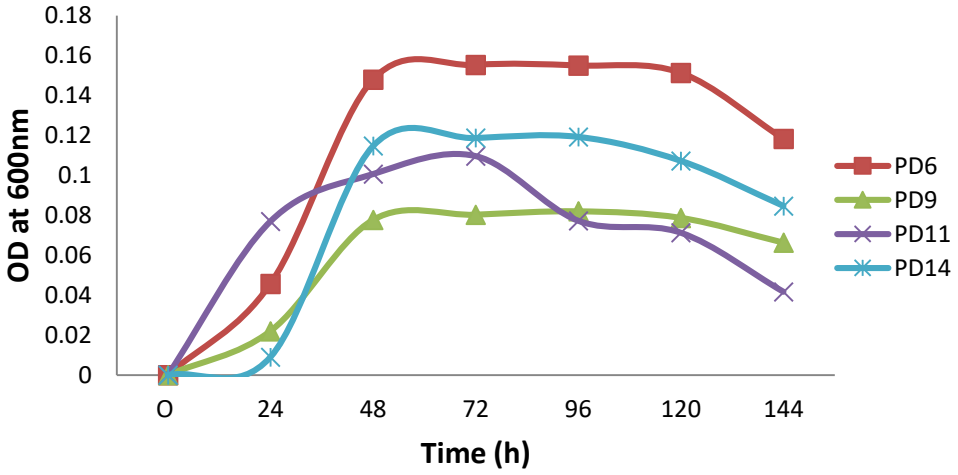

Figure 1.3: Growth curves of bacterial isolates in presence of octadecane

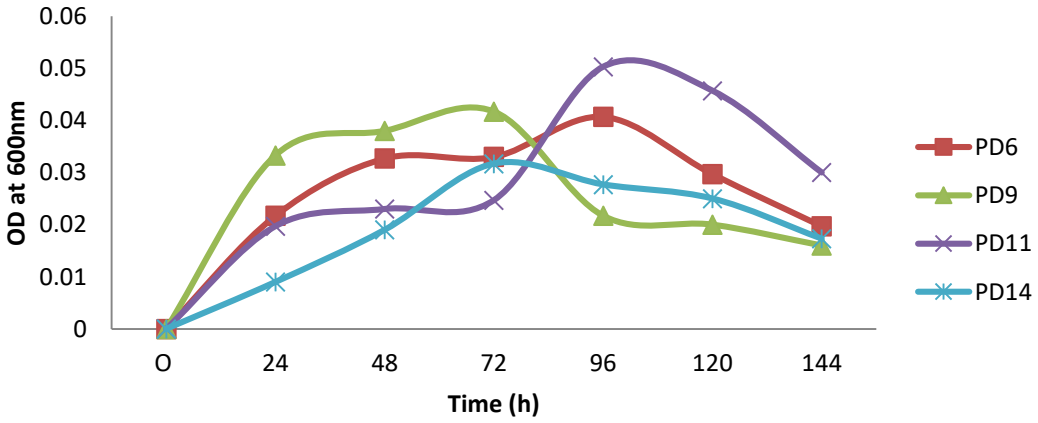

Figure 1.4: Growth curves of bacterial isolates in presence of eicosane

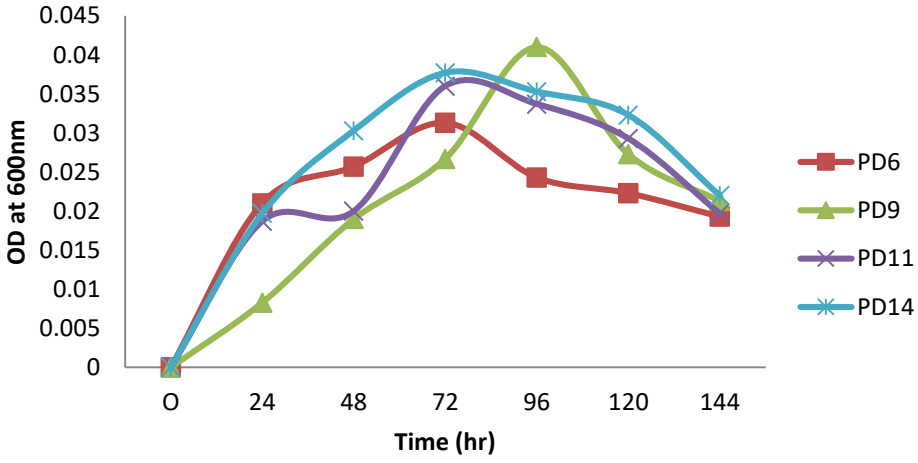

Figure 1.5: Growth curves of bacterial isolates in presence of tetracosan

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BIODEGRADATION OF ALIPHATIC HYDROCARBON COMPOUNDS BY *Bacillus subtilis*PD6*Bacillus sp.* PD9, *Enterobacter sp.* PD11,*Bacillus sp.* PD14

In the present study, the degradation of different hydrocarbons (dodecane, hexadecane, octadecane, eicosane and tetracosane) was studied as a function of bacterial growth. The hydrocarbon degradation efficacy of different bacterial species (selected on the basis of preliminary screening studies) was determined by indirect method wherein, the change in COD (Chemical Oxygen Demand) was determined after a specific time interval (0 day to 6th day).

