DIESEL DEGRADATION STUDIES AND MOLECULAR CHARACTERIZATION OF DIESEL DEGRADING ISOLATES

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TABLE OF CONTENTS

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CERTIFICATE

This is to certify that the work entitled "DIESEL DEGRADATION STUDIES AND MOLECULAR CHARACTERIZATION OF DIESEL DEGRADING ISOLATES" submitted by **Divya Narayan (071552) and Shipra Jain (071704)** in partial fulfillment of for the award of degree of Bachelor of Technology in Bioinformatics of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institution for the award of this or any other degree or diploma.

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Divya Narayan **Shipra Jain**

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

- PHYLIP Phylogeny Inference Package
- BLAST Basic Local Allignment Search Tool
- DCPIP 2,6-Dichlorophenol indophenol
- BH Bushnell Haas
- PCB Polychlorinated biphenyls
- TCE Trichloroethylene
- O.D Optical Density
- dNTP di-Nucleotide Tri Phosphate
- EtBr Ethidium Bromide
- PCR Polymerase Chain Reaction
- DNA Deoxyribonucleic acid
- rRNA Ribosomal ribonucleic acid
- TE Tris-EDTA (ethylenediamine tetraacetate) buffer
- TAE Tris-acetate-EDTA (ethylenediamine tetraacetate)
- SDS Sodium Dodecyl Sulphate
- U.V Ultraviolet
- Kb Kilo basepairs
- aq Aqueous
- B. Bacillus

ABSTRACT OF THE DISSERTATION

Metabolic diversity of bacterial flora is a well-established phenomenon, and a consequence of this diversity is the degradation of various bio-hazardous or persistent anthropogenic compounds by microbial activity. Many bacteria have the capability to utilize hydrocarbon contaminants as sources of carbon and energy due to the production of various catabolic enzymes. Microbial strains isolated from chronic contaminated sites act as potential bioremediators. The response of 15 microbial strains arbitrarily named B1, B2, BL1, BL2, BL3, BL4, D1, D2, DP, DW, HPD, S1, S2, TDD and TDH previously isolated from a site that had been contaminated with diesel for approximately 75 years, was assessed in term of ability or inability to grow in presence of diesel oil and potential to degrade the diesel oil. The range of diesel degradation potential varied from 27% to 43%. Moreover, 16S rRNA universal primer were used to amplify the conserved rRNA which was then sequenced and compared to database containing previously sequenced 16S rRNA genes. Further phylogenetic analyses were performed with the closely related hits to arrive at the identity of the strains. Phylogenetic analysis revealed the identity of the strains broadly belonging to the genus *Bacillus, Enterobacter, Staphylococcus, Leucobacter, Achromobacter* and *Brevundimonas*.

CHAPTER 1

INTRODUCTION

Diesel fuel has been considered as priority pollutants which exert bio-hazardous effects on both human and other living organisms in the environment (Kramer and Van 1990; Refaat *et al.,* 2008; Richardson, 1996). Diesel oil is a complex mixture of normal, branched and cyclic alkanes and aromatic compounds. Fortunately, this mixture represents an excellent substrate in the study of hydrocarbon biodegradation due to its composition (Bicca *et al.*, 1999). Among several clean-up techniques available to remove petroleum hydrocarbons from the soil and groundwater, bioremediation processes are gaining ground due to their simplicity, higher efficiency and cost effectiveness when compared to other technologies (Alexander 1994; Ojo, 2006). These processes rely on the natural ability of microorganisms to carry out the mineralization of organic chemicals, leading ultimately to the formation of CO_2, H_2O and biomass (Duarte and Leite, 2000). The fate of petroleum hydrocarbons in the environment is largely controlled by abiotic factors which influence rates of microbial growth and enzymatic activities that determine the rates of petroleum hydrocarbon utilization (Leahy and Colwell, 1991; Ojo, 1995). The persistence of petroleum pollution depends on the quantity and quality of the hydrocarbon mixture and on the properties of the affected ecosystem. Although light petroleum products, like gasoline are efficiently removed by many physio-chemical methods, heavier fuels like diesel often require other techniques because of their low volatility. Since many naturally occurring microorganisms have the ability to utilize hydrocarbons as the sole source of carbon and are widely distributed (Koren *et al.*, 2003), the biodegradation of these compounds is common in nature. Bacterial degradation of petroleum has been known for over 50 years, responsible bacteria have mostly been isolated from surface areas, such as soils, petroleum storage tanks and oil spills (Huy *et al.*, 1999). There are numerous reports of isolation of petroleum hydrocarbon degrader bacteria from oil exposed areas (Kasai *et al.*, 2001; Tazaki, 2005). Biodegradation of individual hydrocarbon compounds by pure bacterial strains has been studied extensively and metabolic pathways have been described (Heitkamp and Cerniglia, 1988; Gibson and Subramanian, 1984; Rehm and Reif 1981; Stirling and

Watkinson, 1977). Bacteria have evolved a wide range of catabolic pathways for the degradation of hydrocarbon pollutants. These pathways are very complex, comprising of a series of enzymes. The ability to isolate high numbers of certain oil-degrading microorganisms from petroleum-contaminated environment is commonly taken as evidence that these microorganisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003).

1.1 Hydrocarbon Spillage

An oil spill is the release of petroleum hydrocarbons into the environment as a result of human activities like disposal of waste motor oil, leaking of storage tanks and other spillages and accidents during its transport. The term often refers to marine oil spills, where oil is released into the ocean or coastal waters. Oil can refer to many different materials, including crude oil, refined petroleum products (such as gasoline or diesel fuel) or by-products, ships' bunkers, oily refuse or oil mixed in waste. Oil is also released into the environment from natural geologic seeps on the seafloor. Hydrocarbons are released from many different land uses. A study by Shepp (1996) showed that high concentrations of hydrocarbons in storm water are released regularly from automotive intensive-use areas such as convenience store parking lots, gas stations, streets and all-day parking lots. The study also indicated that the hydrocarbons are easily mobilized, even during very frequent light rainfall events. A hazardous material is safe till it is fully contained and maintained at desired parameters during storage, operation and transportation. If not handled properly, hydrocarbons can create a disaster by polluting a large area of land and water. The annual release of crude oil in the ocean is estimated to be around 1.7 to 1.8 metric tons and the impact of this pollution can be severely environmental imbalance (Koch *et al.*, 1991). In India alone, the current hydrocarbon consumption is around 110 million metric tons per annum (PMRC, 2003), which can create a higher risk of hydrocarbon pollution of soil and aquatic ecosystems. Oil refineries, tanner accidents, exploratory well drilling and pipelines pose major risk of hydrocarbon spills. The proposed cross country pipelines in India like Iran-Pakistan-India (IPI) pipeline, Central Asia-Israel-India pipeline etc. can further increase the chances of spills due to various geographical and political factors. In addition to environmental damage, spills

are costly to clean up. Numerous examples exist, including a fuel oil spill in Essex County, New York, in 1985. In this example, 7000 US gallons of fuel oil were spilled during the transfer from a tanker trunk to a storage facility. The final cost for clean-up was $589,000. An additional $106,000 was paid in interest and penalties after arguments went through the court system for 14 years. There is a worldwide concern about the liberation of hydrocarbons in the environment, both from industrial activities and from accidental spills of oil and oil-related compounds. Through research on establishing various physical, chemical and biological methods to remove the spillage is necessary, serious thought should also be paid towards determining the potential cause, risk assessment and management of hydrocarbon spillage. Although studies on these aspects are going on (Zandbergen, 1998; Saterbak *et al.*, 1999; Huang, 1999; Neff *et al.*, 2005; Chen, 2007) yet rigorous statistical and analytical methods are required to analyze the hydrocarbon spillage and possible management strategies to combat them. It is high time that the key to manage the widespread hydrocarbon spillage risk is to have full proof transportation and safe storage facilities so as to prevent the spills at first hand. But in case the spills occur, then quick and accurate risk assessment frame should be employed and decontamination of the polluted sites must be carried out which must involve ecofriendly technologies like bioremediation instead of the other techniques.

1.2 Diesel: A brief overview

The carbon number of diesel oil hydrocarbons is between 11 and 25 (2000 to 4000 hydrocarbons) and the distillation range is between 180 to 380 °C (Durand *et al.*, 1995). Diesel is produced from petroleum and is a hydrocarbon mixture, obtained in the fractional distillation of crude oil between $180^{O}C$ and $380^{O}C$ at atmospheric pressure (Durand *et al.*, 1995). The density of diesel obtained from petroleum is about 850 gram per liter. Diesel oil is a complex mixture of normal, branched and cyclic alkanes and aromatic compounds with the properties of low water solubility, high adsorption coefficient and high stability of the aromatic ring (Dean *et al.*, 2002; Kanaly and Harayama, 2002; Kropp and Fedorak, 1998; Van *et al.*, 2003). It is composed of about 75% saturated hydrocarbons (primarily paraffins including n, iso and cycloparaffins) and 25% aromatic hydrocarbons (including napthalenes and alkylbenzenes) (ATSDR, 1995). The average chemical formula for common diesel fuel is $C_{12}H_{23}$, ranging from approximately $C_{10}H_{20}$ to $C_{15}H_{28}$. When burnt, diesel typically

releases about 40.9 megajoules (MJ) per liter energy, whereas petrol releases 34.8 MJ/L, about 15% less, thereby leading to a better fuel economy. Besides this, diesel is generally simpler to refine from petroleum than petrol. Due to these reason diesel is used extensively in chemical and transportation industries and preferred over other hydrocarbons. During its extensive transport and use, it can be released accidentally to soils and groundwater and represent a significant threat to environment.

1.2.1 Reason for Choosing Diesel in the Present Study

There are many reasons which led us to choose diesel for our studies:

1) It is extensively used as a fuel both in land and water transport all over the world.
2) Due to its higher carbon range, it is a complex molecule to breakdown as compared to other hydrocarbon fuels which are comparatively lighter; for instances petrol is blend of hydrocarbons in the range of C_7 to C_{11}.
3) There is extensive diesel spillage in nearby sites.
4) Due to its various health hazards.
5) It is a mixture of both aliphatic and aromatic hydrocarbon.

1.3 Hazards of Diesel contamination

Hydrocarbon spillage may lead to various environmental hazards. As oil is lighter than water, and does not decompose, it will remain on the surface for a very long time. As it is also flammable, oil spills can often cause ocean fires. Other effects on environment include tarring of beaches, damage to fisheries, water contamination etc. With vast number of people living and depending on coastal areas for fishing and tourism throughout the world, the consequences of oil spills can be serious. Hydrocarbon spillage may also lead to various health hazards. It can lead to permanent genetic mutation on entry through food chain (Cronin & Bickham, 1998). Skin irritation problem can occur if substance gets onto bare or broken skin. Some animal studies suggest that repeated contact with fuel oils may cause liver or skin cancer (Bosch, 2003).

1.4 Possible Method of Dealing with Diesel Contamination

Through physical and chemical methods are also quite effective in dealing with diesel contamination problem, but detoxification of the contaminated sites by conventional chemical or physical methods is expensive and time consuming as compared to bioremediation. Besides this bioremediation technique has added advantage of being an ecofriendly method. All these advantages of bioremediation make it a much preferred technique as compared to the other conventional methods (Table 1.1).

Method	Limitations	Adaptability
a)Natural - Evaporation, Dissolution - Photo-oxidation - Biodegradation - Sedimentation, Dispersion, Spreading and Drifting	 - Limited to low MW fractions - Slow and controlled by environmental factors - Limited Efficiency - Environmentally controlled	 -Restricted -Restricted -Restricted -Restricted and Unacceptable
b)Physical Mechanical recovery (Skimmers, Booms, Suction, Separation)	Clumsy, time consuming, costly	Restricted
c)Chemical Sorbent – inorganic/organic (Vermiculites, Glass wool, Carbon wool, foam) , Surfactants for soil washing, Encapsulation of soil using lime, asphalt etc., vitrification	Requires other physical method Reuse of chemicals/sorbents is impractical	Not ecofriendly
d)Bioremediation Oil degrading bacteria, Bioemulsifier, other mediators	Requires supply of nutrient	Ecofriendly, easy application

Table 1.1: Comparison of various decontamination methods for hydrocarbon polluted sites

Bioremediation, has many advantages over other physical and chemical methods of curing diesel and other hydrocarbon contaminated sites as it causes minimal disruption of the site, has no adverse environmental or health effects and it is a simpler and relatively less costly approach than other physic-chemical technologies. Importantly, bioremediation can also be used in conjugation with a wide range of traditional physical and chemical technologies to enhance their efficacy.

1.5 Project Background

Many bacteria have the capability to utilize hydrocarbon contaminants as source of carbon and energy due to the production of various catabolic enzymes. Microbial strains isolated from chronic contaminated sites act as potential bioremediators. In the previous study, sixteen strains of potential diesel degrading bacteria were isolated from a chronic hydrocarbon contaminated site. The uniqueness of this site lies in its location at an altitude of 2100 meters above sea level, where temperature ranges from 30°C in summers to as low as -4°C in winters. The site has been contaminated with diesel and other hydrocarbons for more than 70 years because of its operation as an automobile workshop. The strain isolated from air (Air) and was identified to be *Pseudomonas aeroginosa* and further studies were performed upon it. The present study is its follow up study in which molecular characterization of these strains was performed and their diesel degrading potential was investigated. These strains were arbitrarily named as: B1, B2, BL1, BL2, BL3, BL4, D1, D2, S1, S2, DP, DW, TDD, TDH, HPD, Air.

1.6 Project Overview

In the present study potential diesel degrading bacteria previously isolated from contaminated sites in Himachal Pradesh have been morphologically and molecularly characterized using various methods like Gram staining, KOH string test and molecular characterization using 16s rRNA universal primers. To affirm that the strains are diesel degrading, a new method of reduction of DCPIP was used in place of the standard flask

method. Further, the diesel degrading potential of the strains was assessed using gravimetric analysis.

1.7 Objectives

- *In vitro* diesel degradation studies using isolated strains (percentage of diesel degradation).
- Molecular characterization of the diesel degrading isolates with 16s rRNA universal primers.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Bioremediation

Diesel and other petroleum hydrocarbon have been a veritable source of economic growth to society from the point of view of its energy and industrial importance. These realizations, which have become more pronounced in the last decade, have resulted in extensive exploration for more oil reserves. The resultant effects of these exploratory activities have been the extensive pollution of the environment. Bioremediation, which exploits the biodegradative abilities of organisms like bacteria, fungi, actinomycetes, cyanobacteria and to a lesser extent plants and their attributes, have proven to be the preferred alternative in the long-term restoration of hydrocarbon polluted system, with the added advantage of cost efficiency and environmental friendliness. The process of removing or at least significantly degrading the contaminants present in soil and ground water at the microbial level is known as bioremediation (Anderson *et al.*, 1993).

