ISOLATION AND CHARACTERIZATION OF DIESEL DEGRADING BACTERIA FROM AIR AND SOIL FOR BIOREMEDIATION OF CONTAMINATED SITES OF HIMACHAL PRADESH

By

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CERTIFICATE

This is to certify that the work entitled, "**ISOLATION AND CHARACTERIZATION OF DIESEL DEGRADING BACTERIA FROM AIR AND SOIL FOR BIOREMEDIATION OF CONTAMINATED SITES OF HIMACHAL PRADESH**" pursued by Prachi Awasthi (031544) and Isha Sood (031558) in partial fulfillment for the award of degree of Bachelor of Technology in Department of Bioinformatics and Biotechnology of Jaypee University of Information Technology is being carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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At the end we would like to dedicate our work to our parents and all the teachers. Their inspiring words will be a guiding force in all our endeavors to attain greater height.

Isha Sood
ISHA SOOD
23rd May, 2007
WAKNAGHAT.

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PRACHI AWASTHI
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LIST OF ABBREVIATIONS

cfu - Colony forming units

CTAB - Hexa decyl methyl ammonium Bromide

EDTA - Ethyline diamine tetra aceticacid

HRTC - Himachal Roadways Transport Corporation

IARC - International Agency for Research on Cancer

OD - Optical Density

ODB - Oil Degrading Bacteria

PVP - Polyvinylpyrrolidone

rpm - Rotations per minute

SDS - Sodium Dodecyl Sulphate

U.V - Ultraviolet

ABSTRACT

A large percentage of soil pollution is attributed to contamination by diesel and other hydrocarbons. Bioremediation is an ecofriendly, cheap and easily applicable natural method for curbing this problem. We selected diesel contaminated sites in Himachal Pradesh, India. Diesel contamination in soil was estimated to be within range of 1% to 9% in soil samples from various sites. Screening of potential diesel degrading microorganisms was done. 11 isolates were obtained. Isolation of diesel degrading bacteria from air was also done by simple and quick method from which one putative diesel degrading isolate was obtained. Effect of varying diesel concentrations on bacterial growth was studied. Diesel utilization studies were also performed at the end of the experiment. 4% was found to be optimum concentration for growth of diesel degrading bacteria. On performing various tests, some strains gave indication of belonging to *Pseudomonas* genus. Biochemical and molecular characterization of the isolates was done using various methods. For detecting unculturable population of microorganisms from diesel contaminated soils, metagenomics approach was used. This relatively new field of genetic research allows the genomic study of organisms that are not easily cultured in a laboratory.

CHAPTER-I

INTRODUCTION

1.1 - Hydrocarbon spillage

An oil spill is the unintentional release of liquid petroleum hydrocarbon into the environment as a result of human activity like disposal of waste motor oil, leaking of storage tanks and other spilllages 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, as along the California coastline. There is world wide concern about the liberation of hydrocarbons in the environment, both from industrial activities and from accidental spills of oil and oil-related compounds. The annual release of crude oil in the oceans is estimated to be around 1.7 to 1.8 metric tons and the impact of this pollution can be severe environmental imbalance (Koch *et al.* 1991).

1.2 - Hazards

Hydrocarbon spillage may lead to various envirnomental 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 numbers of people living and depending on coastal areas for fishing and tourism throughout the world, the consequences of oil spills can be serious (Fig 1.1 a and b).

Hydrocarbon spillage may also lead to various health hazards. It can lead to permanent genetic mutations on entry through food chain. Skin irritation problems 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.

(a) Effect on birds

(b) Effect on turtles

Fig 1.1 Hazards being caused to creatures because of hydrocarbon spillage.

1.3 - Reasons 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) Its carbon range is C_{15} to C_{20} which makes it a complex molecule to breakdown as compared to other hydrocarbons.
3) There is extensive diesel spillage in nearby sites (e.g Taradevi - Himachal Roadways Transport Corporation Workshop, Diesel storage unit of Jaypee University of Information Technology).
4) Due to its various health hazards.
5) It is a mixture of both aliphatic and aromatic hydrocarbons.

1.4 - Possible methods of dealing with diesel contamination

Though 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) **Physical** Mechanical recovery (Skimmers, Booms, Suctions, Separation)	Clumsy, time consuming.	Restricted
b) **Chemical** Sorbents – inorganic/organic (Vermiculites, Glass wool, Carbon wool, foam)	Requires other physical methods. Reuse of chemicals /sorbents is impractical.	Not ecofriendly
c) **Bioremediation** Oil degrading bacteria, Bioemulsifier, other mediators	Requires supply of nutrients.	Ecofriendly, easy application.