DODECANE BIODEGRADATION

From the results, it was observed that the COD (after a period of six days was) for Control (without culture), PD6, PD9, PD11, PD14 was 1187±4, 389±8, 616±6, 627±7, 365±11mg/L respectively. The COD change observed during all the days i.e. day 0 to day 6 is presented in Figure1.6. The comparative assessment of the COD values obtained on different days was carried out using analysis of variance procedure. The results indicated that the COD change (as a function of growth of different bacterial species) during the different days was significantly ($P<0.01$) different. However, the overall dodecane (C12 aliphatic compound) degradation efficiency (after 6 days) by selected bacterial species revealed that *Bacillus subtilis* PD6, *Bacillus sp.*PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 was 67.2%, 48.1% and 47% , 69.6% respectively (Figure1.7). Hence, it was evident that culture PD14 had the best efficiency amongst the isolated bacterial species to degrade dodecane.

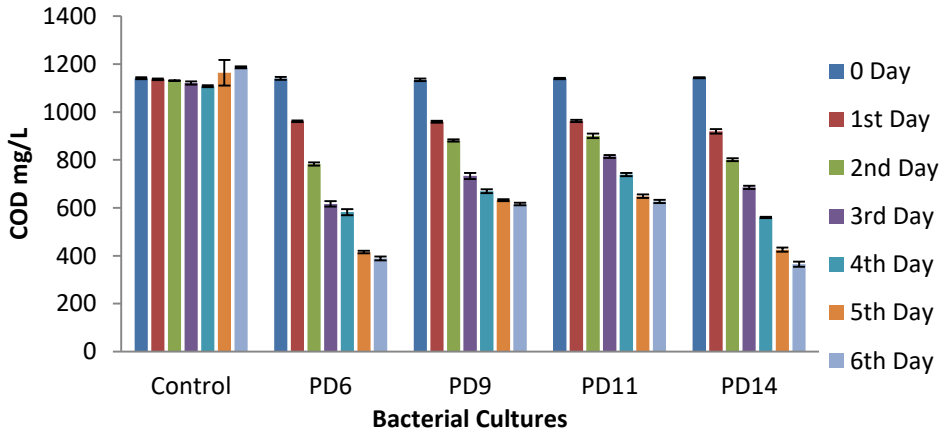

Figure 1.6: Biodegradation of dodecane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14

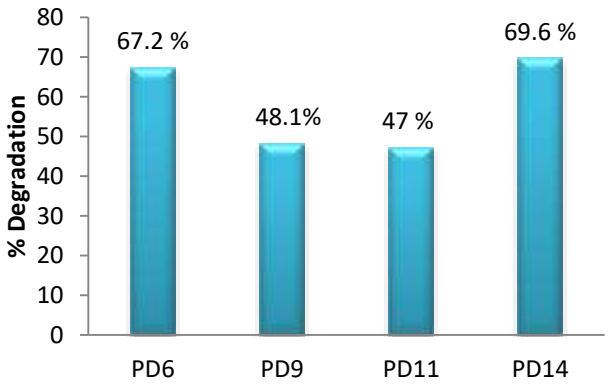

Figure 1.7: Changes in COD during biodegradation of dodecane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14

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HEXADECANE BIODEGRADATION

From the results obtained for hexadecane degradation, it was observed that the COD (after a period of six days was) for Control (without culture), PD6, PD9, PD11, PD14 was 1152±2, 451±2, 778±3, 705±56, 182±2mg/L respectively. The COD change observed during all the days i.e. day 0 to day 6 is presented in Figure1.8. The comparative assessment of the COD values obtained on different days was carried out using analysis of variance procedure. The results indicated that the COD change (as a function of growth of different bacterial species) during the different days was significantly ($P<0.01$) different. However, the overall hexadecane (C16 aliphatic compound) degradation efficiency (after 6 days) by selected bacterial species revealed that *Bacillus subtilis* PD6, *Bacillus sp.*PD9, *Enterobacter sp.* PD11 was respectively 60.8%, 32.5%, 36.4 % and 84.2% (Fig. 1.9). Hence, it was evident that culture PD14 had the best efficiency amongst the isolated bacterial species to degrade hexadecane.