Petroleum and coal contain a class of molecules known as hopanoids, which are commonly found in bacterial cell walls (Gold, 1985). Gold (1985) indicated that these fuels at some point originated at least in part from microorganisms and that biodegradation of these fuels has always been occurring to some extent. The 'biological evidence' within these hydrocarbons could be the reason for the adaptation of microorganisms to degrade them so readily upon technological industrialization of the earth and why biodegradation is such an applicable method for polluted soil reclamation (Surridge, 2007). Biodegradation of hydrocarbons by natural populations of microorganisms allows for the conversion of hazardous substances into forms that are less or non-toxic and represents one of the primary mechanisms by which petroleum and diesel products are removed from the environment inexpensively (Atlas, 1981; Leahy and Colwell, 1990; Lidderdale, 1993).These organisms may be naturally occurring or laboratory cultivated. These organisms either eat up the contaminants or assimilate within them all harmful compounds from the surrounding area, thereby, rendering the region virtually contaminant-free. Generally, the substances that are eaten up are organic compounds while those, which are assimilated within the organism, are

heavy metals. The catabolic pathways for the degradation of complex compounds like hydrocarbons are mainly plasmid borne but can also be present on the chromosomes and the clustering of these catabolic genes varies among the bacteria (Cerniglia 1984). Bioremediation harnesses this natural process by promoting the growth and/or rapid multiplication of these organisms that can effectively degrade specific contaminants and convert them to non-toxic by-products. Some approaches can make bioremediation even more effective. These include biostimulation i.e. stimulation of the indigenous microorganisms, by introducing nutrients and oxygen into the soil and bioaugmentation i.e. inoculation of an enriched mixed microbial consortium into the soil (Bento *et al.*, 2004). The ability to degrade hydrocarbon substrates including diesel is exhibited by a wide variety of bacterial and fungal genera. A few such bacteria genera are *Pseudomonas, Acinetobacter, Bacillus, Alcaligenes, Flavobacterium, Nocardia sp.* etc. (Labana *et al.*, 2005; Pfender *et al.*, 1997; Mrozik *et al* 2003; Desouky, 2003; Bento *et al.*, 2003). Example of fungi which have hydrocarbon degrading ability are *Aspergillus niger, Rhizopus, Talaromyces, Psuesallescheria boydii, Candida, Pichia guilliermondii* etc. (Cerniglia, 1997; Silva *et al.*, 2004; Adekunle & Adebambo, 2007; April *et al.*,1998). But since the diesel is heterogeneous, it is practically impossible to degrade it by any single microbial species. This is because no single microbial species has the genes to metabolize more than two or three classes of compounds typically found in diesel (Westlake, 1982). A mixed microbial population that has broad substrate specificity is required to perform this task. Consortia of putative diesel or crude oil degrading microorganism have been proved to be better as compared to pure cultures to pure cultures in bioremediation studies (Foght *et al.* 1998; Hamer, 1992). Till now, the most highly publicized use of bioremediations was for the treatment in 1989-90 of the affected beaches in Prince Wlliam Sound, Alaska during Exxon Valdez oil spill disaster.

2.2 Molecular Biology Approach to Bioremediation

Application of bioremediation for the decontamination of hydrocarbon polluted sites was in its infancy in the early 80's, but due to the revolution brought about by molecular biology, it has been evolved now. Molecular biology tools that allow organisms to be identified on the

basis of their genetic composition present an exciting opportunity for bioremediation as these are far less time consuming and more reliable than conventional morphological and biochemical characterization methods. Information on the phylogenetic diversity of microbial communities can be obtained by molecular methods, like fingerprinting or cloning and sequencing of PCR-amplified rRNA genes. This is the reason why nowadays almost all researchers use molecular approaches in their bioremediation studies (Chang *et al.*, 2005; Nikolausz *et al.*, 2005). Molecular tools like PCR have also provided a cultivation independent approach of predicting the bioremediation potential of the microbial population which has proved to be a significant improvement over the standard cultivation based methods which are time intensive and are subject to several well known biases. This is achieved by the use of primers specific for hydrocarbon degrading genes present in microorganisms (Hamann *et al.*, 1999; Brusa *et al.*, 2001). Such catabolic gene probes that are designed from specific genes involved in key enzymatic steps in the microbial degradation pathways for environmental pollutants can be used to examine the environments to determine the presence of organisms having specific functional capacities.

Molecular methods along with the genetic engineering techniques can also pave the way for the creation of organisms specifically designed for bioremediation. For instance, the bacterium *Deinococcus radiodurans* has been modified to consume and digest toluene from radioactive nuclear waste environments by firstly isolating toluene degrading gene using molecular approaches and then introducing it into this bacterium (Brim *et al.*, 2000).

The plasmids inherently containing hydrocarbon degrading genes as well as the ones in which these genes have been artificially introduced can be put into the desired strains and effective bioremediation can be achieved. Notable work in this area was done in 1971 by Dr. Anand Mohan Chakraborty, an India borne American scientist. He successfully synthesized an oil eating superbug by introducing 4 plasmids named as OCT, XYL, CAM and NAH from the four different strains having the genes for degradation of octane, hexane, decane, xylene, toluene, camphor and naphthalene into a single cell of *Pseudumonas putida*. For this work he was awarded the first patent in the history for a new life form by the U.S patent office in 1980.

2.2.1 16S rRNA Sequencing

Bacterial communities from several petroleum-contaminated sites have been effectively characterized by culture-independent molecular techniques using 16S rRNA gene sequences (Kebria *et al.,* 2009; Boboye *et al.*, 2009; Zanaroli *et al*, 2010).

The rRNA is the most conserved (least variable) gene in all cells. Portion of the rRNA sequence from distantaly- related organism are remarkably similar. This mean that sequence from distantly related organism can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA have been extensively used to determine taxonomy, phylogeny (evolutionary relationship) and to estimate the rates of species divergence among microorganisms. This work was pioneered by Carl Woese. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons (Patel, 2001). These reasons include:

(i) its presence in almost all bacteria, often existing as a multigene family, or operons;

(ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and

(iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes .

In 1980 in the *Approved Lists*, 1,791 valid names were recognized at the rank of species. Today, this number has ballooned to 8,168 species. The explosion in the number of recognized taxa is directly attributable to the ease in performance of 16S rRNA gene sequencing studies. The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%), with from 1 to 14% of the isolates remaining unidentified after testing (Drancourt, 2000; Mignard and Flandrois. 2006; Woo *et al.*, 2003). Difficulties encountered in obtaining a genus and species identification include the recognition of novel taxa, too few sequences deposited in nucleotide databases, species sharing similar and/or identical 16S rRNA sequences, or nomenclature problems arising from multiple genomovars assigned to single species or complexes.

In another work performed by Kebria *et al.,* 2009, a study was conducted on the isolation and characterization of commercial diesel-degrading bacterium from Iranian soils. Two isolates out of ten were able to degrade 86.67% and, 80.60 % of diesel fuel at 500 ppm. Only one strain was capable to degrade 85.20 % of diesel fuel at the same time (15 days) at 10000 ppm. Phenotype and phylogeny analysis of this strain was characterized and identified as diesel degrading bacteria, based on gram staining, biochemical tests, 16S rRNA gene sequence analysis and results indicate that this new strain was *Bacillus sp.* and could be considered as *Bacillus Cereus* or *B. thuringiensis* with 98% 16S rRNA gene sequence similarity.

In 2009 a study was carried out by Boboye *et al.*, to isolate hydrocarbon-degrading bacteria associated with environmental samples from Ilaje coastal area, Nigeria. The samples were analyzed by morphological and biochemical processes, and their biodegrading activity on hydrocarbons was assessed. The microbial growths were determined using spectrophotometer blanked at 600 nm. The nine bacteria isolated from environmental samples were *Enterobacter aerogenes, Pseudomonas aeroginosa, Aerococcus viridian, Clostridium sporogenes, Staphylococcus aureus, Lactobacillus acidophilus, Micrococcus luteus, Streptococcus faecalis* and *Bacillus* sp. It was observed from the result that the length of incubation had significant effect on degradation as well as the cell load.

In a study by Zanaroli *et al.*, 2010, two microbial consortia, i.e., ENZ-G1 and ENZ-G2, were enriched from ENZYVEBA (a complex commercial source of microorganisms) on Diesel (G1) and HiQ Diesel (G2), respectively, and characterized in terms of microbial composition and hydrocarbon biodegradation capability and specificity where, ENZ-G1 and ENZ-G2 exhibited a comparable and remarkable biodegradation capability and specificity towards n-C10 to n-C24 linear paraffins by removing about 90% of 1 g l-1 of diesel fuel applied after 10 days of aerobic shaken flask batch culture incubation at 30°C. Through 16S rRNA sequencing approach, both consortia were found to consist of bacteria belonging to the genera *Chryseobacterium, Acinetobacter, Psudomonas, Stenotrophomonas, Alcaligenes* and *Gordonia* along with the fungus *Trametes gibbosa*. However, only the fungus was found to grow and remarkably biodegrade G1 and G2 hydrocarbons under the same conditions. The biodegradation activity and specificity and the microbial composition of ENZ-G1 and ENZ-

G2 did not significantly change after cryopreservation and storage at -20°C for several months.

2.3 Advantages of Bioremediation

Contamination of environment with petroleum hydrocarbons is the cause of serious concern to many countries. Scientists have conducted research on cost effective clean up techniques with minimal long term damage to the environment (Mehrasbi *et al.*, 2003). Among the technologies available to deal with contaminated soils, bioremediation based on metabolic activity of microorganisms has certain advantages (Exner, 1994)

- Bioremediation is a natural process and is therefore perceived by the public as an acceptable hazardous waste treatment process for contaminated material such as soil. Microbes able to degrade the contaminant increase in numbers when the contaminant is present; when the contaminant is degraded, the biodegradative population declines. The residues for the treatment are usually harmless complete product and include carbon dioxide, water, and cell biomass.
- Theoretically, bioremediation is useful for the complete destruction of a wide variety of contaminants. Many compounds that are legally considered to be hazardous can be transformed to harmless products. This eliminates the chance of future liability associated with treatment and disposal of contaminated material.
- Instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, the complete destruction of target pollutants is possible.
- Bioremediation can often be carried out on site, often without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation.
- Bioremediation can prove less expensive than other technologies that are used for clean-up of hazardous waste.

If we see the other side of bioremediation, it is limited to compounds which are biodegradable and the process is highly specific that depends upon environmental parameters like temperature, soil properties, moisture level, pH, percentage of contaminants on site. (Vidali, 2001)

2.4 Process of Diesel Utilization

When microorganisms are used to degrade the oil, we meet with another problem i.e. most of the oil hydrocarbons remain on the water surface due to their low solubility. Low water availability greatly limits their availability. To assimilate the hydrocarbons, biosurfactants (surface active molecules) are produced by hydrocarbon degrading microorganisms (Ilori *et al.*, 2005). Biosurfactants have both polar and non-polar domains, they are able to partition at water-oil interface, emulsify hydrocarbons and thus reduce the surface tension. Surfactants increase the surface area of hydrophobic contaminants in soil or water and increase aqueous solubility and consequently increase their microbial degradation. The type of biosurfactants include lipopeptides synthesized by many *Bacilli* and other species, glycolipids synthesized by *Pseudomonas* species and phospholipids synthesized by *Thiobacillus thioaxidans*, polysaccharide-lipid complexes synthesized by *Acinetobacter* species, or even the microbial cell surface itself (Youssef *et al.*, 2004). For degradation it is necessary that bacteria and the contaminant be in contact. This is not easily achieved, as neither the microbes nor contaminants are uniformly spread in the soil. Some bacteria are mobile and exhibit a chemotactic response, sensing the contaminant and moving toward it. Other microbes such as fungus grow filamentous form toward the contaminant. It is possible to enhance the mobilization of the contaminant utilizing some surfactants such as sodium dodecyl sulfate (SDS) (Vidali, 2001).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Diesel

The diesel used in the present study was procured from diesel storage unit of JUIT, Waknaghat, Solan.

3.1.2 Chemicals

The chemicals used in the present study were procured from S.D fine chemicals Limited, Merc Limited and Qualigens fine chemicals Limited. The various media used in the study were obtained from HiMedia Laboratories Pvt. Ltd.

3.2 Methods

3.2.1 Revival of Strains

A loop full culture from glycerol stocks was taken and inoculated in five milliliter of nutrient broth and was incubated at 28°C for 24 hrs. The cultures so obtained were then streaked on nutrient agar plates to obtain isolated colonies after an incubation of 24 hours at 28°C. The plates were restreaked twice.

3.2.2 Ascertaining that strains are Diesel degrading

A rapid and simple technique has been developed for screening potential crude oil degrading bacteria using the redox indicator 2,6-Dichlorophenol indophenols in Bushnell and Haas

medium with diesel oil and a microtitre plate (Hanson *et al.*, 1993) . The inoculums were prepared by shake cultivation (rotary shaker, 120 rpm) of cells in nutrient broth at 28°C for 24 h. Cells were centrifuged (5000 g, 10 min.), washed with saline and the cell density was adjusted to O.D. = 1 at 600 nm (3.5 mg dry wt/ml) and 25 µl of cell suspension thus obtained was then inoculated into sterile microtitre plates, each well containing 1.5 ml Bushnell and Haas (BH) medium (consisting of magnesium sulphate 0.20 g/L, calcium chloride 0.02 g/L, monopotassium phosphate 1 g/L, dipotassium phosphate 1 g/L, ammonium nitrate 1 g/L, ferric chloride 0.05 g/L, pH 7), 10 µl JUIT diesel and 1.5 µg 2,6-dichlorophenol indophenol (DCPIP).The plates were incubated at 28°C and observed after 24 hr.

3.2.3 Preparation of Glycerol stocks

All the strains were preserved for long duration by preparing glycerol stocks. 80% glycerol was prepared and autoclaved. For each glycerol stock, 1ml of 80% glycerol and 1ml culture were put in a cryopreservation tube and was stores at -20°C and -80°C for short and long term preservation respectively.

3.2.4 Morphological Characterization

Gram nature of all the 16 strains was studied using conventional gram staining method and KOH string test. Moreover the colony characteristics were also studied.

3.2.4.1 Conventional Gram Staining

It was done by taking a drop of liquid cultures of the strains on a slide and leaving for air drying. The slide was flooded with crystal violet and left for 1 min. It was then rinsed with water for 5s. Then the smear was covered with gram's iodine for 1 min. It was then rinsed for 5s with water to remove the excessive stain. The smear was then decolorized with 95% ethanol for 15-30s. The slide was rinsed for 5s with water. Counter staining with safranine was done for 1 min. Slide was rinsed again for 5s with water. It was then blot dried with filter

paper and viewed under a microscope using oil emulsion objective. Gram negative bacteria appeared pink due to counter stain i.e. safranin whereas gram positive bacteria appeared purple-violet in color.

3.2.4.2 KOH string test

For KOH string test, a visible amount of growth from a colony was picked and mixed continuously with a drop of 3% aq. KOH on a glass slide. If such a suspension gels or becomes viscous and strings out when the loop is lifted (positive KOH reaction), the isolate is gram negative. Gram positive cells do not string out (negative KOH method).

2.2.4.3 Colony Characterization

Each strain was streaked on nutrient agar plates and the colony characteristics of each strain were assessed. After quadrant streaking being performed, the plates were observed after an incubation of 48hrs at 28°C. The size of colony, the types of edges, and the color, the consistency of the colony by touching it with a loop, the colony elevation, the optical characteristics and the presence of pigments were studied for each strain.

3.2.5 Molecular Characterization

Initial molecular characterization of all the previously isolated diesel degrading bacterial strains arbitrarily named as BL1, BL2, BL3, BL4, B1, B2,D1,D2,S1,S2,HPD,TDD,TDH, DW, DP and AIR was done by isolating their genomic DNA and then carrying out the PCR using 16s rRNA universal primers. The products were sent for sequencing to reveal the identity of the isolates.