Table 1.1 Comparison of Physical, Chemical and Bioremediation techniques.

1.5 - Bioremediation

Bioremediation consists of using living organisms (usually bacteria, fungi, actinomycetes, cyanobacteria and to a lesser extent, plants) to reduce or eliminate toxic pollutants. The process of removing or at least significantly degrading the contaminants present in soils and ground water at the microbial level is known as bioremediation (Anderson *et al.* 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. 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. Bioremediation, has many advantages over other physical and chemical methods of curing diesel contaminated sites such 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 mechanical technologies. Importantly, bioremediation can also be used in conjunction with a wide range of traditional physical and chemical technologies to enhance their efficacy. 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. The ability to degrade hydrocarbon substrates including diesel is exhibited by a wide variety of bacterial genera. Some of these are *Pseudomonas, Acinetobacter, Arthrobacter, Bacillus, Alcaligenes, Flavobacterium, Nocardia* spp. *etc.* However when we use microorganisms to degrade the oil, we met 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 nonpolar domains, they are able to partition at water-oil interphases, emulsify hydrocarbons and thus reduce

the surface tension. Surfactants increase the surface area of hydrophobic contaminations 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).

1.6 - Project Overview

In the present study we isolated potential diesel degrading bacteria from contaminated sites in Himachal Pradesh. The percentage diesel contamination in the soil samples under study was calculated by gravimetric analysis. We also devised a new and simple method of isolating diesel degrading bacteria directly from air.12 isolates obtained from both the sources were morphologicaly and biochemicaly characterized using various methods like Gram staining, KOH string test, catalase test, spore staining test and growth on King's media etc. The strain isolated from air was selected for further studies. A set of experiments were conducted in order to check the diesel degrading potential of this isolate. The growth was monitored by performing microbiuret test and measuring pH on a daily basis. Biosurfactant analysis was also done by checking emulsification activity daily. Gravimetric analysis was done in order to quantify the diesel utilized by the bacteria. For detecting unculturable population of microorganisms from diesel contaminated soils, metagenomics approach is being used. This relatively new field of genetic research allows the genomic study of organisms that are not easily cultured in a laboratory. Early molecular work in the field was conducted by Norman R. Pace and colleagues, who used PCR to explore the diversity of ribosomal RNA sequences from organisms present in uncultured environmental samples (Handelsman *et al.* 1998).

1.7 - Objectives

1. Hydrocarbon estimation in problem soils, before and after the execution of the experiment.
2. Isolation of diesel degrading microbial strains from air and contaminated soil.
3. Characterization of diesel degrading microbial strains.

4. *In vitro* diesel degradation studies of microbial strains.
5. Estimation of biosurfactant production of microbial strains.
6. Metagenomics of diesel contaminated sites.

CHAPTER -2

MATERIALS AND METHODS

2.1 - MATERIALS

2.1.1 - Diesel

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

2.1.2 - Soil

The soil samples from the two diesel contaminated sites were collected using quadrant method i.e. were collected from different areas in the sites and were then pooled together so that final sample represents the whole area. These samples were then put in aseptic plastic bags and were then taken to laboratory for further processing.

2.1.3 – Chemicals

The chemicals used in the present study were obtained from S.D fine chemicals Limited, Merk Limited and Qualigens fine chemicals Limited. The various media used in the study namely Nutrient Broth, Nutrient Agar and Bushnell-Hass were manufactured by HiMedia Laboratories Pvt. Ltd.

2.2 - METHODS

2.2.1 - Estimation of Diesel contamination in the soil samples

Percentage of diesel contamination in soil was estimated using gravimetric analysis. 5g of gravel free soil was mixed with approximately 30ml of hexane and was kept on shaker for 20 minutes so that all of the diesel gets extracted by hexane. The mixture was filtered using a vacuum filter. The filterate was poured in a pre-weighed dish and was allowed to evaporate for about 8-10 hours after which the dish was weighed again. Increase in the weight of the dish gave the estimate of diesel contamination in 5g of soil.