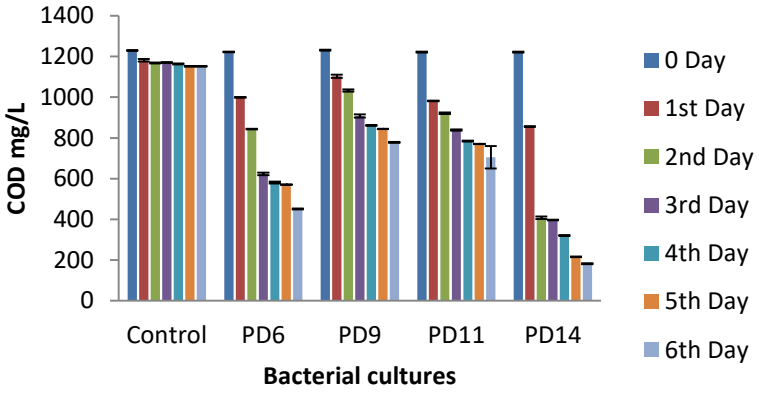

Figure 1.8: Biodegradation of hexadecane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14

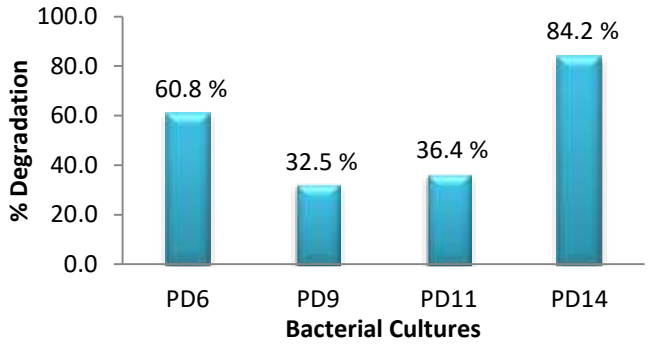

Figure 1.9: Changes in COD during biodegradation of hexadecane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and*Bacillus sp.* PD14

OCTADECANE BIODEGRADATION

From the results obtained for octadecane degradation, it was observed that the COD (after a period of six days was) for Control (without culture), PD6, PD9, PD11, PD14 was 1517±4, 671±4, 976±5, 926±6, 626±4mg/L respectively. The COD change observed during all the days i.e. day 0 to day 6 is presented in Figure1.10. The comparative assessment of the COD values obtained on different days was carried out using analysis of variance procedure. The results indicated that the COD change (as a function of growth of different bacterial species) during the different days was significantly ($P<0.01$) different. However, the overall octadecane (C18 aliphatic compound) degradation efficiency (after 6 days) by selected bacterial species revealed that *Bacillus subtilis* PD6, *Bacillus sp.*PD9, *Enterobacter sp.* PD11 *Bacillus sp.* PD14 was respectively 55.8%, 35.8%, 38.8 % and 58.8% (Figure1.11). Hence, it was evident that culture PD14 had the best efficiency amongst the isolated bacterial species to degrade octadecane

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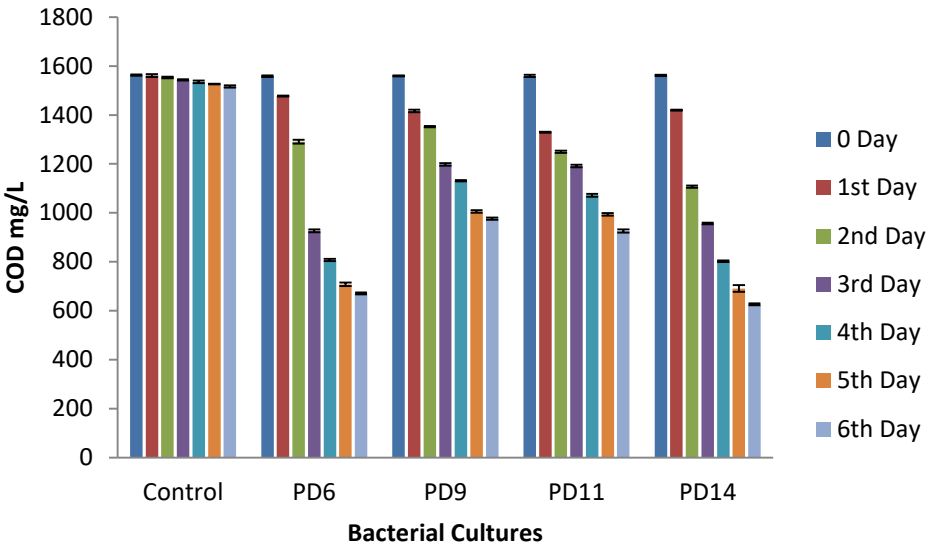

Figure 1.10: Biodegradation of octadecane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14