3.2.5.1 Bacterial Genomic DNA Isolation

An overnight grown rich broth culture (5ml) was taken and centrifuged at 8000 rpm for 5min. The pellet was then re suspended in 1ml TE buffer. It was then centrifuged at 6000rpm for 3min thrice more. Then 400 µl of TE plus 40 µl of 10% SDS was added and then incubation was performed at 37°C for 30 min Equal volumes of phenol and chloroform was added and mixed well until phases were completely mixed. The solution was then spun at 10,000 rpm for 10mins. The upper layer was then transferred to a new tube using pipette. Then $1/10^{th}$ volume of Sodium Acetate was added and further 0.6 volume of isopropanol was added and mixed gently. The solution was allowed to stand at -20°C for an hour. Again centrifugation was performed for 3 min and the supernatant was discarded. The pellet was then washed 70% ethanol. The pellet was then allowed to dry for 5 min. 50µl of TE was then added to make up the volume (Sambrook *et al.*, 1989).

3.2.5.2 PCR using universal 16s RNA primers:

Universal 16S rRNA primers having 5'-CCAGCAGCCGCGGTAATACG-3' as forward primer sequence and 5'-ATCGG(C/T)TACCTTGTTACGACTTC-3' as reverse sequence were used in this study.

Reaction Conditions:

The PCR mixture contained 2.5 µl of 10XPCR buffer (containing 15 mM $MgCl_2$), 0.5 µl of dNTP, 1 µl of DNA sample, 1 µl of each primer(Forward and Reverse), 0.25µl Taq Polymerase (Intron) and distilled water was added to achieve a final volume of 25µl.

PCR amplification was carried out using the following program:

After initial denaturation for 4mins at 95°C, 30 cycles each consisting of 30sec at 94°C, 1min at 52°C and 1min at 72°C were completed. A final extension of 7mins at 72°C was applied following which the samples were subjected to cooling at 4°C.

3.2.5.3 Agarose Gel Electrophoresis

1% agarose gel was prepared using 1X TAE buffer. After boiling the solution until it becomes clear, 0.5µg/ml of ethidium bromide was added to it. Whole mixture was then allowed to polymerize in a gel casting tray for about 45min, following which loading of the PCR amplified product was done. 1kb and 100bp molecular marker were also loaded along with the samples and allowed to run at 100mA/80V in an electrophoresis tank. When 60% movement of the bands had occurred, gel was visualized under 260nm U.V. light.

3.2.5.4 *In silico* identification of strains

3.2.5.4.1 *In silico* Tools Used

3.2.5.4.1.1 PHYLIP (Phylogeny Inference Package)

PHYLIP is a package of programs for inferring phylogenies (evolutionary trees). It was written by Joseph Felsenstein at the University of Washington and has been distributed since 1980. It has over 15,000 registered users, making it the most widely distributed package of phylogeny programs. It is available free, from its web site: http://evolution.gs.washington.edu/phylip.html

PHYLIP is available as source code in C, and also as executables for some common computer systems. It can infer phylogenies. It can also compute consensus trees, compute distances between trees, draw trees, resample data sets by bootstrapping, edit trees, and compute distance matrices.

Each program is controlled through a menu, which asks the users which options they want to set, and allows them to start the computation. The data is read into the program from a text file, which the user can prepare using any word processor or text editor. Some sequence analysis programs such as the ClustalX alignment program can write data files in the PHYLIP format.

Output is written onto files with names like outfile and outtree. Trees written onto outtree are in the Newick format, an informal standard agreed to in 1986 by authors of a number of major phylogeny packages

The programs used by us were:

- **DNADIST :** Program to compute distance matrix from nucleotide sequences

This program uses nucleotide sequences to compute a distance matrix. It can also compute a table of similarity between the nucleotide sequences. The distance for each pair of species estimates the total branch length between the two species. As the distances are computed, the program prints on your screen or terminal the names of the species in turn, followed by one dot (".") for each other species for which the distance to that species has been computed. When the distance matrix has been written out to the output file, the user is notified of that. The output file contains on its first line the number of species. The distance matrix is then printed in standard form, with each species starting on a new line with the species name, followed by the distances to the species in order. The distance matrix is square with zero distances on the diagonal. In general the format of the distance matrix is such that it can serve as input to any of the distance matrix programs.

- **NEIGHBOR- Neighbor-Joining and UPGMA methods**

This program implements the Neighbor-Joining method of Saitou and Nei (1987) and the UPGMA method of clustering. The program was written by Mary Kuhner and Jon Yamato. NEIGHBOR constructs a tree by successive clustering of lineages, setting branch lengths as the lineages join. The tree is not rearranged thereafter. The tree does not assume an evolutionary clock, so that it is in effect an unrooted tree. The program cannot evaluate a User tree, nor can it prevent branch lengths from becoming negative. It is particularly effective in its place for large studies or for bootstrap or jackknife re-sampling studies which require runs on multiple data sets. The output consists of an tree (rooted if UPGMA, unrooted if Neighbor-Joining) and the lengths of the interior segments. The Average Percent Standard Deviation is not computed or printed out. The major advantage of NEIGHBOR is

its speed: it requires a time only proportional to the cube of the number of species. Thus NEIGHBOR is well-suited to bootstrapping studies and to analysis of very large trees.

- **SEQBOOT:**

It is a general bootstrapping and data set translation tool. It is intended to allow you to generate multiple data sets that are resampled versions of the input data set. Since almost all programs in the package can analyze these multiple data sets, this allows almost anything in this package to be bootstrapped.

Bootstrapping was invented by Bradley Efron in 1979, and its use in phylogeny estimation was introduced by Felsenstein, 1985. It involves creating a new data set by sampling *N* characters randomly with replacement, so that the resulting data set has the same size as the original, but some characters have been left out and others are duplicated. The random variation of the results from analyzing these bootstrapped data sets can be shown statistically to be typical of the variation that you would get from collecting new data sets. The method assumes that the characters evolve independently, an assumption that may not be realistic for many kinds of data. It is a popular way of evaluating the reliability of an inferred phylogenetic tree.

The first step in a bootstrap analysis is to re-sample the alignment columns with replacement. i.e., in the re-sampled alignment, a given column in the original alignment may occur two or more times, while some columns may not be represented in the new alignment at all. The re-sampled alignment represents an estimate of how a different set of sequences from the same genes and the same species may have evolved on the same tree. By re-sampling a number of times it is possibly to put reliability weights on each internal branch of the inferred tree. If the data was bootstrapped a 100 times, a bootstrap score of 100 means that the corresponding branch occurs in all 100 trees made from re-sampled alignments. Thus, a high bootstrap score is a sign of greater reliability.

- **CONSENSE -- Consensus tree program**

CONSENSE reads a file of computer-readable trees and prints out (and may also write out onto a file) a consensus tree. At the moment it carries out a family of consensus tree methods called the M_l methods (Margush and McMorris, 1981). These include strict consensus and majority rule consensus. Basically the consensus tree consists of monophyletic groups that occur as often as possible in the data. If a group occurs in more than a fraction l of all the input trees it will definitely appear in the consensus tree. The tree printed out has at each fork a number indicating how many times the group which consists of the species to the right of (descended from) the fork occurred. The majority rule consensus tree consists of all groups that occur more than 50% of the time.

3.2.5.4.1.2 NCBI-BLAST(Basic Local Alignment Search Tool)

The BLAST algorithm and tool is possibly one of the most popular and important bioinformatics tools. It is the tool most frequently used for calculating sequence similarity NCBI implementation of BLAST is a set of similarity search programs designed to explore all of the available sequence databases. BLAST comes in variations for use with different query sequences against different databases.

3.2.5.4.1.3 ClustalX

The most widely used programs for global multiple sequence alignment are from the Clustal series of programs. The first Clustal program was written by Des Higgins in 1988 and was designed specifically to work efficiently on personal computers, which at that time had feeble computing power by today's standards. It combined a memory-efficient dynamic programming algorithm with the progressive alignment strategy developed by Feng and Doolittle and Willie Taylor. The multiple alignment is built up progressively by a series of pairwise alignments, following the branching order in a guide tree.

The program displays the multiple alignment in a scrollable window and all parameters are available using pull-down menus. Within alignments, conserved columns are highlighted

using a customizable colour scheme and quality analysis tools are available to highlight potentially misaligned regions. ClustalX is easy to install, is user-friendly and is portable.

3.2.5.4.1.4 Tree View

Tree View provides a simple way to view the contents of a NEXUS, PHYLIP, Hennig86, Clustal, or other format tree file.

3.2.5.4.2 Steps Followed

- The sequences of the 16s rRNA was procured from Ocimum Biosolutions. The sequences received were in the fasta format and had been sequenced using both the forward and reverse primer. Then a consensus sequence was built using ClustalX software. A search of the GenBank nucleotide library for sequences similar to those determined was made by using BLAST, through the National Center for Biotechnology Information (NCBI) internet site (http://www.ncbi.nlm.nih.gov/BLAST). The results of the BLAST program were then analyzed to determine if there was absolute identity to any organism or not. In most of the cases the results of the BLAST had shown high identity to two or more species of the same genera. In such a case, the top hits showing very high similarity to the query were taken up for phylogenetic analysis. (Figure 3.1)

- A multiple alignment of 16S rRNA gene sequences of the selected top hits which were close matches to the query strain were retrieved from GenBank and were aligned using ClustalX with the PHYLIP output option. The alignment was observed for any obvious mis-alignments. (Figure 3.2)

Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
HQ202545.1	Bacillus sp. RHH8 16S ribosomal RNA gene, partial sequence	1622	1622	98%	0.0	96%	
EU260045.1	Bacillus subtilis strain X2 16S ribosomal RNA gene, partial sequence	1620	1620	98%	0.0	96%	
EU257700.1	Bacillus subtilis strain DC2-2 16S ribosomal RNA gene, partial sequenc	1620	1620	98%	0.0	96%	
HQ693082.1	Bacillus subtilis strain 1-3 16S ribosomal RNA gene, partial sequence	1618	1618	97%	0.0	96%	
FJ763648.1	Bacillus subtilis strain S64 16S ribosomal RNA gene, partial sequence	1618	1618	98%	0.0	96%	
GQ180177.1	Bacillus sp. JR56 16S ribosomal RNA gene, partial sequence	1618	1618	97%	0.0	96%	
EU334106.1	Bacillus subtilis strain EXWB2-14 16S ribosomal RNA gene, partial seq	1618	1618	98%	0.0	96%	
HQ202544.1	Bacillus subtilis strain MTH29 16S ribosomal RNA gene, partial sequen	1616	1616	98%	0.0	96%	
HQ698267.1	Bacillus sp. TC16 16S ribosomal RNA gene, partial sequence	1616	1616	98%	0.0	96%	

Figure 3.1: Showing screen shot of BLAST result strain i.d. TDH

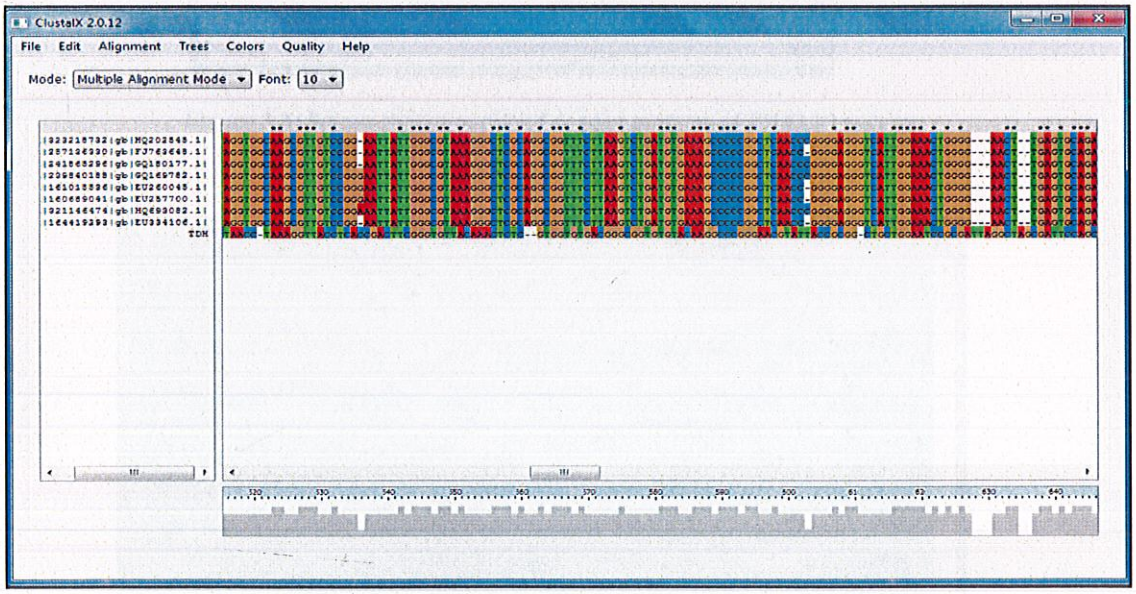


Figure 3.2: Screen shot showing multiple alignment of strain i.d. TDH with its top BLAST hits using ClustalX

- A phylogenetic tree was then constructed by using PHYLIP, version 3.62 (Felsenstein, 1985). Evolutionary distance matrices for the neighbour-joining/UPGMA methodology were computed using the DNADIST algorithm program. The program read in nucleotide sequences and wrote an output file containing the distance matrix. The model of nucleotide substitution was that of Kimura (1980). Phylogenetic tree was inferred by using the NEIGHBOR program in PHYLIP package.

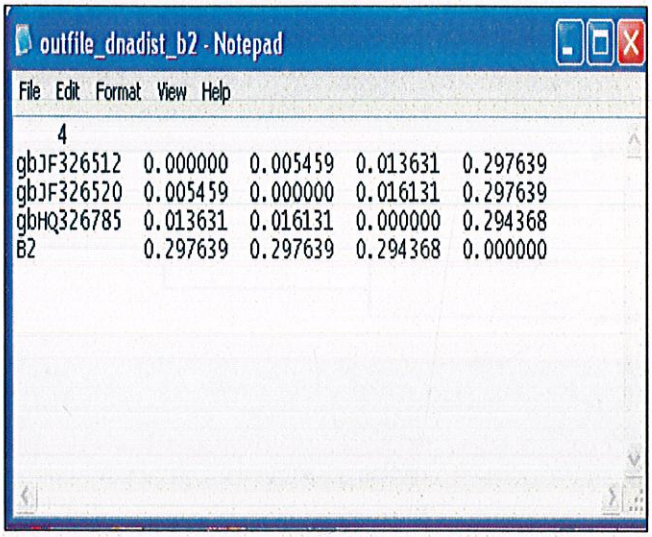


Figure 3.3: Screen shot of outfile of dnadist program of PHYLIP package

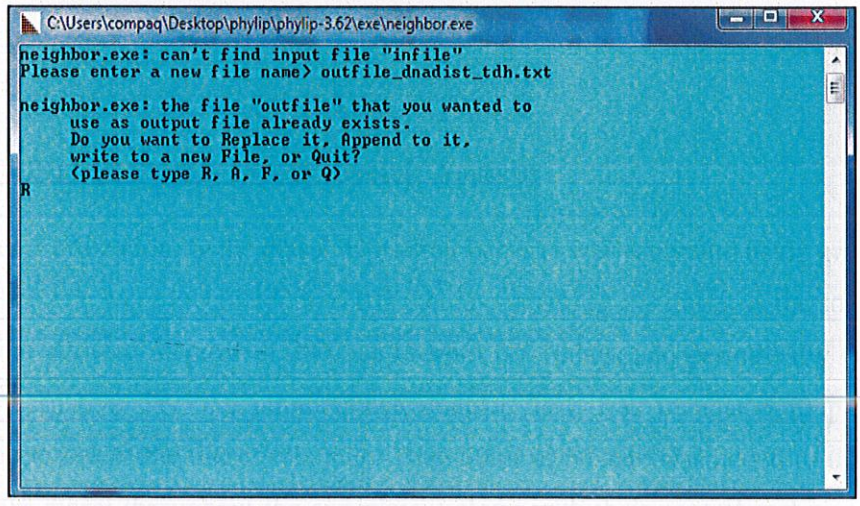


Figure 3.4: Screen shot of the neighbor program of PHYLIP package

- With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1000 bootstraps by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the topologies found using a family of consensus tree methods called the MI methods using the CONSENSE program and the tree was viewed using Tree View (Shukor *et al.*).