2.2.2 - Screening of potential diesel degrading microorganisms from soil using enrichment culture technique

Fresh soil (1g) was inoculated in 250 ml autoclaved flasks with 100ml of Bushnell-Hass media ($MgSO_4$ 0.20 g/l, $CaCl_2$ 0.02 g/l, K_2HPO_4 1g/l, NH_4NO_3 1g/l, $FeCl_3$ 0.05 g/l, KH_2PO_4 1g/l) (Bushnell and Hass,1941) supplemented with 1% diesel (v/v) obtained from the nearby filling station. The flasks were incubated at 28°C with continuous shaking at 150 rpm. The growth was sub cultured (1ml inoculum) every alternate day in fresh medium (100ml) with 1% diesel for 12 days and then plated on the nutrient agar.11 bacterial colonies obtained were observed which were further characterized.

2.2.3 - Isolation of diesel degrading microorganisms from air

A less tedious and newer method of isolating diesel degrading strains directly from air was employed. The experiment was set up in 250 ml flasks containing 100 ml Bushnell Hass media, supplemented with increasing concentrations of diesel (2% to 8%) and left open for isolating strains from air against a blank which was well covered. The flasks were left open for 10 days and manual shaking was done at regular intervals. These cultures were then plated on nutrient agar and incubated at 28°C overnight after which one isolate was obtained.

2.2.4 - Characterization of bacterial strains obtained from air and soil

Gram nature of all the 12 strains was studied using conventional gram staining (Gregersen *et al.* 1978) method and KOH string test (Powers EM 1995). Conventional gram staining was done by taking a drop of the liquid cultures of the strains on a slide and leaving for air drying. The slide was flooded with crystal violet and left for 1 minute. It was then rinsed with water for 5 seconds. Then the smear was covered with gram's iodine for 1 minute. It was then rinsed for 5 seconds with water to remove the excessive stain. The smear was then decolorized with 95% ethanol for 15-30 seconds. The slide was rinsed for 5 seconds with water. Counter staining with safranin was done for 1 minute. Slide was rinsed again for 5 seconds with water. It was then blot dried with filter paper and viewed under a microscope using oil emulsion objective. Gram negative bacteria

appear pink due to counter stain i.e safranin whereas gram positive bacteria appear purple- violet in color.

For KOH string test, a visible amount of growth from a colony was picked and mixed continuously with a drop of 3% aqueous 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).

Further characterization of bacteria was done using catalase test (Maehly AC and Chance B, 1954) and spore staining method. A visible amount of bacterial growth was taken on a slide to which 3-4 drops of 3% H_2O_2 were added. Catalase breaks down hydrogen peroxide into water and oxygen. Hence bubbling indicates catalase positive nature of the bacterial strains.

Under spore staining procedure we took a drop of overnight grown bacterial culture on a slide and dried it over a flame. The smear was then covered with 5% malachite green and heated over a spirit lamp for 1-2 minutes till vapors appear without letting the stain boil off. Slide was allowed to cool. It was then rinsed with water for 30 seconds. Counter staining with safranin was done for 15-30 seconds. Slide was washed briefly to remove safranin, blot dried and examined under oil emulsion lens. In Spore forming bacteria green oval shaped or spherical objects within or outside of vegetative cells could be seen.

Bacterial strains were tested for Flourescence activity by plating them on King's media: (peptone 20 g/l, glycerol 10g/l, K_2HPO_4 1.5g/l, $MgSO_4.7H_2O$ 1.5 g/l, agar 15g/l ; pH 7.2. (King *et al.* 1954) and then visualized in U.V light.

2.2.5 - Determining the optimum diesel concentrations for air isolate

12-16 hours grown bacterial culture of air isolate was centrifuged at 10,000 rpm for 10 minutes and the pellet was washed thrice with 0.9% NaCl to completely remove traces of any carbon source. The pellet was then resuspended in 1 ml of 0.9% NaCl and spread on nutrient agar plate whose cfu/ml was calculated. The pellet was further resuspended in 8 tubes in 1 ml 0.9% NaCl each and inoculated in Bushnell Hass medium, supplemented with varying concentrations (2% to 8%) of diesel by performing the experiment in duplicates. The temperature was maintained at 28°C on shaker at 150 rpm. Growth was estimated by performing various tests on the cultures on a daily basis.