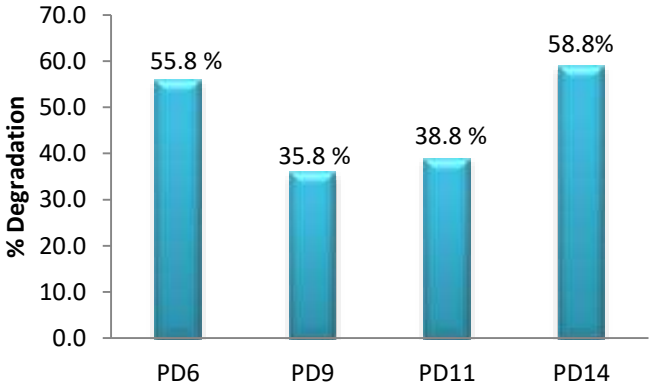

Figure 1.11: Changes in COD during biodegradation of octadecane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and*Bacillus sp.* PD14

EICOSANE BIODEGRADATION

From the results obtained for eicosane degradation, it was observed that the COD (after a period of six days was) for Control (without culture), PD6, PD9, PD11, PD14 was 1123±2, 498±7, 617±5, 591±7, 600±15mg/L respectively. The COD change observed during all the days i.e. day 0 to day 6 is presented in Figure 1.12. The comparative assessment of the COD values obtained on different days was carried out using analysis of variance procedure. The results indicated that the COD change (as a function of growth of different bacterial species) during the different days was significantly ($P<0.01$) different. However, the overall eicosane (C22 aliphatic compound) degradation efficiency (after 6 days) by selected bacterial species revealed that *Bacillus subtilis* PD6, *Bacillus sp.*PD9, *Enterobacter sp.* PD11 *Bacillus sp.* PD14 was respectively 55.6%, 44.9%, 47.6 % and 47.2% (Figure 1.13). Hence, it was evident that culture PD11 had the best efficiency amongst the isolated bacterial species to degrade eicosane.

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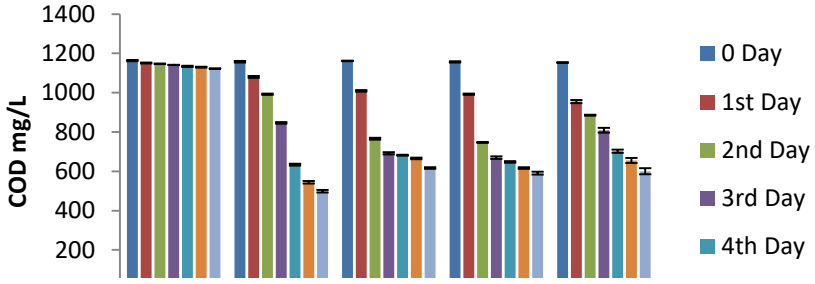

Figure 1.12: Biodegradation of eicosane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14

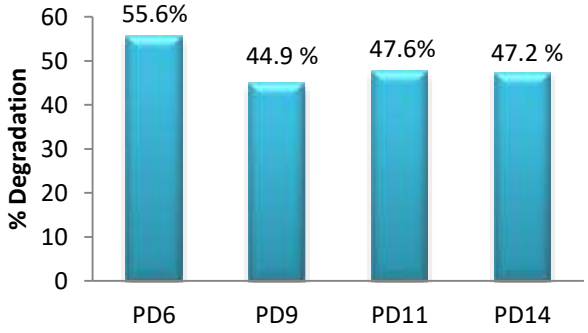

Figure 1.13: Changes in COD during biodegradation of eicosane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and*Bacillus sp.* PD14

TETRACOSANE BIODEGRADATION

From the results obtained for tetracosane degradation, it was observed that the COD (after a period of six days was) for Control (without culture), PD6, PD9, PD11, PD14 was 1071±3, 618±5, 540±7, 518±16, 585±5mg/L respectively. The COD change observed during all the days i.e. day 0 to day 6 is presented in Figure1.14. The comparative assessment of the COD values obtained on different days was carried out using analysis of variance procedure. The results indicated that the COD change (as a function of growth of different bacterial species) during the different days was significantly ($P<0.01$) different. However, the overall tetracosane (C22 aliphatic compound) degradation efficiency (after 6 days) by selected bacterial species revealed that *Bacillus subtilis* PD6, *Bacillus sp.*PD9, *Enterobacter sp.* PD11 *Bacillus sp.* PD14 was respectively 42.3%, 49.9%, 51.3 % and 45.5% (Figure1.15). Hence, it was evident that culture PD11 had the best efficiency amongst the isolated bacterial species to degrade tetracosane.