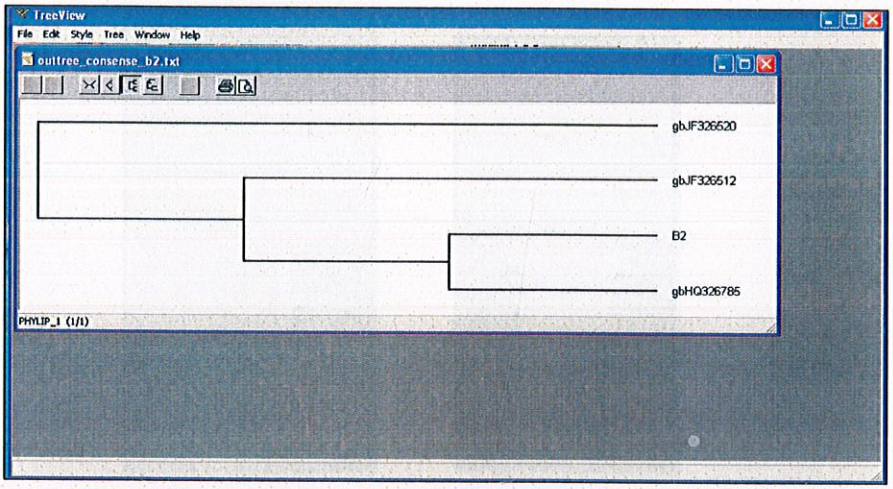


Figure 3.5: Screen shot of Tree View showing the consensus tree of strain i.d. B2

3.2.6 Diesel Utilization Studies by Gravimetric Analysis

Percentage diesel utilization by the diesel degrading bacteria was estimated using gravimetric analysis. The bacteria were inoculated into BH media containing 3% diesel as the sole carbon source and were allowed to grow at 28°C at 120rpm for 10days and periodically O.D. was taken every alternative day. An uninoculated control flask was incubated in parallel to monitor abiotic losses of the diesel substrate. Then 50ml of hexane was added to the culture flask having 3% diesel throughout the experiment along with their control and was kept on shaker for about an hour so that the entire diesel gets extracted by hexane. The solution was

then poured in a separating funnel and the upper layer of hexane containing diesel was separated into a pre weighed dish and was allowed to evaporate for about 10-12 hrs after which the dish was again weighed. Increase in the weight of dish was due to the diesel left in the flask at the end of the experiment and hence gave the estimate of the diesel utilization by the bacteria.

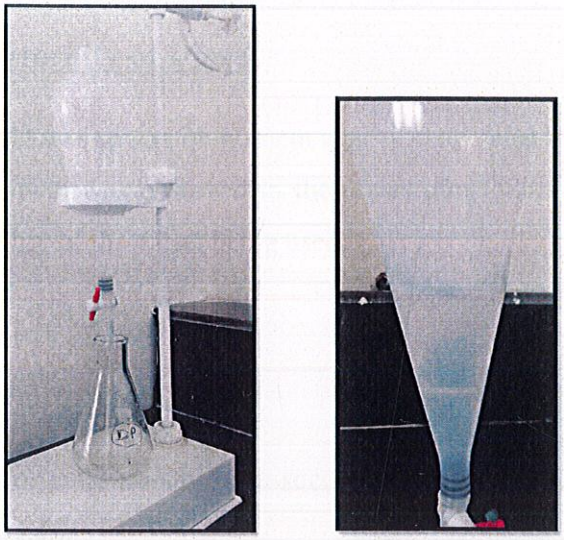


Figure 3.6: Separating Diesel from the rest of the media by Separating Funnel. Notice the upper layer of hexane which has diesel dissolved in it discretely separated from the lower layer of media containing cells

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Checking the sterility of Diesel

The filter sterilized diesel was spread on nutrient agar plates to check for the presence of any microorganisms. No growth was observed in the plates after 48hrs of incubation at 28°C. This confirmed the sterility of the diesel being used.

4.1.2 Revival of the cultures

16 strains out of the 17 original isolates were successfully revived and were further purified. They are namely (strain i.d.):

BL1, BL2, BL3, BL4, B1, B2, D1, D2, S1, S2, HPD, TDH, TDD, DP, DW and Air.

Strain Air was not further used for the study as it has already been studied over extensively.

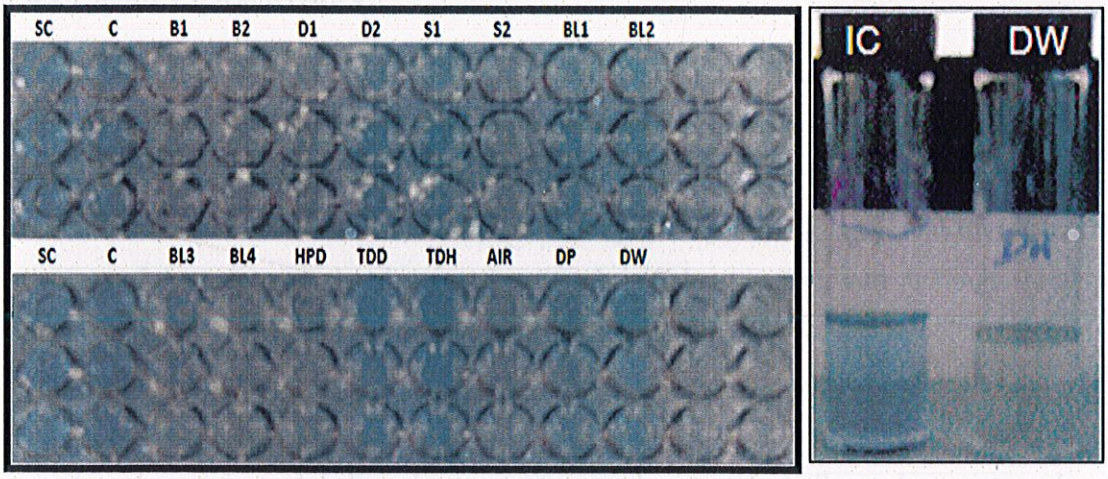
(a) (b)

Figure 4.1: (a) Isolated colonies of strain i.d. D1 obtained following revival
(b) Isolated colonies of strain i.d. BL1 obtained following revival

4.1.3 Ascertaining the strains so revived were Diesel degrading

During the microbial oxidation of hydrocarbons, electron are transferred to electron acceptor such O_2, nitrates and sulphates (Hutchins *et al.*, 1991; Beller *et al.*, 1992). By incorporating an electron acceptor such as DCPIP, it is possible to ascertain the ability of the microorganism to utilize hydrocarbon substrate by observing the color change of DCPIP from blue (oxidize) to colorless (reduced).

Bacteria that had diesel degrading potential were found to give a color lighter than the control well and eventually turned colorless when observed after about 24 hours. So we ascertained that all the strains revived by us were all diesel degrading. (Figure 4.2).



(a) (b)

Figure 4.2: (a) Decolorization of 2,6-DCPIP by different bacterial strains compared to substrate control (SC) and inoculums control (C) in microtitre plate ; (b) Difference in the color of strain i.d. DW(colorless) compared to Inoculum Control (IC) after 24 hours

4.1.4 Morphological Characterization

4.1.4.1 Gram Nature of Isolates

Once the bacteria were ascertained to be diesel degrading, their nature was studied using conventional gram staining procedure (Table 4.1) and the results were confirmed by using

the KOH method, which is a simple and non staining method for the determination of bacterial gram nature . The basis of KOH string test is that, the thick peptidoglycan layer that makes up the cell wall of Gram-positive bacteria is much more resistant to chemical digestion than that of Gram-negatives. Therefore, treatment with a dilute base such as 3% potassium hydroxide (KOH) affects these two classes of bacteria differently. The thin-walled Gram-negatives will lyse in 3% KOH, releasing cell contents, including DNA, which is very viscous. The thick-walled Gram-positives do not lyse and therefore do not affect the viscosity of the KOH on the slide. The results were then correlated with that of the actual gram nature of the strains once they were identified. All the results were in consensus.

4.1.4.2 Colony Characteristics

Bacteria show characteristic type of growth on solid media under appropriate cultural conditions and the colony morphology can be used in presumptive identification. The colonies can be varying in size and diameter, in outline (circular, wavy, rhizoid etc.) elevation (flat, raised, convex, etc.) and translucency (transparent, opaque, and translucent). The colors of the colony or the changes that they bring about in their surroundings are also used as diagnostic tools in the tentative identification of the bacteria. Colony characteristics provide useful information about the different organisms but these cannot be used as the sole criteria for classification. Further, biochemical, serological and specific tests are often required to be performed before bacteria are clearly identified. Some bacteria produce brilliant pigments, If stable and distinct, the pigmentation is used as an aid in identifying the organism. The colony characteristics of the strains have been enlisted in Table 4.2. None of the strains was observed to be producing any pigments.

Table 4.1: Showing the gram nature of different strains

Strain i.d.	Strain Name	Gram Staining	KOH test
B1	*Leucobacter aridicolis*	+ve	+ve
B2	*Brevundimonas terrae*	-ve	-ve
BL1	*Staphylococcus pasteuri*	+ve	+ve
BL2	*Enterobacter sp*	-ve	-ve
BL3	*Staphylococcus epidermidis*	+ve	+ve
BL4	*Enterobacter hormaechei*	-ve	-ve
D1	*Leucobacter aridicolis*	+ve	+ve
D2	*Enterobacter cancerogenus strain M123*	-ve	-ve
DP	*Bacillus sp.*	+ve	+ve
DW	*Bacillus subtilis*	+ve	+ve
HPD	*Achromobacter xylosoxidans*	-ve	-ve
S1	*Enterobacter sp*	-ve	-ve
S2	*Bacillus cereus*	+ve	+ve
TDD	*Bacillus subtilis/ Bacillus amyloliquefaciens*	+ve	+ve
TDH	*Bacillus subtilis*	+ve	+ve

Table 3.2: Showing Colony Characteristics of the strains

Strain i.d.	Strain Name	Size (mm)	Margin/ Edges	Surface Texture	Elevation	Consis-tency	Optical Feature	Color of Colony
B1	*Leucobacter aridicolis*	2	Circular	Rough (Matty)	Convex	Stringy	Opaque	Creamy
B2	*Brevundimonas terrae*	1-2	Circular	Rough	Convex	Viscous	Opaque	White
BL1	*Staphylococcus pasteuri*	0.5	Circular	Rough	Thin-Convex	Stringy	Opaque	White
BL2	*Enterobacter sp*	3	Circular	Mucoid	Convex	Butyrous	Opaque	Cream
BL3	*Staphylococcus epidermidis*	1-2	Circular	Smooth	Convex	Mucoid	Opaque	Yellowish white
BL4	*Enterobacter hormaechei*	1	Circular	Smooth Shiny Waterous	Convex	Viscous	Opaque	Creamy white
D1	*Leucobacter aridicolis*	1-2	Circular	Rough	Convex	Viscous	Opaque	Cream-Orangish Tint
D2	*Enterobacter cancerogenus strain M123*	0.2	Circular	Mucoid Shiny	Convex	Stringy	Opaque	Cream
DP	*Bacillus sp.Y39/ B. pumilus*	4-5	Irregular Radial	Rough	Flat	Butyrous	Transp-arent	No Color
DW	*Bacillus subtilis*	1-2	Irregular	Rough	Flat	Mucoid	Transl-ucent	Off white
HPD	*Achromobacter xylosoxidans*	3	Circular	Rough	Thin	Viscous	Opaque	Cream
S1	*Enterobacter sp*	2	Circular	Shiny	Convex	Stringy	Transl-ucent	No color
S2	*Bacillus cereus*	2	Circular	Smooth	Raised	Stringy	Opaque	Cream
TDD	*Bacillus subtilis/ Bacillus amyloliquefaciens*	1-2	Irregular	Rough	Flat	Mucoid	Transl-ucent	Off white
TDH	*Bacillus sp RHH8*	1-2	Circular	Rough	Flat	Butyrous	Transl-ucent	Creamy white

4.1.5 Molecular characterization Studies of the Revived strains

4.1.5.1 Bacterial Genomic DNA Isolation

By carrying out agarose gel electrophoresis of extracted genomic DNA of the revived strains, it was confirmed that DNA for all the isolates had been isolated successfully and genomic DNA sizes of the isolates were greater than 10Kb (Figure 4.3).

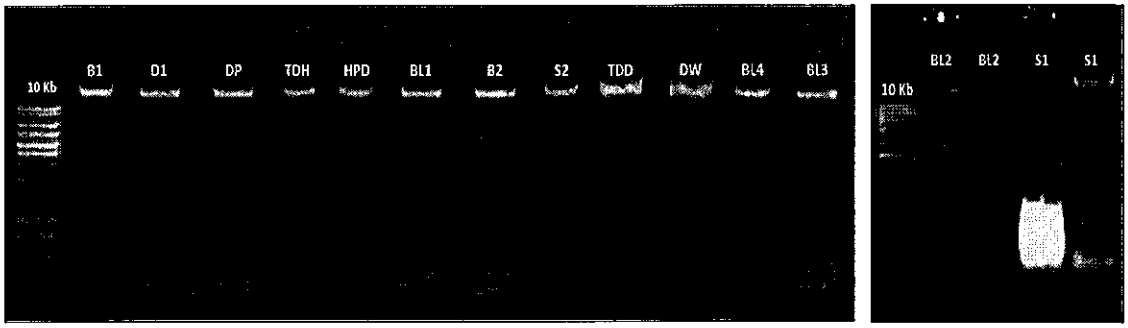


Figure 4.3: Gel pictures showing genomic DNA of all the strains

4.1.5.2 Amplification of 16s rRNA gene

On using universal 16s rRNA, the corresponding genomic DNA fragments of all the isolated got amplified (Figure 4.4). This is because 16s rRNA sequences are conserved sequences which are present in all the hierarchical domains. The amplified PCR product obtained was sent for sequencing in order to characterize the bacterial.