2.2.6 - Growth estimation studies for the air isolate

Microbiuret test (Gornall *et al.* 1949) was performed for measuring the protein content of the bacterial cultures. 1ml of overnight grown culture from each of the flasks containing varying concentrations of diesel was taken and centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with pH 7 buffer. The pellet was then resuspended in 2ml of pH 7 buffer. Blank consisted of just 2ml of pH 7 buffer. To each sample 1ml of biuret reagent (1% copper sulphate and 10% sodium hydroxide solution) was added to make the volume 3ml. The reaction mixture was incubated for 10 minutes. The concentration of the protein was calculated from the absorbance value obtained at 310 nm against the standard solution of bovine serum albumin (Itzhaki and Gill, 1964). Daily record of pH was also kept for estimating growth.

2.2.7 - Biosurfactant analysis

The surfactant activity in the culture fluid was determined using xylene emulsification method (Banat *et al*, 1991). A sample of the culture (35µl) or Tris buffer (2.0mM, pH 8.0) as control and an equal volume of xylene were added to the surface of 5ml of Tris buffer in a clean glass test tube. After vigorous mixing for 45 seconds, the mixture was allowed to stand at room temperature for 20 min. in the cuvette before reading the optical density at the aqueous phase at 660nm against the blank, using a spectrophotometer. High OD indicated the presence of surface active compounds causing dispersion of oil in the buffer.

2.2.8 - Metagenomic studies of diesel contaminated soils

Metagenomic studies of diesel contaminated soils were carried out using Soft lysis method in order to get whole soil DNA so that DNA of unculturable microbial population can also be obtained. To disrupt microorganisms by solely enzymatic and chemical means a modified protocol of Zhou *et al.* (1996) was used. 1g diesel contaminated soil samples were homogenized by vortexing in 750 µl lysis buffer [100 mM Tris-HCl, 100 mM sodium EDTA, 1.5 M NaCl, 1 % CTAB; pH 8; to which 2% PVP, 5% β-mercaptoethanol and 0.12% Sodium Sulfite were added to prevent diesel degradation.] at maximum speed for 5 min in 2-ml screw-cap tubes. 40 µl lysozyme (50 mg/ml) and 10 µl

proteinase K (10 mg/ml) were added before incubation at 37°C for 30 min. After addition of 200 µl SDS (20 %), mixtures were incubated at 65°C for 2 h with vigorous shaking by hand after every 30 min. The supernatants were collected by centrifugation at 12,500 rpm for 10 min at room temperature, and the pellets were re-extracted twice by adding 500 µl of lysis buffer, vortexing for a few seconds, and incubation at 65°C for 10 min. Centrifugation was carried out as before. The combined supernatants were extracted with an equal volume of chloroform before precipitating the DNA from the recovered water phase by addition of 0.6 volumes of isopropanol and overnight incubation at 4°C. The precipitates were collected by centrifugation at 13,000 rpm, washed with 70 % ethanol, and suspended in a total volume of 50 µl TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA; pH 8). The DNA was stored at -20°C for future use. The DNA so obtained was run on 8% agarose gel and was visualized under U.V light.

CHAPTER -3

RESULTS AND DISCUSSION

3.1 - Selection of diesel contaminated sites.

We selected two sites for carrying out this study. One of these was JUIT diesel storage unit (Fig3.1(a)) and the other was a 50 year old HRTC workshop in Taradevi (Fig3.1(b)). These sites were selected because soils present there are getting contaminated with diesel since many years and chances of obtaining diesel degrading bacteria from these were very high.

Fig.3.1(a) JUIT diesel storage unit

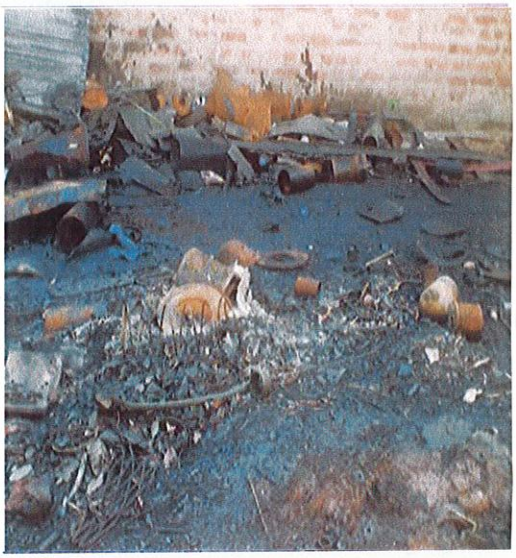


Fig.3.1(b) HRTC Taradevi workshop

3.2 - Estimation of diesel contamination in the soils studied

The first set of experiments was a simple gravimetric analysis of the soil samples for the estimation of percentage diesel contamination (Table 3.1). The soil sample collected from the drain of a local diesel storage unit was found to be the most contaminated one with 9% diesel contamination. The number of diesel degrading microorganisms isolated from the drain soil were very less which indicates that higher percentage of diesel is not