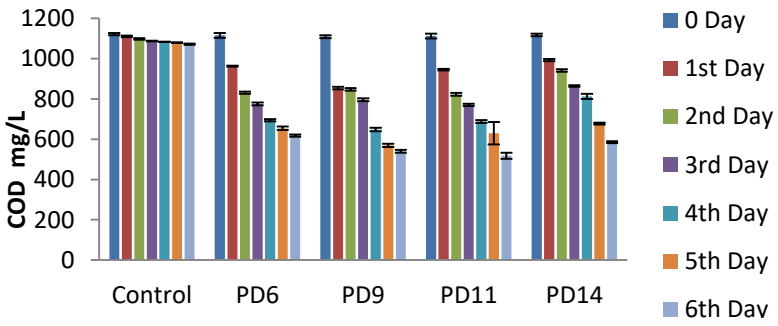

Figure 1.14: Biodegradation of tetracosane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14

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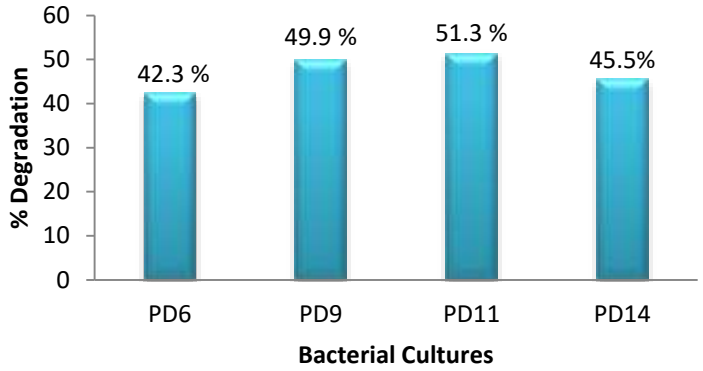

Figure 1.15: Changes in COD during biodegradation of tetracosane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14

TARGETING*alk*B GENE IN THE*Bacillus subtilis*PD6,*Bacillus sp.* PD9, *Enterobacter sp.* PD11,*Bacillus sp.* PD14.

Presence of aliphatic hydrocarbon degrading capability was assessed by amplifying 910 bp Alkane Hydroxylase (*alk* B) gene by the application of Polymerase Chain Reaction (PCR) technique. DNA samples from *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 were extracted and subjected to PCR with primers for amplification of *alk*B gene wasF 5′CCGCTCCAGAGTACGTAGATAAAA3′ and R 5′GAGTGCCGCTGAAGGTGGAACA 3′.The reaction mix contained 5 µl template solution, 1X PCR buffer; (50 mM KCl and 10 mM Tris-HCl, pH8.3), 200 µM of each of the dNTPs, 3.0 mM $MgCl_2$, 50 pmol of each of the primers and 2.5U AmpliTaq DNA polymerase (Perkin Elmer, USA) in a final volume of 50 µl.The following temperature program for the amplification was used: initial denaturation at 94ºC for 2min, 35 cycles of 94ºC for 1 min; primer annealing at 60ºC for 1 minute, extension at 72ºC for 1 minute and final extension at 72ºC for 5 min. The PCR program was performed in an Applied Biosystem thermocyler. After completion of PCR program amplified products were analysed by agarose gel electrophoresis as mentioned in the Chapter II, Section 2.6.4.8 with 1.5% concentration of gel.The PCR amplification of *alk*B gene in the isolates is shown in Figure1.16. Appearance of band at the specific location as given by *Pseudomonas Oleovorans*near to 910bp indicated the presence of *alk*B gene as was seen in case of isolates PD6, PD9 and PD14. Absence of band at specific position in PD11 indicated absence of *alk*B gene locus in this bacterial isolate.

DISCUSSION

Water in its pure form is boon for the living system and becomes curse when it is contaminated by unwanted agents, either chemical or biological. Water has been contaminated with variety of pollutants present in the effluents from different industries. Aliphatic hydrocarbons present in the petroleum products such as oils, grease contributes significant COD load in the water used for the washing purpose of automobile vehicles (Paxéus, 1996).Though n-alkanes are less hazardous than aromatic and polycyclic aromatic hydrocarbons, but its presence makes water absolutely non-potable and unwholesome for certain types of living systems. In the present study five aliphatic hydrocarbon compounds viz. Dodecane (Makula *et al.*, 1968), Hexadecane (Mehboob, 2009; Stewart 1959), Octadecane, Eicosane and Tetracosane were chosen randomly, as all these compounds are also present in the petroleum mixture as n-alkane fraction (Head *et al.* 2006; Whyte *et al.*, 1998).