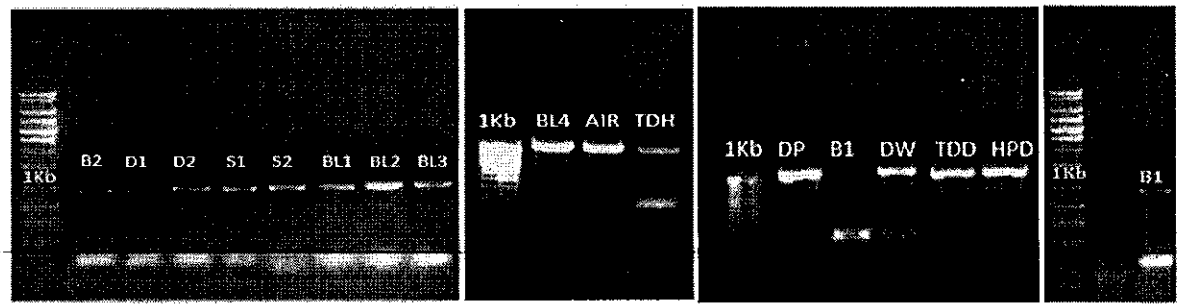


Figure 4.4: Gel Pictures showing 16 s rRNA of all the strains

4.1.5.3 *In silico* identification of strains

Once the sequences had been received, a consensus sequence was constructed and BLAST was performed against the non-redundant database of nucleotide of NCBI. In most of the cases the hits gave same Genus with a few varying species. The top hits were picked for phylogenetic analysis and phylogenetic trees were constructed. The robustness of the tree was indicated by the bootstrap values.

The Figures 4.5-4.18 show the trees constructed by neighbor joining method and the numbers at the branching point or nodes refer to the bootstrap value based on 1000 resampling performed.

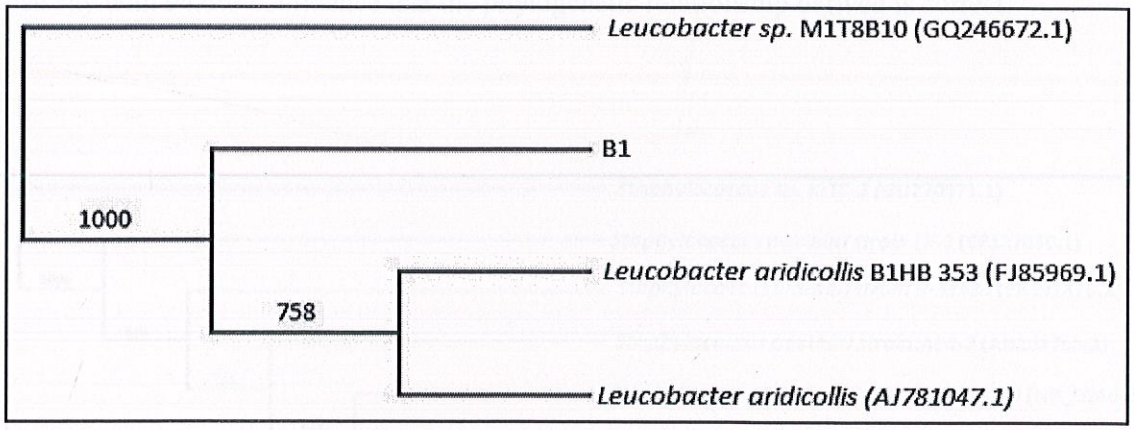


Figure 4.5: A phylogram (neighbour-joining method) showing genetic relationship between strain B1 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.5 it was inferred that the strain i.d. B1 is *Leucobacter aridicollis* which shows 95% identity with it. The high bootstrap value of 1000 indicates that we can say with 100% confidence that the phylogenetic relationship derived is correct.

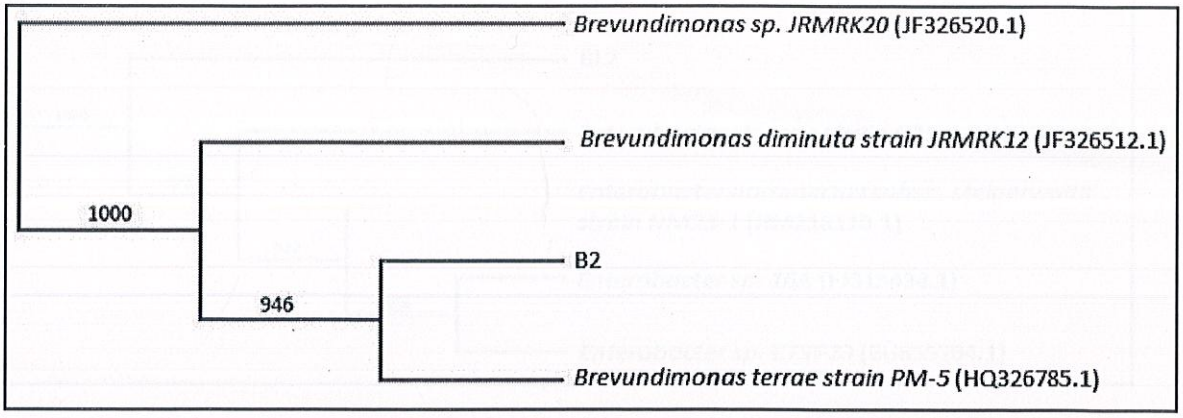


Figure 43.6: A phylogram (neighbour-joining method) showing genetic relationship between strain B2 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.6 it was inferred that the strain i.d. B2 is *Brevundimonas terrae* which shows 94% identity with it. The high bootstrap value of 946 indicates that we can say with 94.6% confidence that the phylogenetic relationship derived is correct.

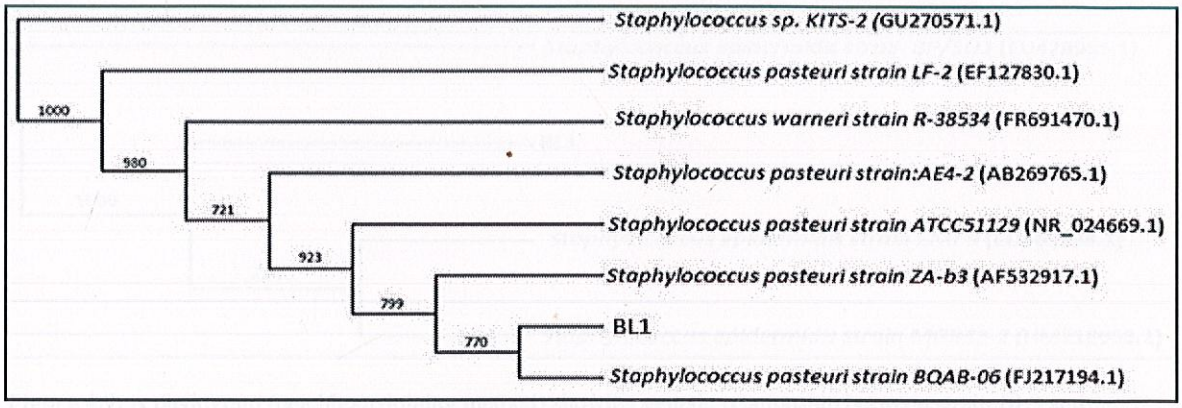


Figure 4.7: A phylogram (neighbour-joining method) showing genetic relationship between strain BL1 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.7 it was inferred that the strain i.d. BL1 is *Staphylococcus pasteuri* which shows 95% identity with it. The high bootstrap value of 770 indicates that we can say with 77% confidence that the phylogenetic relationship derived is correct.

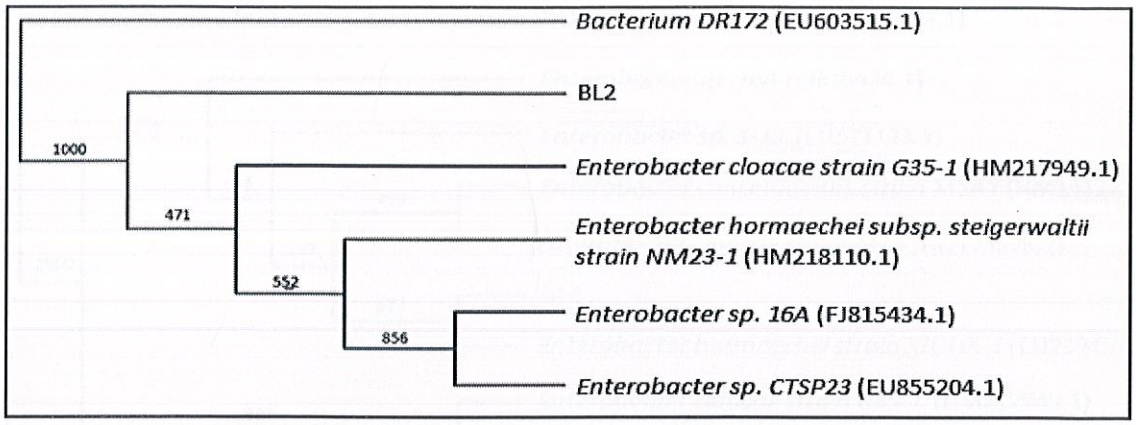


Figure 4.8: A phylogram (neighbour-joining method) showing genetic relationship between strain BL2 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.8 it was inferred that the strain i.d. BL2 is *Enterobacter sp*. In this case the genus has been confirmed to be *Enterobacter* but the species cannot be confirmed. All the BLAST results consensus over the genus with 93% identity. For the characterization of the specific species, biochemical tests would be needed to be performed.

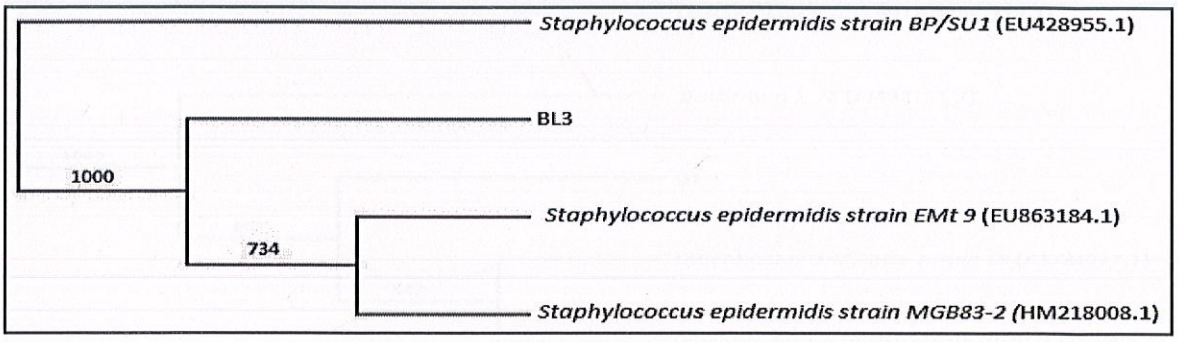


Figure 4.9: A phylogram (neighbour-joining method) showing genetic relationship between strain BL3 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.9 it was inferred that the strain i.d. BL3 is *Staphylococcus epidermidis* which shows 94% identity with it. The high bootstrap value of 734 indicates that we can say with 73.4% confidence that the phylogenetic relationship derived is correct.

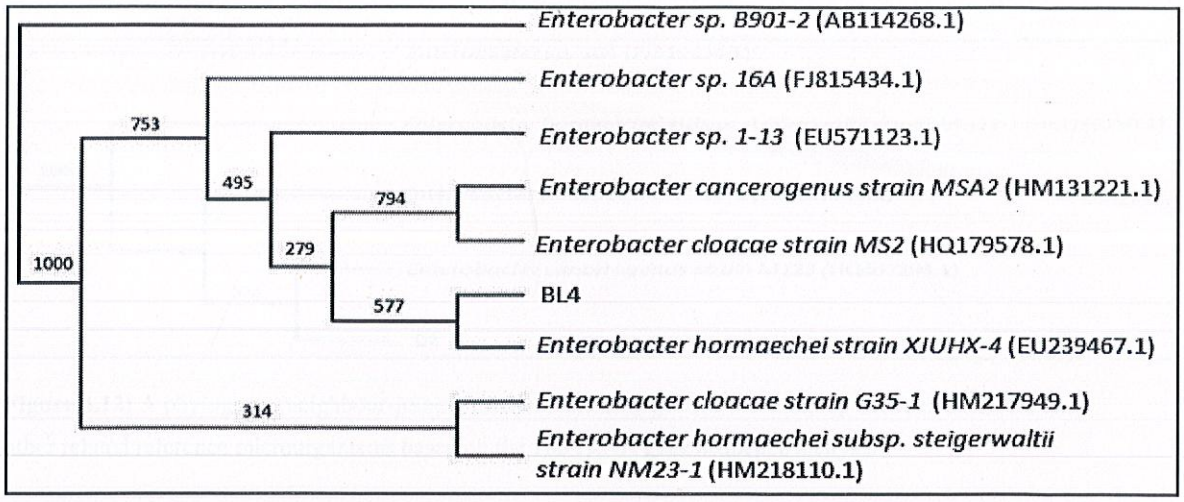


Figure 4.10: A phylogram (neighbour-joining method) showing genetic relationship between strain BL4 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.10 it was inferred that the strain i.d. BL4 is *Enterobacter hormaechei* which shows 96% identity with it. The high bootstrap value of 577 indicates that we can say with 57.7% confidence that the phylogenetic relationship derived is correct.

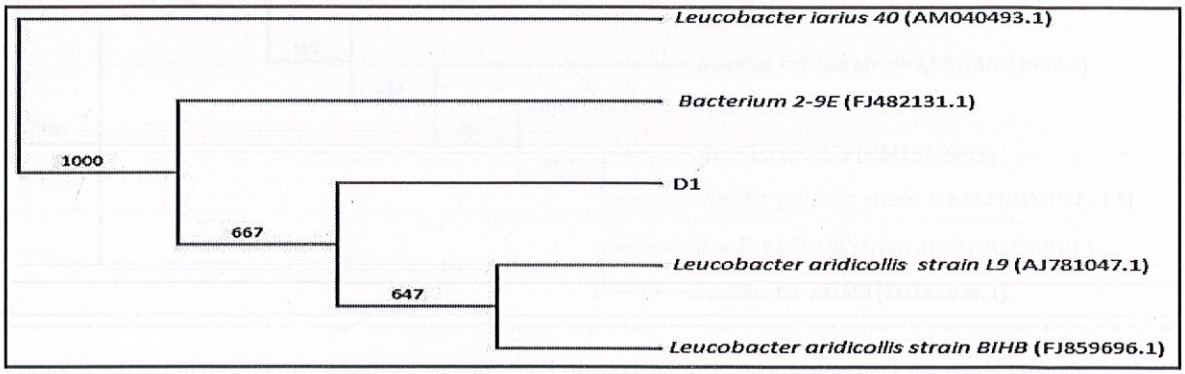


Figure 4.11: A phylogram (neighbour-joining method) showing genetic relationship between strain D1 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.11 it was inferred that the strain i.d. D1 is *Leucobacter aridicollis* which shows 96% identity with it. The high bootstrap value of 647 indicates that we can say with 64.7% confidence that the phylogenetic relationship derived is correct.