favorable for many diesel degrading bacteria. This may be due to unavailability of nutrients or due to production of toxic intermediates which inhibit the growth of diesel degrading microorganisms. We could isolate 2 strains of diesel degrading bacteria D1 and D2 (arbitrary names) from drain soil. The ability of these organisms to grow at such high percentage of diesel indicate that these bacteria have a high diesel degrading potential and tolerance range.

PLACE NAME	SAMPLE	% DIESEL
JUIT (university diesel storage unit)	Soil	5%
	Soil (drain)	9%
TARADEVI (H.R.T.C. Workshop)	Soil(black in colour)	6%
	Soil(brown in colour)	1%

Table 3.1 – Diesel contamination in soil samples used in the study.

3.3 - Bacterial isolation from air

The first set of experiments was simple isolation of diesel degrading bacteria from air at room temperature. All the flasks containing varying concentrations of diesel were showing turbidity after a few days, hence were indicating bacterial growth. Flasks having 2% and 4% diesel concentration indicated observable growth after 4 days. After 6 days, other flasks containing higher percentage of diesel were also showing growth and emulsification but lesser as compared to the ones with lower concentration of diesel. This observation led to the conclusion that lower concentrations of diesel are more favorable for growth of the isolate. The temperature at which the experiment was carried out varied between 19°C to 32°C. Isolation at such variable temperature indicated that our strain had a wide range of thermal tolerance. Plating of the cultures from flasks having variable diesel concentrations on nutrient agar gave identical colonies which indicated towards the presence of a single strain in all the flasks. The flask containing 2% diesel concentration which was showing the best growth was chosen for further studies.

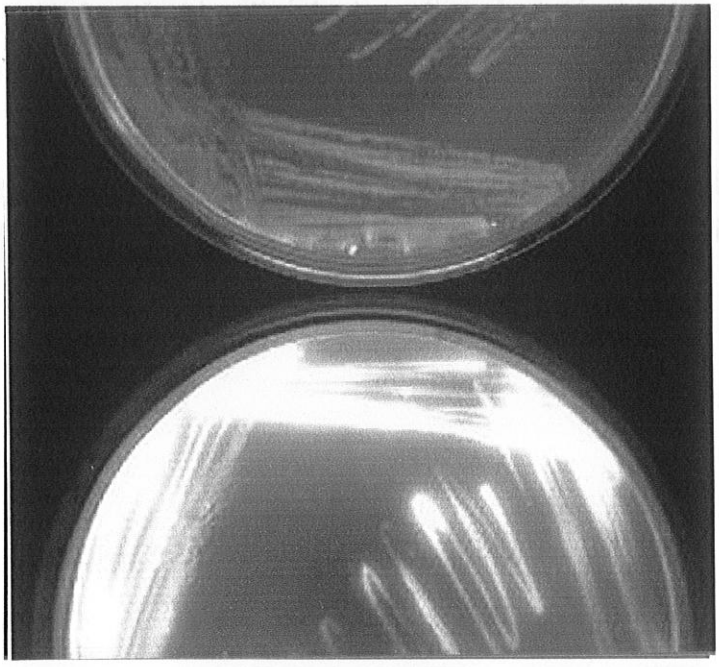
3.4 - Biochemical and morphogical studies of the isolated strains

The isolates obtained from both the sources i.e air and diesel contaminated soils were morphologicaly and biochemicaly characterized using various methods. Gram nature of the isolates was studied using conventional gram staining procedure and KOH method (Table 3.2). The results obtained from the data concluded that the isolates from air and soil show similar characteristics and hence may belong to same species or even may be the same strain. All the isolates obtained from Taradevi soil along with the air isolate gave indication of belonging to *Pseudomonas* species as they showed fluorescence (Fig 3.2a, 3.2b). Further studies to identify these isolates will be carried out in future.