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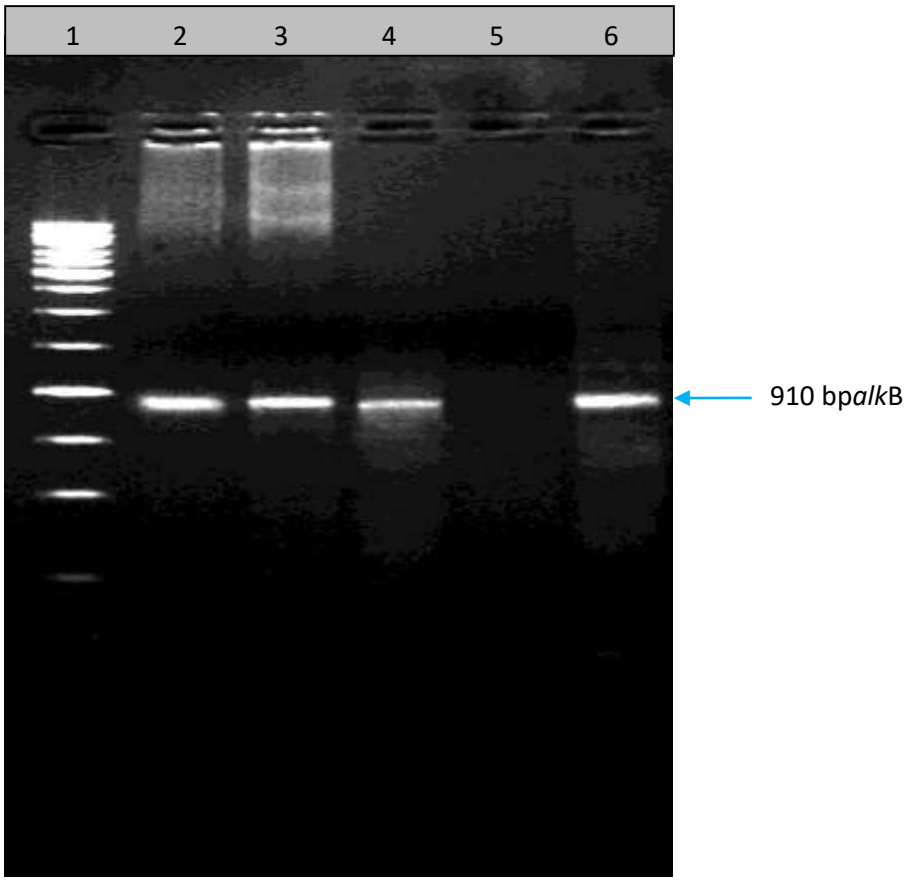

Figure 1.16: Gel photograph showing PCR amplification products of *alk* B gene in *Bacillus subtilis* PD6, *Bacillus sp.* PD9 and *Bacillus sp.* PD14. *Enterobacter sp.* PD11 did not show amplification. In this figure Lane 1 shows 1Kb Ladder, Lane 2 shows +ve control (*Pseudomonas oleovorans*), Lane 3 shows *alk*B amplified product of *Bacillus subtilis* PD6 , Lane 4 shows a*lk*B amplified product of *Bacillus sp.* PD9, Lane 5 shows *Enterobacter sp.* PD11 and Lane 6 shows *alk*B amplified product of *Bacillus sp.* PD14

Observation made in the laboratory about physical state of the compound shows that the Dodecane and Hexadecane are in liquid state at room temperature whereas Eicosane and Tetracosane are solid. Octadecane has shown intermediate behaviour at room temperature and needs a little higher temperature for conversion into liquid state. Here it has been tried to isolate and assess the biodegradation potential of the bacterial culture which posses wide range of catabolic potential towards different n-alkanes, since aliphatic hydrocarbons from C1 to C40 are present in the petroleum mixture.

GROWTH ANALYSIS

Bacterial cells utilize hydrocarbon compounds as a sole source of carbon (Breuil *et al.*, 1978) and energy resulting exponential growth in the medium (Alupoae*et al.*,2003;Regina., 2006). In the minimal medium where carbon is a limiting factor, increase in the number of bacterial cells can be considered as utilization of carbon compound by bacterial cells for growth and development. Bacterial cells utilize specific hydrocarbon compound added in the minimal medium as carbon source thereby causing its degradation. Increase in number of cells can be measured by measuring optical density of medium (Widdel, 2007), or by measuring colony forming units (CFUs) on nutrient agar plates.

All the selected bacterial cultures *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and*Bacillus sp.* PD14 showed growth on five selected aliphatic hydrocarbon compounds Dodecane, Hexadecane, Octadecane, Eicosane and Tetracosane.

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The utilization of aliphatic hydrocarbons by selected bacterial cultures, as depicted by Figures 1.1, 1.2, 1.3, 1.4 and 1.5, indicates that, hexadecane and octadecane are favoured substrates for the selected bacterial cultures.

Growth and development of bacteria depend on presence of essential nutrients. Organic compounds serve as carbon source and this fact is responsible for the elimination of organic compounds from the growth medium. Keeping organic compound as a limiting factor substrate utilization potential of bacteria can be assessed by carrying out growth analysis. Increase in optical density of growth medium due to growth of bacteria indicates elimination of organic compound by utilizing it as carbon source (Widdel, 2007). Utilization of all the five selected aliphatic hydrocarbon compounds were analysed by growth analysis. Growth analysis in presence of dodecane, hexadecane, octadecane, eicosane and tetracosane by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 provided crucial information about substrate utilizing capability of all these cultures. Differential substrate utilizing capability was observed during growth analysis. *Bacillus subtilis* PD6 was found to be excellent dodecane, hexadecane and octadecane user whereas *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 were found to be average user of these three substrates (Figure 1.1 to 1.3). *Enterobacter sp.* PD11 was found to be an efficient eicosane user which showed rapid increase in OD after 72 h. In case of *Enterobacter sp.* PD11 slow but steady increase in growth till 72 h indicates acclimatization and preparatory phase. Different bacterial strains possess different enzymes for the utilization of different compounds belonging to same class (Ortiz-Hernanez *et al.*, 2010).