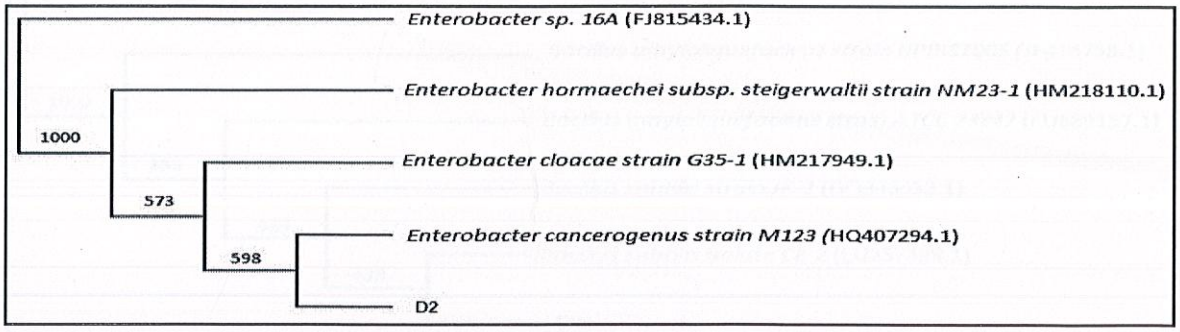


Figure 4.12: A phylogram (neighbour-joining method) showing genetic relationship between strain D2 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.12 it was inferred that the strain i.d. D2 is *Enterobacter cancerogenus* which shows 97% identity with it. The high bootstrap value of 598 indicates that we can say with 59.8% confidence that the phylogenetic relationship derived is correct.

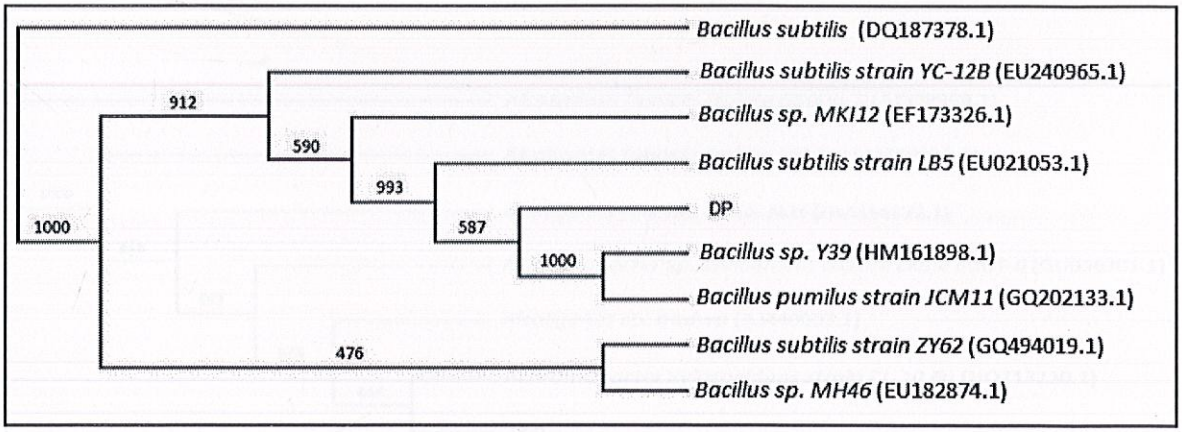


Figure 4.13: A phylogram (neighbour-joining method) showing genetic relationship between strain DP and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.13 it was inferred that the strain i.d. DP is either *Bacillus sp. Y39* or *Bacillus pumilus,* both of which show 98% identity with it. The high bootstrap value of 587 indicates that we can say with 58.7% confidence that the phylogenetic relationship derived is correct. To ascertain the species, further biochemical tests need to perform.

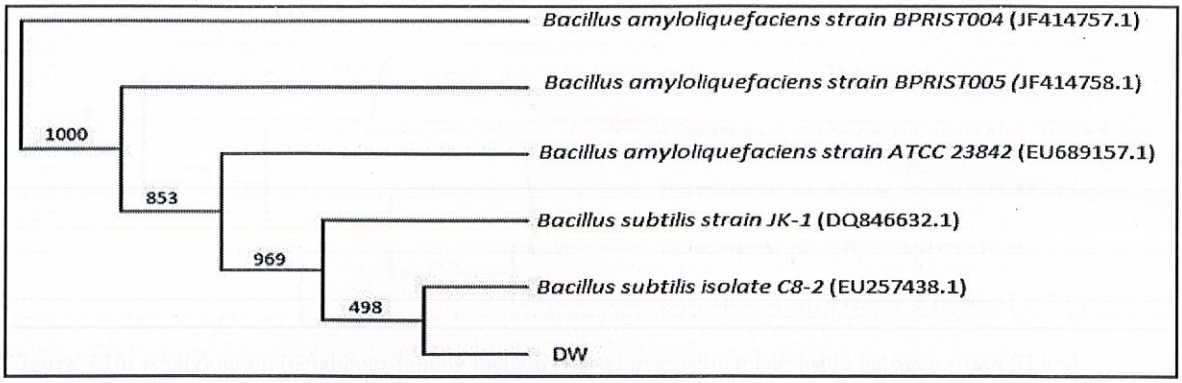


Figure 4.14: A phylogram (neighbour-joining method) showing genetic relationship between strain DW and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.14 It was inferred that the strain i.d. DW is *Bacillus subtilis* which shows 99% identity with it. The high bootstrap value of 498 indicates that we can say with 49.8% confidence that the phylogenetic relationship derived is correct.

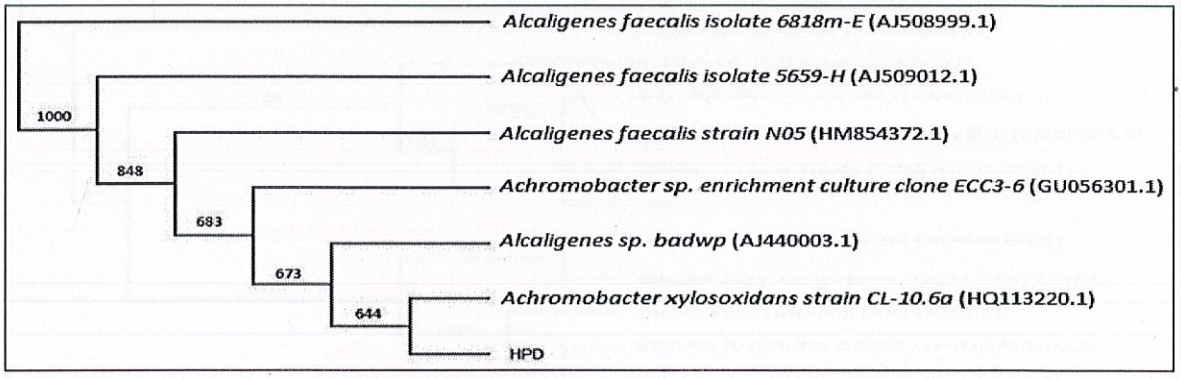


Figure 4.15: A phylogram (neighbour-joining method) showing genetic relationship between strain HPD and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.15 it was inferred that the strain i.d. HPD is *Achromobacter xylosoxidans* which shows 96% identity with it. The high bootstrap value of 644 indicates that we can say with 64.4% confidence that the phylogenetic relationship derived is correct.

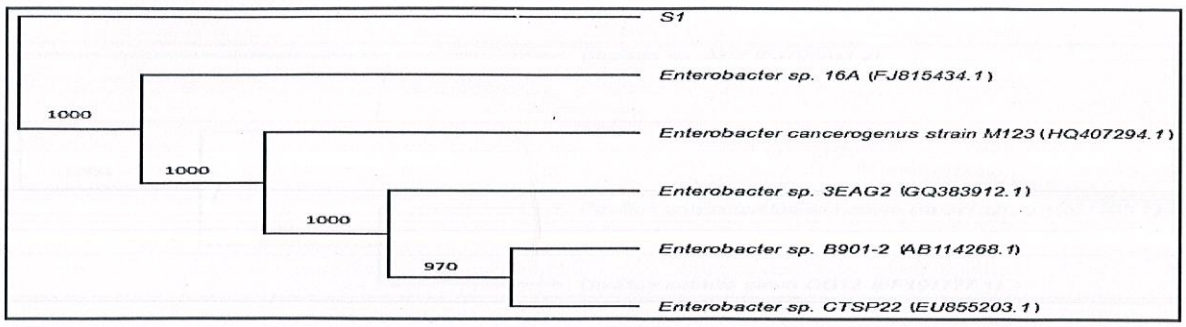


Figure 4.16: A phylogram (neighbour-joining method) showing genetic relationship between strain S1 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.16 it was inferred that the strain i.d. S1 is *Enterobacter sp.* which shows 96% identity with it. In this case the genus has been confirmed to be *Enterobacter* but the species cannot be confirmed. All the BLAST results consensus over the genus with 96% identity. For the characterization of the specific species, biochemical tests would be needed to be performed.

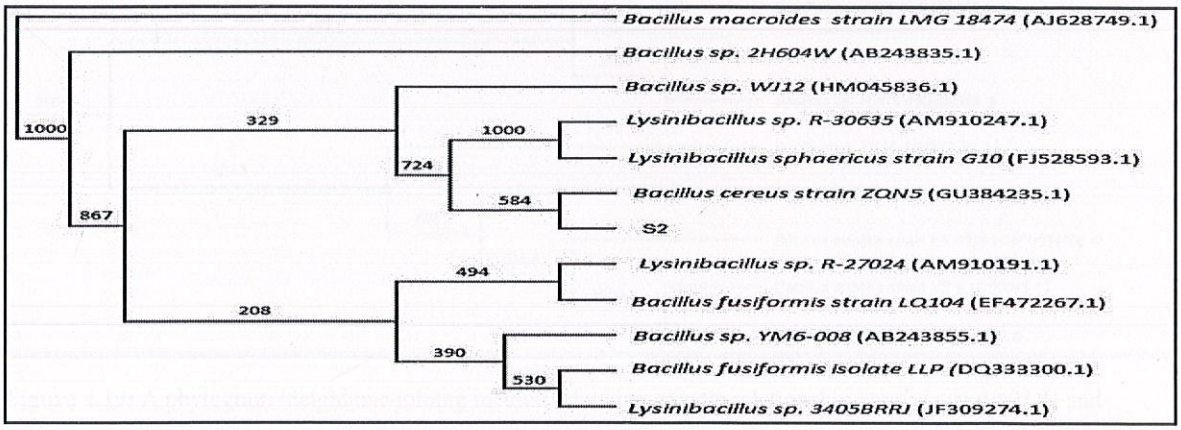


Figure 4.17: A phylogram (neighbour-joining method) showing genetic relationship between strain S2 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.17 it was inferred that the strain i.d. S2 is *Bacillus cereus* which shows 97% identity with it. The high bootstrap value of 584 indicates that we can say with 58.4% confidence that the phylogenetic relationship derived is correct.

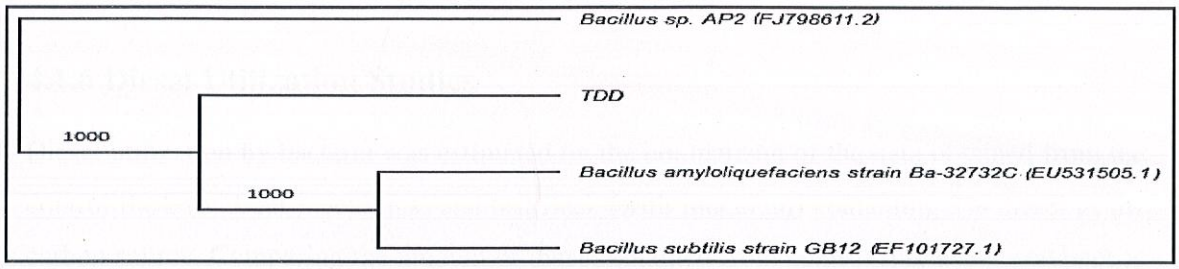


Figure 4.18: A phylogram (neighbour-joining method) showing genetic relationship between strain TDD and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.18 it was inferred that the strain i.d. TDD is either *Bacillus amyloliquefaciens* or *Bacillus subtilis.* which shows 99% identity with it. The high bootstrap value of 1000 indicates that we can say with 100% confidence that the phylogenetic relationship derived is correct.

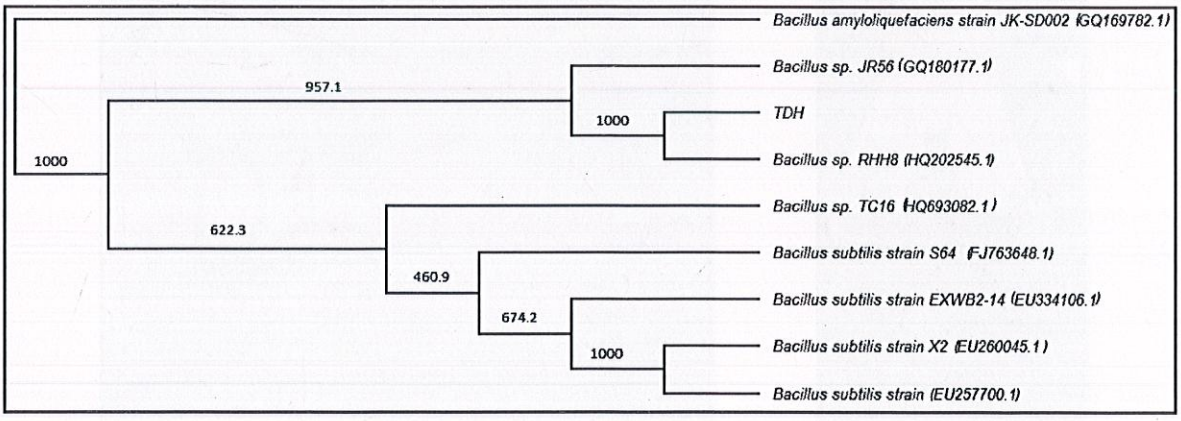
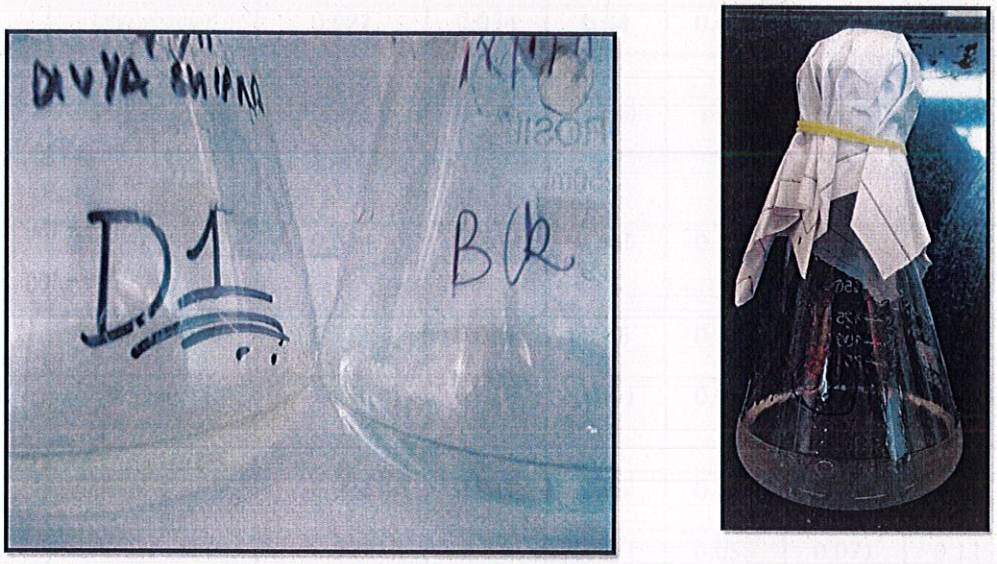


Figure 4.19: A phylogram (neighbour-joining method) showing genetic relationship between strain TDH and other related reference microorganisms based on the 16S rRNA gene sequence analysis

From the tree given in Figure 4.19 it was inferred that the strain i.d. TDH is *Bacillus sp. RHH8* which shows 96% identity with it. This strain has not yet been characterized. The high bootstrap value of 1000 indicates that we can say with 100% confidence that the phylogenetic relationship derived is correct.