Strain name	**Gram nature (conventional method)**	**Gram's nature (KOH test)**	**Catalase test**	**Flourescence (on King's media)**
A1 (air)	**-ve**	**-ve**	**+ve**	**present**
S1 (soil)	-ve	-ve	+ve	absent
S2 (soil)	-ve	-ve	+ve	absent
S3 (soil)	-ve	-ve	+ve	absent
D1 (soil)	-ve	-ve	+ve	absent
D2 (soil)	-ve	-ve	+ve	absent
B1 (soil)	-ve	-ve	+ve	present
B2 (soil)	-ve	-ve	+ve	present
BL1 (soil)	-ve	-ve	+ve	present
BL2 (soil)	-ve	-ve	+ve	present
BL3 (soil)	-ve	-ve	+ve	present
BL4 (soil)	-ve	-ve	+ve	present

Table 3.2 – Characterization of different isolates on the basis of gram staining, KOH string test, catalase test and flourescence on King's media.

a) Air isolate showing fluorescence



b) One of the soil isolates being compared with non - fluorescence showing soil isolate.

Fig 3.2 - Fluorescence on King's media.

3.5 - Study of growth pattern of air isolate on varying concentrations of diesel

To test the diesel degrading potential of the air isolate an experiment was setup which continued for 9 days. Growth was monitored on a daily basis at an interval of 24 hours, by performing various tests like pH, biuret test and emulsification activity.

The daily pH measurements (Fig 3.3) showed the shift of pH towards alkaline side with the growth of bacteria at all diesel concentrations. Such a pH pattern in the growth phase may be due to the secretion of some alkaline product by this bacterium. Chemical analysis of the bacterial secretions may be done in future.

The data obtained by the growth pattern of isolate suggested that our isolate exhibited best growth at 2% and 4% diesel concentrations followed by 6% and 8% (Fig 3.4). The growth in 2% and 6% diesel concentrations peaked around 168 hours and declined thereafter whereas the culture in flask containing 4% diesel was still in growth phase which indicates that 4% is the optimum concentration for the growth of this isolate. Even at 8% concentration, the strain was growing but at a low pace. This result strengthened the earlier observations which suggested that low and moderate diesel concentrations favor better growth of this diesel degrading bacterium as compared to higher concentrations.

3.6 - Emulsification activity of diesel degrading bacterial strain isolated form air

4% diesel concentration was showing best emulsification activity (Fig 3.5).The emulsification activity was determined by xylene emulsification test. Emulsification activity was seen in all the flasks containing different concentrations of diesel (2 to 8%). This emulsification activity is probably due to biosurfactant released by the diesel degrading bacterium isolated form air. Many researchers have reported the production of biosurfactant from various bacterial strains including *Pseudomonas aeruginosa*. And these biosurfactants were useful in enhancing the removal of hydrocarbons from contaminated sites (Harvey *et al.*1990, Bragg *et al.* 1994).

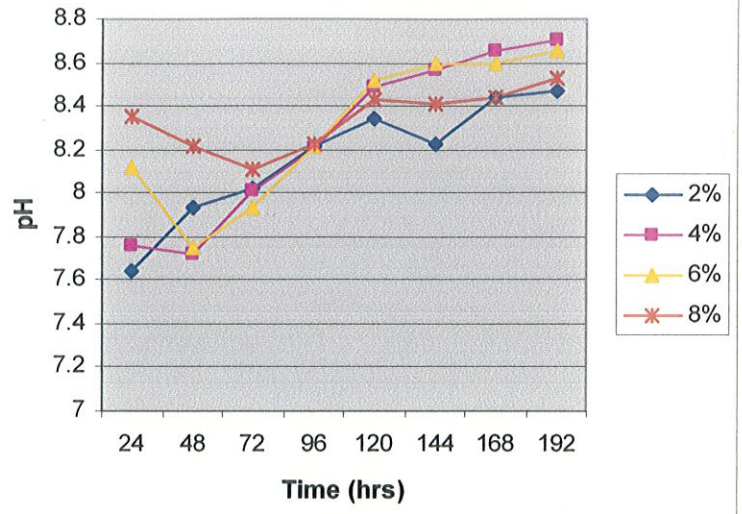


Fig 3.3 pH change during the growth of air isolate on increasing percentage of diesel in the medium