In case of tetracosane (Figure 1.5) PD6 showed least increase in growth compared to other three cultures whereas PD9 showed maximum increase in OD at 96 h. PD14 was also found to be excellent user of tetracosane with maximum OD at 72 h. Growth analysis results suggested that hexadecane and octadecane were preferred substrate for utilization as carbon source since all cultures showed increase in OD ranging from 0.07 to 0.15 which was found to be ranging below 0.04 to 0.06 OD in case of other three substrates. State of the substrate also affects its utilization by microorganisms. Eicosane and tetracosane remain solid at room temperature and become less available to utilization by bacterial cultures when added to the growth medium. Hence low increase of OD was observed in case of these substrates indicating less utilization.

COD ANALYSIS

In the present study apart from performing growth analysis, COD removal by individual bacterial culture for the degradation of specific hydrocarbon compound was also carried out to corroborate the results, obtained by growth analysis. Studies performed on the biodegradation of hydrocarbons indicated that all the four bacterial cultures *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 selected on the basis of screening test, possessed catabolic potential towards all five aliphatic hydrocarbons but all differed in the degradation rates of selected hydrocarbons.

Bacillus subtilis PD6 showed decrease in COD from initial value 1140 ± 5.7 mg/l to 389 ± 7.5 mg/l which corresponded to dodecane degradation of 67.2% in 6 days (Figure 1.6). Slightly higher COD removal of 69.6% was observed in case of *Bacillus sp*. PD14. COD removal was comparatively lower in case of the remaining two cultures *Bacillus sp.* PD9 (48.1%) and *Enterobacter sp*. PD11 (47%) (Figure 1.7).

Bacillus sp. PD14 displayed decrease in COD from initial value 1222± 2.1 mg/l to 182± 2.1 mg/l which correspond to hexadecanedegradation84.2% in six days whereas *Bacillus subtilis* PD6 showed hexadecane degradation of 60.8% in six days. COD removal was comparatively lower in case of the remaining two cultures *Bacillus sp.* PD9 (32.5%) and *Enterobacter sp.* PD11 (36.4 %) (Figures1.8,1.9).

Bacillus sp. PD14 showed decrease in COD from initial value 1562 ± 2.5mg/l to 626 ± 4.0mg/l which corresponds to octadecanedegradation of 58.8% in six days. Slightly lower COD removal of 55.8% was observed in case of *Bacillus subtilis* PD6. COD removal was comparatively lower in case of the remaining two cultures *Bacillus sp.* PD9 (35.8%) and *Enterobacter sp.* PD11 (38.8 %) (Figure 1.10, 1.11).

Bacillus subtilis PD6 showed decrease in COD from initial value 1157 ± 4.5mg/l to 498 ± 6.6mg/l which corresponds to eicosane degradation of 55.6% in six days. COD removal was comparatively lower in case of the remaining three cultures *Bacillus sp.* PD9 (44.9%), *Enterobacter sp.* PD11 (47.6 %) and *Bacillus sp.* PD14 (47.2%) (Figure 1.12, 1.13).

Enterobacter sp. PD11 displayed decrease in COD from initial value 1112 ± 11.5mg/l to 518 ± 15.6 mg/l which corresponds to tetracosane degradation of51.3 % in six days. COD removal was comparatively lower in case of *Bacillus subtilis* PD6 (42.3%),

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Bacillus sp. PD9 (49.9%), and *Bacillus sp.* PD14 (45.5%) (Figures1.14, 1.15). In control flasks of all selected aliphatichydrocarbons little or no change in COD was observed.

During biodegradation of all five selected aliphatic hydrocarbon compounds the order of cultures with respect to biodegradation was found to be *Bacillus sp.* PD14 (**69.6%**) >*Bacillus subtilis* PD6 (67.2%) >*Bacillus sp.* PD9 (48.1%) >*Enterobacter sp.* PD11(47%) for Dodecane; *Bacillus sp.* PD14 (**84.2%)**>*Bacillus subtilis* PD6 (60.8%) >*Enterobacter sp.* PD11(36.4 %) >*Bacillus sp.* PD9 (32.5%) for Hexadecane; *Bacillus sp.* PD14 (**58.8%**) >*Bacillus subtilis* PD6 (55.8%) >*Enterobacter sp.* PD11(38.8 %) >*Bacillus sp.* PD9 for Octadecane (38.8%); *Bacillus subtilis* PD6 (**55.6%**)>*Enterobacter sp.* PD11(47.6 %)>*Bacillus sp.* PD14 (47.2%)>*Bacillus sp.* PD9 (44.9%) for Eicosane and *Enterobacter sp.* PD11(**51.3 %**) >*Bacillus sp.* PD9 (49.9%) >*Bacillus sp.* PD14 (45.5%) >*Bacillus subtilis* PD6 (42.3%) for Tetracosane.