4.1.6 Diesel Utilization Studies

Diesel utilization by bacteria was estimated by the comparison of the data obtained from the control flask (without inoculums) and test flask (with inoculum) containing 3% diesel as the carbon source. Comparing the amount of diesel obtained from control flak using gravimetric analysis, with the fresh 1.5 ml diesel sample (total volume of media = 50 ml) , the evaporation loss was calculated and found to be almost negligible in the control. This may be attributed to diesel being a viscous liquid and the ambient temperature used in the studies, at which diesel cannot vaporize. From the result obtained by gravimetric analysis, the percentage diesel utilization is shown in Table 4.8, with the maximum percentage of 43% (approx.) by DP (*Bacillus sp.*) in 10 days thereby suggesting their use in bioremediation purpose.



(a) (b)

Figure 4.20: (a) Showing the difference in the turbidity demonstrated by Strain i.d. D1 and BLNK i.e. control flask
(b) Showing turbidity and emulsification activity demonstrated by strain i.d. S2

Strains i.d.	Strain	Day 0	Day 1	Day 2	Day 4	Day 6	Day 8	Day 10
Blnk	Blank	0.021	0.026	0.03	0.033	0.039	0.04	0.043
B1	*Leucobacter aridicolis*	0.021	0.078	0.116	0.135	0.175	0.19	0.215
B2	*Brevundimonas terrae*	0.02	0.051	0.082	0.101	0.116	0.123	0.132
BL1	*Staphylococcus pasteuri*	0.023	0.033	0.04	0.045	0.056	0.061	0.068
BL2	*Enterobacter sp*	0.02	0.039	0.048	0.057	0.064	0.065	0.069
BL3	*Staphylococcus epidermidis*	0.021	0.029	0.039	0.05	0.059	0.063	0.068
BL4	*Enterobacter hormaechei*	0.024	0.043	0.06	0.083	0.1	0.109	0.114
D1	*Leucobacter aridicolis*	0.022	0.034	0.34	0.069	0.08	0.088	0.093
D2	*Enterobacter cancerogenus strain M123*	0.022	0.036	0.059	0.08	0.102	0.111	0.121
DP	*Bacillus sp. Y39 / Bacillus pumilus*	0.024	0.06	0.165	0.188	0.203	0.212	0.222
DW	*Bacillus subtilis*	0.021	0.044	0.06	0.076	0.087	0.092	0.1
HPD	*Achromobacter xylosoxidans*	0.02	0.048	0.061	0.076	0.085	0.089	0.094
S1	*Enterobacter sp*	0.022	0.038	0.05	0.064	0.084	0.09	0.098
S2	*Bacillus cereus*	0.021	0.04	0.051	0.058	0.071	0.125	0.136
TDD	*B. subtilis / B. amyloliquefaciens*	0.024	0.031	0.039	0.045	0.062	0.071	0.089
TDH	*Bacillus sp RHH8*	0.021	0.034	0.054	0.083	0.107	0.119	0.133

Table 4.3: Showing the optical densities of the control (blank) and various other strains up to the period of 10 days

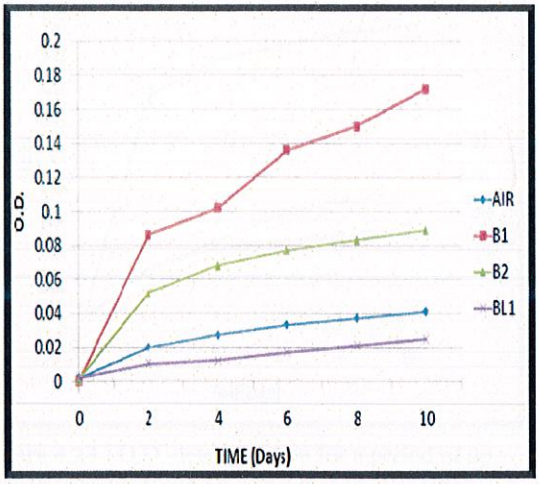


Figure 4.21: O.D. determination for a period of up to 10 days for strain i.d. AIR, B1, B2, BL1

TIME(Days)	AIR	B1	B2	BL1
0	0.002	0	-0.001	0.002
2	0.02	0.086	0.052	0.01
4	0.027	0.102	0.068	0.012
6	0.033	0.136	0.077	0.017
8	0.037	0.15	0.083	0.021
10	0.041	0.172	0.089	0.025

Table 4.4: Showing the values of the optical densities for a period of up to 10 days for strain i.d. AIR, B1, B2 and BL1

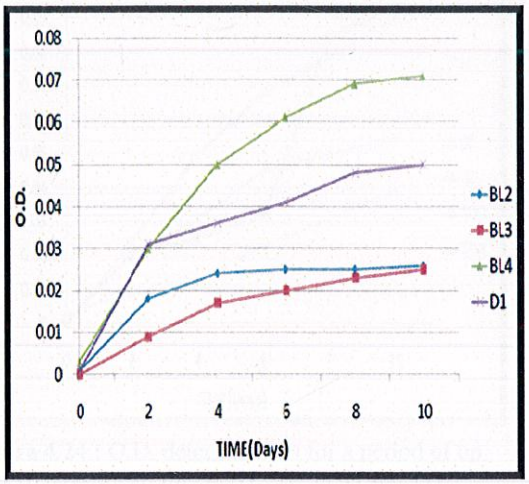


Figure 4.22: O.D. determination for a period of up to 10 days for strain i.d. BL2, BL3, BL4 and D1

TIME (Days)	BL2	BL3	BL4	D1
0	-0.001	0	0.003	0.001
2	0.018	0.009	0.03	0.031
4	0.024	0.017	0.05	0.036
6	0.025	0.02	0.061	0.041
8	0.025	0.023	0.069	0.048
10	0.026	0.025	0.071	0.05

Table 4.5: Showing the values of the optical densities for a period of up to 10 days for strain i.d. BL2, BL3, BL4 and D1

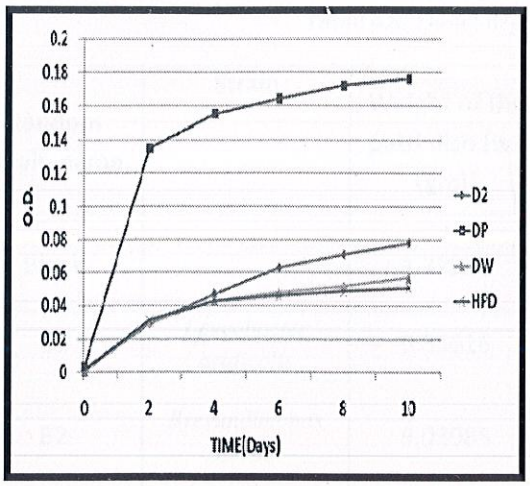


Figure 4.23 : O.D. determination for a period of up to 10 days for strain i.d. D2, DP, DW and HPD

TIME (Days)	D2	DP	DW	HPD
0	0.001	0.003	0	-0.001
2	0.029	0.135	0.03	0.031
4	0.047	0.155	0.043	0.043
6	0.063	0.164	0.048	0.046
8	0.071	0.172	0.052	0.049
10	0.078	0.176	0.057	0.051

Table 4.6: Showing the values of the optical densities for a period of up to 10 days for strain i.d. D2, DP, DW and HPD

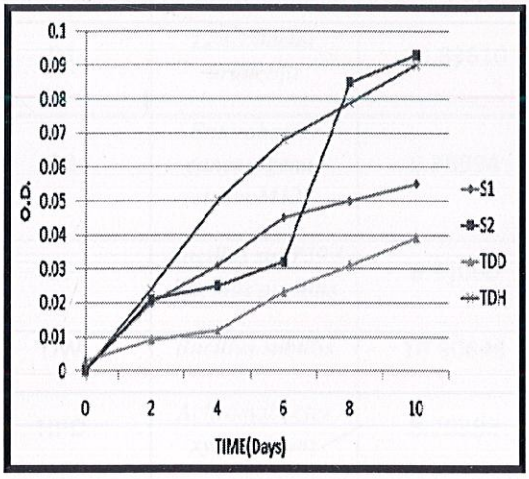


Figure 4.24 : O.D. determination for a period of up to 10 days for strain i.d. S1, S2, TDD and TDH

TIME(Days)	S1	S2	TDD	TDH
0	0.001	0	0.003	0
2	0.02	0.021	0.009	0.024
4	0.031	0.025	0.012	0.05
6	0.045	0.032	0.023	0.068
8	0.05	0.085	0.031	0.079
10	0.055	0.093	0.039	0.09

Table 4.7: Showing the values of the optical densities for a period of up to 10 days for strain i.d. S1, S2, TDD and TDH

Table 4.8: Diesel degrading potential of strains

Random Strain name	Strain	Weight of the petri dish [w1 (gm)]	Weight of the petri dish with diesel [w2 (gm)]	Weight of the diesel [w2-w1 (gm)]	% diesel degradation
Blank		1.22041	2.44970	1.22929	-
B1	*Leucobacter aridicolis*	9.35616	10.13523	0.77907	36.62439
B2	*Brevundimonas terrae*	9.03085	9.82914	0.79829	35.06089
BL1	*Staphylococcus pasteuri*	11.00318	11.87541	0.87223	29.04603
BL2	*Enterobacter sp*	8.96639	9.80007	0.83368	32.18199
BL3	*Staphylococcus epidermidis*	10.94228	11.75532	0.81304	33.86101
BL4	*Enterobacter hormaechei*	10.93815	11.78999	0.85184	30.70472
D1	*Leucobacter aridicolis*	10.89610	11.79536	0.89926	26.8472
D2	*Enterobacter cancerogenus strain M123*	9.38994	10.18793	0.79799	35.08529
DP	*Bacillus sp. Y39 / Bacillus pumilus*	8.94060	9.63661	0.69601	43.38114
DW	*Bacillus subtilis*	10.90646	11.75330	0.84684	31.11145
HPD	*Achromobacter xylosoxidans*	8.70383	9.51911	0.81528	33.67879
S1	*Enterobacter sp*	8.89149	9.72812	0.83663	31.94202
S2	*Bacillus cereus*	8.87569	9.63148	0.75579	38.51817
TDD	*B. subtilis / B. amyloliquefaciens*	11.12808	11.93786	0.80978	34.1262
TDH	*Bacillus sp RHH8*	10.78314	11.63498	0.85184	30.70472

4.2 Discussion

By performing Gram staining on the strains we concluded that five strains (B2, BL2, BL4, D2 and HPD) are Gram negative while the other ten strains (B1, BL1, BL3, D1, DP, DW, S1, S2, TDD and TDH) are Gram positive. It was thought that Gram-negative bacteria (*Acinetobacter, Pseudomonas, Alkanivorax* and related genera) are often dominant in microcosms after oil spill simulations (Cho *et al.*, 1997; MacNaughton *et al.*, 1999; Margesin *et al.*, 2003; Kasai *et al.*, 2005; Brakstad and Bonaunet, 2006) and in hydrocarbon contaminated Environments after biostimulation (Kaplan and Kitts, 2004; Röling *et al.*, 2004). These groups of bacteria are associated with a fast petroleum-degradation phase and their large quantity is positively correlated to total petroleum hydrocarbons (TPH) (Kaplan and Kitts, 2004; Röling *et al.*, 2004; Yakimov *et al.*, 2005). According to Kaplan and Kitts (2004), Gram positive bacteria, when detected in environment, are never prevailing. The role of Gram positive bacteria is largely unknown and it can be hypothesized that they belong to minor communities having a role in the degradation of more specific hydrocarbon classes. Very few Gram positive bacteria, mainly *Rhodococci,* have been described as having the capability of degrading alkanes.

But recently, Gram positive bacteria have been shown to have a great potential for the biotransformation and biodegradation of organic compounds (Larkin *et al.*, 2005). Gram positive bacteria particularly *Bacillus* sp. has been attracting interest in both environmental bioremediation strategies. The dominance of Gram positive bacteria in this study is not surprising, since these bacteria have a stronger cell envelope and are more tolerant to high levels of hydrocarbons due to their resistant endospores than Gram negative bacteria, which allow them to thrive in the highly variable diesel contaminated environment (Zhuang *et al.*, 2002).

There is growing evidence that isolates belonging to the *Bacillus* sp. are effective in clearing oil spills (Ghazali *et al.*, 2004). More recently, *Bacillus, Geobacillus* (phylum *Firmicutes*) and *Thermus* (phylum *Deinococcus-Thermus*) isolates were found to degrade alkanes (Marchant *et al.*, 2006; Meintanis *et al.*, 2006). Recently, Obuekwe *et al.* (2009) identified *Bacillus* sp. And *Paenibacillus* sp. as two of the most prominent crude-oil degraders in the Kuwait desert environment. The dominant isolation of these spore-forming Gram positive

bacteria was predicated on their ability to survive the prevalent high soil temperature (40 - 50°C) and tailings from oil-recovery operations (e.g. water flooding) (Ganesha *et al.*, 2009). Even in our present study, the strains with the highest diesel degradation potential are Gram positive being *Bacillus sp.* which can be considered to be *Bacillus pumilus* and *Bacillus cereus* with 43% and 38% diesel degradation potentials respectively.

The strains so identified by us have previously been implicated in the process of hydrocarbon degradation in several other studies conducted elsewhere. In a study conducted by Kebria *et al.*, 2009, *Bacillus Cereus* was isolated from oil refinery field in Tehran, Iran and was found to degrade 86.67% diesel at 500 ppm and 85.20% at 10000 ppm. In another study conducted by Singh *et al.*, 2010, *Bacillus pumilus* was found to degrade 85.7% diesel. In our study the top degrader also happened to be *Bacillus sp.*.

Another genus identified in our study happened to be that of *Enterobacter,* which common gut colonizer is in healthy human and also animals. The enteric bacteria in the family enterobacteriaceae are mainly regarded as inhabitants of animal guts (Daiz *et.al.*, 2001), but *Leclercia adecarboxylata* was noticed in oily-sludge contaminated soil . In another study conducted by Rajasekar *et al.*, 2007, *Serratia marcescens* had been isolated in a cross country pipeline in North India and had diesel degradation potential. Saadoun, 2002, has isolated and characterize the bacteria from crude petroleum oil contaminated soil that happened to be *Enterobacter.*

Other four genuses namely *Staphylococcus, Brevundimonas, Achromobacter and Leucobacter* have also been previously found to be involved in diesel degradation activities. Rodríguez *et al.*, 2009, studied *Brevundimonas*, previously isolated from United States Air Force aviation fuel tanks, for its capability of degrading polycyclic aromatic hydrocarbons. Shukor *et al.*, 2009 has characterized a diesel-degrading strain isolated from a hydrocarbon-contaminated site. The isolate was tentatively identified as *Staphylococcus sp.*. Taske *et al.*, 2011, isolated eight bacteria capable of using diesel fuel as the sole carbon source from both a bunker oil-saturated soil sample and from a gasoline-contaminated water sample. And one isolate was found to be a *Achromobacter sp.*. Wang W, 2010 has found alkane hydroxylase (alkB) genes in the in Leucobacter sp. isolated from subtropical seawater of Xiamen Island.