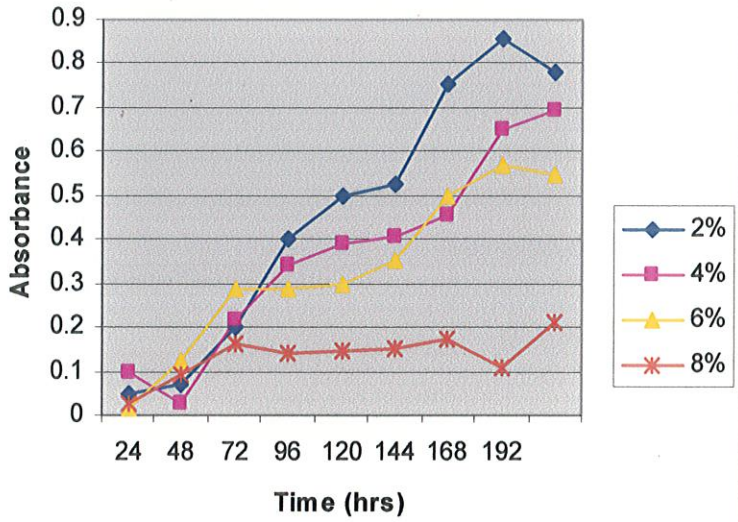
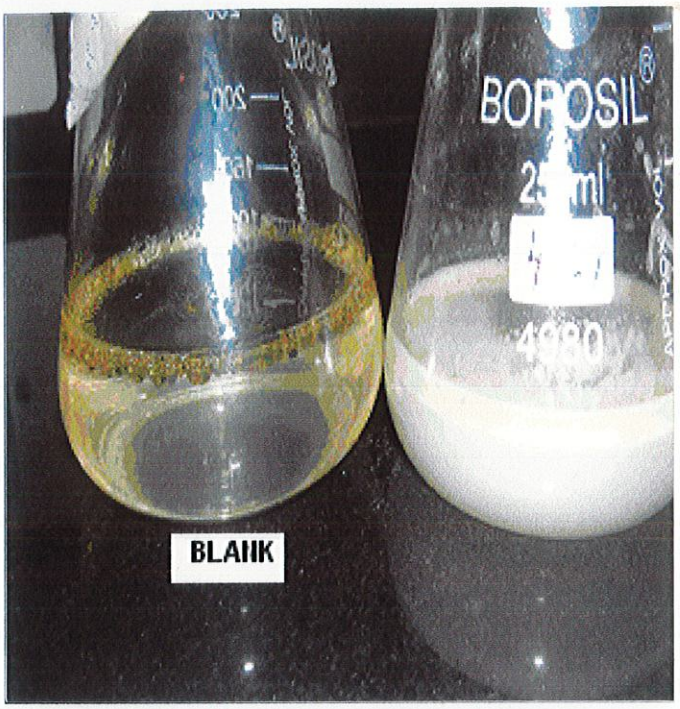
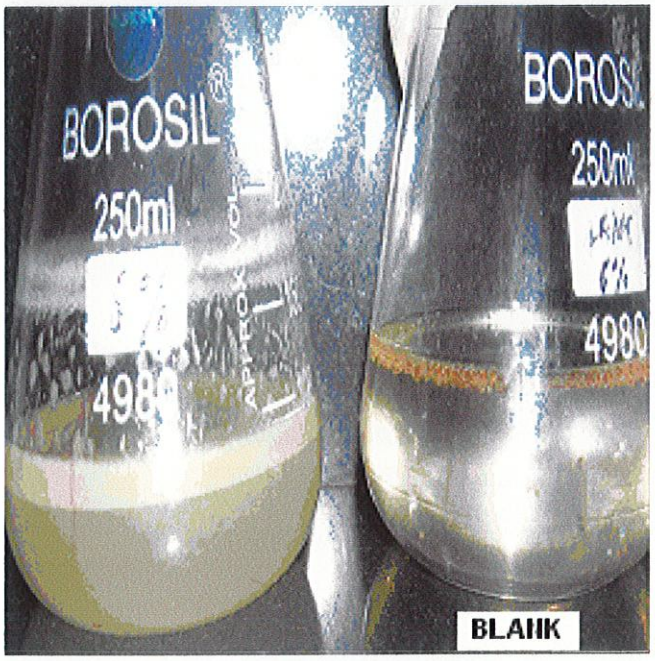


Fig 3.4 Growth of air isolate on increasing percentage of diesel in the medium



a) 4% diesel concentration



b) 6% diesel concentration

Fig 3.5 Flasks showing diesel degradation and emulsion formation by the A1 air isolate at different diesel concentrations against blank.