Uptake or biodegradation of any compound by bacteria depends on the type of bacterial species used for biodegradation and availability of the appropriate catabolic enzyme system in the bacterial cell and other factors such as temperature, moisture, pH of the medium, nutrient level, load of carbon source and culture concentration (Mulkins-Phillips *et al*., 1974;Atlas, 1981). Biodegradation pattern obtained for five aliphatic hydrocarbon compounds clearly indicates that, though the compounds belong to same class and contain same type of chemical structures, the four selected bacterial cultures behaved differently and exhibited different degradation pattern. Biodegradation of any compound is also dependant on the physical state of the compound. If the compound is readily soluble in water chances of its utilization by the specific bacterial species is more. This could be because of rapid uptake of solubilised compound by bacterial cells. If the compound is less soluble in water and aggregates as a clump in the medium, fewer surfaces becomes available for bacterial action and hence less degradation occurs. One option to increase the bioavailability of the compound is its dissolution by using synthetic surfactants or biosurfactants (Kosaric,2001; Tabatabaee *et al*., 2005; Syakti *et al*., 2009). In this study though all cultures were found to degrade selected five aliphatic hydrocarbon compounds, *Bacillus subtilis* PD6 , *Bacillus sp.* PD9 and *Enterobacter sp.* PD11 showed significant degradation of Eicosane and Tetracosane within six days.

MOLECULAR ANALYSIS

As shown in the Figure 1.16 presence of aliphatic hydrocarbon degradation capability in the selected four bacterial cultures was substantiated by the PCR amplification of *alk*B genetic loci in three out of four cultures. *Bacillus subtilis*PD6,*Bacillus sp.* PD9 and *Bacillus sp.* PD14 exhibited successful amplification of *alk*B gene by PCR whereas *Enterobacter sp.* PD11did not give amplification product. Successful amplification of *alk*B gene loci in*Bacillus subtilis*PD6,*Bacillus sp.* PD9 and *Bacillus sp.* PD14 indicates that, these cultures are potential aliphatic hydrocarbon degraders and possess required genetic arsenal for degradation of n-alkanes. Non-amplification of *alk*B in *Enterobacter sp.* PD11 did not mean that it does not possess genetic capability for n-alkane degradation, instead COD analysis and growth curve analysis results strongly suggest the presence of degradation potential in it. On the basis of COD and growth curve analysis results, it can be inferred that there can be presence of variant of *alk*B gene loci in *Enterobacter sp.* PD11 or for successful amplification of *alk*B locus condition needs to be optimized further.

In the present study aliphatic hydrocarbon degradation was studied with *Bacillus* and *Enterobacter sp*. *Bacillus sp.* PD14 was found to be the most efficient culture exhibiting strong catabolic potential towards all selected aliphatic hydrocarbon compounds followed by *Bacillus subtilis* PD6 and *Enterobacter sp.* PD11.

The presence of aliphatic hydrocarbon degrading capability in the *Bacillus* and *Enterobacter sp*. has been well studied in case of crude oil and engine oil biodegradation (Jain *et al.*, 2010, 2011). Thus the results obtained in the present study regarding presence of catabolic potential of *Bacillus* and *Enterobacter sp.* are in congruence with the results obtained by various studies performed worldwide(Churchill*et al.*, 1999; Jain *et al.*, 2010, 2011).

CONCLUSION

In the present study assessment of catabolic potential of *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 towards five aliphatic hydrocarbon compounds dodecane, hexadecane, octadecane, eicosane and tetracosane wascarried out. Growth analysis indicated that, these four bacterial cultures utilized selected five aliphatic hydrocarbon compounds as a carbon and energy source. In growth curve analysis, *Bacillus subtilis* PD6 was found to be predominant culture for aliphatic hydrocarbon utilization but COD analysis results gave predominance to the *Bacillus sp.* PD14.

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All four bacterial cultures degraded five aliphatic hydrocarbon compounds significantly (P<0.01). *Bacillus sp.* PD14 and*Bacillus subtilis*PD6 werefound to be excellent degraders ofDodecane, Hexadecane and Octadecane while degradative potential of *Enterobacter sp.* PD11 and*Bacillus sp.* PD9also showed comparable removal of COD for Eicosane and Tetracosane.

Successful amplification of *alk*B gene loci in the three bacterial cultures confirmed the presence of genetic capability in the selected bacterial cultures for the degradation of n-alkanes but degradation potential in *Enterobacter sp.* PD11 was confirmed by COD and growth analysis results.

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IJCSRR @ 2023
www.ijcsrr.org

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