CONCLUSION

Among the several clean up techniques available to remove petroleum hydrocarbons from the soil and groundwater, bioremediation processes are gaining ground due to their simplicity, high efficiency and cost effectiveness when compared to other technologies. Degradation of hydrocarbons by environmental microflorae involves microorganisms having specialized metabolic capacities. In polluted environments, specialized microorganisms are abundant because of the adaptation of the microflorae to pollutant. It has also been shown that bacteria are the most predominant microorganism among other microorganisms in either in-situ or ex-situ bioremediation processes, indicating that bacteria are the main agents responsible for the degradation of diesel fuel (Kebria Y.D. *et al,* 2009). In the previous study sixteen bacterial strains were isolated from diesel contaminated site which had hydrocarbon degrading potential. In the present study fifteen strains out of the sixteen had been successfully revived and they were checked for their diesel degradation potentials. Strains could degrade diesel as high as 43% by *Bacillus sp.* (which can be considered to be *Bacillus pumillus* with 98% identity). High degradation capacities indicate the potential of these strains to be used for bioremediation of sites contaminated with diesel oil. For the determinative characterization of these strains, molecular characterization was done using 16S rRNA universal primers. The sequencing of 16S rRNA followed by *in silico* comparison with the 16S rRNA sequences present in Genbank (NCBI database) and further phylogenetic analysis revealed the identity of the strains broadly belonging to the genus *Bacillus, Enterobacter, Staphylococcus and Leucobacter.* One strain also belonged to the genera *Achromobacter* and one to *Brevundimonas.* After the identification of the hydrocarbon-degrading genes present in the strains, it will be possible to make a microbial consortia consisting of strains which have high diesel degradation potential.

Bibliography

- Adekunle A.A., Adebambo O.A., "*Petroleum hydrocarbon utilization by fungi isolated from Detarium Senegalense (J.F Gmelin) seeds.*", J. Am. Sci., 3:69-79, 2000.
- Amann R. I., Ludwig W., Schleifer K.H.," *Phylogenetic identification and in situ detection of individual microbial media with water-insoluble substrates*", Microbiol. Rev., 59: 143-169, 1995.
- Anderson T.A., "*Bioremediation.*", Environ. Sci. Technol., 27:2630-2637, 1993.
- April T.M., Abbott S.P., Foght J.M., Currah R.S., "*Degradation of hydrocarbons in crude oil by the ascomycete Pseudalleacheria boydii (Microascaceae)*", Can. J. Microbiol., 44:270-278, 1998.
- Atlas R.M., "*Microbial degradation of petroleum hydrocarbons: an environmental perspective*", Microbiol. Rev., 45: 180-209, 1981.
- Awasthi P., Sood I., Syal S., "*Isolating diesel degrading bacteria from air*", Curr. Sci. 94:178-180, 2008.
- Bento F.M., Camargo F.A., Okeke B., Frankenberger W.T., "*Bioremediation of soil contaminated by diesel oil*", Braz. J. Microbiol., 34:65-68, 2003.
- Bosch X., "*Exposure to oil spill has detrimental effect on clean-up workers' health.*", The Lencet, 361:147, 2003.
- Brim H., McFarlan S.C., Fredrickson J.K., Minton K.W., Zhai M., Wackett L.P., Daly M.J., "*Engineering Deinococcus radiodurans for metal remediation in radioactive mixed waste environments*", Nature Biotechnol., 18:85-90, 2000.
- Brusa T., Borin S., Ferrari F., Sorlini C., Corselli C., Daffonchio D., "*Aromatic hydrocarbon degradation patterns and catechol 2,3-dioxygenase genes in microbial cultures from deep anoxic hypersaline lakes in the eastern Mediterranean sea*", Microbiol Res., 156: 49-58, 2001.
- Clement B.G., Kehl L.E., DeBord K.L., Kitts C.L., "*Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities*", Microbiol. Methods, 31: 135-142, 1989.

- Cerniglia C.E., "*Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future application in bioremediation*", J. Ind. Microbiol. Biotechnol., 19:324-333, 1997.
- Cerniglia C.E., "*Microbial degradation of polycyclic aromatic hydrocarbons*", Adv. Appl. Microbiol., 30:31-71, 1984.
- Chang W., Um Y., Hoffman B., Holoman T.R.P. "*Molecular characterization of polycyclic aromatic hydrocarbon (PAH) – degrading methanogenic communities*", Biotechnol. Prog., 21:682-688, 2005.
- Chen H.W., " *Distribution and risk assessment of polycyclic aromatic hydrocarbons in household drinking water*", Bull. Environ, Contam. Toxicol., 78:201-205, 2007.
- Chatterjee D.K., Kellogg S.T., Hamada S., Chakrabarty A.M., "*Plasmid specifying total degradation of 3-chlorobenzoate by a modified ortho pathway*". J. Bacteriol., 146:636-646,1981.
- ChristopherK., Bruno E., "*Identification of Bacterial Species*", Association for Biology Laboratory Education (ABLE), http://www.zoo.utoronto.ca/able, 2003.
- Cronin M.A., Bickham J.W., "*A population genetic analysis of the potential for a crude oil spill to induce heritable mutation and impact natural populations*", Ecotoxicol., 7:259-278, 1998.
- Diaz E., Fernandez A., Prieto M. A., Garcia J. L., "*Bioremediation of aromatic compounds by Escherichia coli*", Microbiol. Mol. Biol., 65:523–569, 2001.
- De Silva M., Esposito E., Moody J.D., Canhos V.P., Cernigilia C.E., "*Metabolism aromatic hydrocarbons by the filamentous fungus Cyclothyrium sp.*", Chemosphere., 57:943-952, 2004.
- Drancourt, M., Bollet C., Carlioz A., Martelin R., Gayral J. P., Raoult D., "*16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates*", J. Clin. Microbiol.38:3623–3630, 2000.
- Desouky A., "*Acinetobacter: Environmental and biotechnological application*". Afr. J. Biotechnol., 2:71:74, 2003.
- Dojka M.A., Hugenholtz P., Haack S.K., Pace N.R., "*Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation*", Appl. Environ. Microbiol.64: 3869-3877,1988, 1998.

- Exner J.H., "Bioremediation: Field experience", edited by Lewis Pulishers, Boca Raton, F.L, USA, 1994.
- Ficker M., Krastel K., Orlicky S., Edwards E., "Molecular characterization of a toluene-degrading methanogenic consortium", Appl.Environ. Microbiol., 65: 5576-5585, 1999.
- Foght J., Semple K., Westlake D.W.S., Blenkinsopp S., Sergy G., Wang Z., Fingas M. "*Development of a standard bacterial consortium for laboratory efficacy testing of commercial freshwater oil spill bioremediation agent*", J. Ind. Microbiol. Biotechnol., 21:322-330, 1998.
- Halebian S., Harris B., Finegold S.M., Rolfei R.D., "*Rapid method that aids in distinguishing gram-positive from gram-negative anaerobic bacteria*", J. Clinical Microbiol.,13: 444-448, 1981.
- Hamann C., Hegemann J., Hilderbrandta A. "*Detection of polycyclic aromatic hydrocarbon degradation genes in different soil bacteria by polymerase chain reaction and DNA hybridization*". FEMS Microbiol. Lett., 173:255-263, 1999.
- Hamer G., "*The functioning of a microbial consortium for bioremediation*", Ann. N.Y. Acad. Sci., 665:435-448, 1992.
- Hanson K.G, Desai J.D., Desai A.J., "*A rapid and simple screening technique for potential crude oil degrading microorganisms.*", Biotechnol. techniques 7: 745- 748, 1993.
- Haung G.H., Chen Z., Tontowachwuthikul P., Chakma A., " *Environmental risk assessment for underground storage tanks through an interval parameter fuzzy relation analysis approach*", Energy sources Part A, 21:75-96, 1999.
- Higgins D.G., Thompson J.D., Gibson T.J., "*Using CLUSTAL for multiple sequence alignments*", Methods Enzymol., 266:383-402, 1996.
- Giovannoni S.J., DeLong E.F., Olsen G.J., Pace N.R., "Phylogenetic group-specific oligo-deoxynucleotide probes for identification of single microbial cells", Bacteriology, 170: 720-726,1998.
- Gold T., "The origin of natural gas and petroleum, and the prognosis for future supplies", Annu. Rev. Energy, 10: 53-77, 1985.
- Ilori M.O., Amobi C.G., Odocha A.C., "*Factors affecting biosurfactant production by oil degrading Aeromonas sp. isolated from a tropical environment*", Chemosphere., 61:985-992, 2005.

- Janda J.M., AbbottS.L., "*16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls*", J Clin Microbiol., 45: 2761–2764, 2007.
- Koch A., Kappeli O., Fiechter A., Reiser J., "*Hydrocarbon assimilation and biosurfactant production in Pseudomonas aerunginosa mutants*", J. Bacterio., 11:4212-4219, 1991.
- Labana S., Singh O.V., Basu A., Pandey G., Jain. R.K., "*A microcosm study on bioremediation of p-nitrophenol-contaminated soil using Arthrobacter protophormiarae RKJ100*" Appl. Microbiol. Biotechnol., 68:417-424, 2005.
- Leahy J.G., Colwell R.J.L., "*Microbial degradation of hydrocarbons in the environment*", Microbiol. Rev. 54: 305-315, 1990.
- Lidderdale T., "*Demand, supply, and price outlook for low-sulfur diesel fuel. In Energy Information Adminismtion. Short term energy outlook annual supplement*", DOE-E&0202 (93),1993.
- Lovely D.R., "*Cleaning up with genomics: applying molecular biology to bioremediation*", Nature Rev. Microbiol., 1:35-44, 2003.
- Margesin R.,Labbé D., Schinner F., Greer C.W., Whyte L.G., "*Characterization of Hydrocarbon-Degrading Microbial Populations in Contaminated and Pristine Alpine Soils*", Applied and Environmental Microbiology., 69:3085-3092, 2003.
- Mrozik A., Piotrowska-Seget Z., Labuzek S., "*Bacterial Degradation and bioremediation of polycyclic aromatic hydrocarbon*", Pol. J. Environ. Stud., 12:15-25, 2003.
- Mignard, S., Flandrois J. P., "*16S rRNA sequencing in routine bacterial identification: a 30-month experiment*", J. Microbiol. Methods, 67:574–581, 2006.
- Mehrasbi M. R., Haghighi B., Sharial M., Naeri S & Naddafi K, "*Biodegradation of petroleum hydrocarbons in soil*", Iran J Pub Health, 32: 2999.
- Neff J.M., Stout S.A., Gunster D.G., "*Ecological risk assessment of polycyclic Aromatic hydrocarbon in sediments: identifying sourced and ecological hazards*", Inter. Environ. Assess. Manage., 1:22-33, 2005.
- Nikolausz M., Kappelmeyer U., Nijenhuis Z., Kastner M., "*Molecular characterization of dichloromethane – degrading Hyphomicrobium strains using 16*

srRNA and DCM dehalogenase gene sequences", Syst. Appl. Microbiol., 28:582-587, 2005.

- Patel, J. B., "*16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory*", Mol. Diagn. 6:313–321, 2001.

- Philip J.C., Atlas R.M., "*Bioremediation of contaminated soils and aquifers, In Atlas RM and Philip J (ed.), Bioremediation applied microbial solutions for real-world environmental cleanup*", Am. Soc. Microbiol. Washington D.C., 140-190, 2005.

- Pfender W.F., Maggard S.P., Gander L.K., Watrud L.S., " *Comparision of three bioremediation agents for mineralization and transformation of pentachlorophenol in soil*", Bull. Environ. Contam. Toxicol., 59:230-237, 1997.

- Project Review and Monitoring Committee (PMRC), "*Road map for oil spill management for India. Office of principal scientific adviser to the government of India, New Delhi, India*", http://psa.gov.in/writereaddata/11913308371_File1_ROAD_MAP.pdf, 2003.

- Sacchi C.T., Whitney A.M., Mayer L.W., Morey R., Steigerwalt A., Boras A., Weyant R.S., Popovic T., "*Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of Bacillus anthracis*", Emerging Infectious Diseases., 8:1117-1123, 2002.

- Saterbake A.,Toy R.J., Wong D.C.L., McMain B.J., Dorn P.B.,Brzuzy L.P., Chai E.Y., Salanitro J.P., "*Ecotoxicoogical and analytical assessment of hydrocarbon-contaminated soil and application to ecological risk assessment*", Environ. Toxicol. Chem., 18:1591-1607, 1999.

- Shepp D.L., "*Petroleum Hydrocarbon Concentrations Observed in Runoff from Discrete, Urbanized Automotive-Intensive Land Uses*", Metropolitan Washington Council of Government, Watershed'96, 1996.

- Surridge A.K.J., "*Denaturing gradient gel electrophoresis characterisation of microbial communities in polycyclic aromatic hydrocarbon and polychlorinated biphenyl contaminated soil*", PhD Thesis. University of Pretoria, South Africa. pp. 1-133, 2007.

- Shukor M.Y., Hassan N.A.A, Jusoh A.Z., Perumal P., Shamaan N.A., MacCormack W.P., Syed M.A., "*Isolation and characterization of a Pseudomonas diesel-degrading strain from Antarctica*", J. Environ. Biol., 30 : 1-6, 2009.

- Shukor M.Y., Hassan N.A.A, Jusoh A.Z., Perumal P., Shamaan N.A., MacCormack W.P., Syed M.A., "*Characterization of a diesel-degrading strain isolated from a hydrocarbon-contaminated site*", J. Environ. Biol., 30 : 145-150, 2009.

- Vidali M., "*Bioremediation. An overview*", Pure Appl. Chem., 73:1163-1172, 2001 .

- Westlake D.W.S., "*Microorganisms and the degradation of oil under northern marine condition. In: Oils and dispersants in Canadian seas – research appraisal and recommendations*", Sprague J.B., Vandermeulen J.H., Wells P.G. (eds), Ottawa, Canada, pp., 47-50, 1982.

- Woo, Ng P. C. Y., Lau K. H. I., S. K. P., Yip K.-T., Fung A. M. Y., Leung K.-W., Tam D. M. W., Que T.-L., Yuen K.-Y., " *Usefulness of the MicroSeq 500 16S ribosomal DNA-based identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles*", J. Clin. Microbiol., 41:1996–2001, 2003.

- Yousefi D.K., Khodadadi A., Ganjidoust H., Badkoubi A., Amoozegar M.A., "*Isolation and characterization of a novel native Bacillus strain capable of degrading diesel fuel*", J. Environ. Sci. Tech., 6:435-442, 2009.

- Youssef N.H., Dumcan K.E., Nagle D.P., Savage K.N., Knapp R.M., Mclnerney M.J., "*Comparison of methods to detect biosurfactant production by diverse microorganisms*", J. Microbiol. Methods., 56:339-347, 2004.

- Zandbergen P.A., "*Urban watershed ecological risk assessment using GIS: A case study of the Brunette river watershed in British Columbia, Canada*", J. Hazard. Mater., 61:163-173, 1998.

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