3.7 - Diesel mineralization studies of the air isolate

Diesel utilization before and after the commencement of the experiment was studied for each of the flasks containing varying diesel concentrations. From the data (Table 3.3) it can be seen that maximum diesel utilization by the air isolate has taken place in the flask containing 4% diesel, followed by 2% concentration. A significant percentage of diesel has also been utilized in the flask containing 6% diesel, but the percentage degradation is lesser as compared to 2% and 4%. Minimum diesel utilization has taken place in the flask containing higher percentage of diesel i.e. 8%.The flask containing 4% diesel was showing very good growth and was still in the growth phase when the experiment was terminated. The flask also showed maximum emulsification as compared to other concentrations hence maximum diesel utilization had taken place in this flask (66%) which is further evident from the data of diesel utilization studies. The flask containing 8% diesel concentration was showing growth at a slow pace as compared to other concentrations and the percentage diesel utilization recorded in this flask was just 7% as evident from data shown in table 3.3, though it was still showing increase in cell biomass when the experiment was terminated.

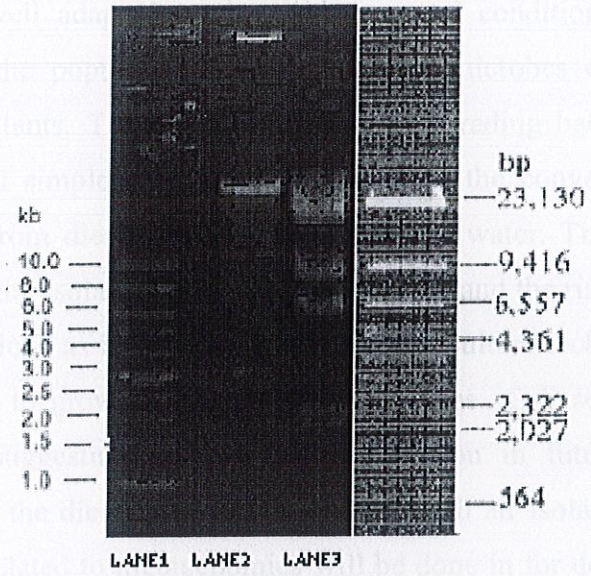
% Diesel added	% Diesel utilized
2%	49
4%	66
6%	36
8%	7

Table 3.3 - Percentage diesel utilization by air isolate while growing on increasing concentrations of diesel.

In the above experiment diesel utilization by the isolate was estimated by subtracting the loss of diesel due to evaporation from the control flask. Data suggested that, the isolate can be used for bioremediation of diesel contaminated zones along with potential diesel degrading strains isolated from soil.

3.8 - Metagenomic studies of diesel contaminated soil samples

This approach is used for detecting unculturable population of microorganisms from diesel contaminated soils. We have started to isolate large environmental genomic fragments from diesel contaminated soil samples (Fig 3.6).



Lane1 - 1 Kb ladder
Lane2 - Diesel Contaminated Soil DNA
Lane3 - Hind III digested λ DNA (ladder)

Fig 3.6 U.V visualization of the agarose gel containing DNA bands of whole soil microbial population.

The agarose gel having diesel contaminated soil DNA bands was visualized in U.V light using Geldoc software. This DNA will be cloned in plasmid vectors and random sequencing of these genomic clones will be done in future. This environmental genomic approach will set a basis to study the physiological, metabolic and genetic features of the uncultivated microorganisms. Metagenomics study will indicate a significant amount of organisms which have so far resisted classical cultivation approaches. Metagenomic approaches will expand our view of natural microbial diversity.

CONCLUSION

Bioremediation is a highly effective method of curing diesel contaminated sites. In the present study, by using enrichment culture technique we were able to isolate a large number of potential diesel degrading bacteria from contaminated soils which can further be utilized for the bioremediation of the diesel contaminated sites of Himachal Pradesh. As these bacteria are well adapted to the environmental conditions in this area, an immediate increase in the population density of these microbes could ensure rapid degradation of the pollutants. The isolation of diesel degrading bacteria from air has proved to be a new and simple method as compared to the conventional method of isolating such bacteria from diesel contaminated soil and water. This is because time taken to obtain the potential isolates is comparatively lesser and the risk of loss of certain strains due to serial dilutions as in the case of enrichment culture technique is not there. The air isolate was able to grow on various concentrations of diesel and was a good biosurfactant producer suggesting its possible exploitation in future bioremediation processes. We optimized the diesel concentrations on which air isolate showed the best growth. Further studies related to metagenomics will be done in for decontaminating the polluted zones using potential diesel degrading culturable microbial consortia.

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